TAXONOMY AND PHYLOGENY OF FUNGI CAUSING TAR SPOT ON *Ficus religiosa* IN CHIANG MAI PROVINCE

IRISH CLAIRE LITERATUS

MASTER OF SCIENCE
IN PLANT PATHOLOGY

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IN PLANT PATHOLOGY

Examination Committee :	**Advisory Committee :**
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16 May 2023

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Irish Claire Literatus

หัวข้อวิทยานิพนธ์	อนุกรมวิธานและวงศ์วานวิวัฒนาการของราสาเหตุใบจุดนูนดำบนโพธิ์ *Ficus religiosa* ในจังหวัดเชียงใหม่
ผู้เขียน	นางสาว ไอริช แคลร์ ลิตเทอราตัส
ปริญญา	วิทยาศาสตรมหาบัณฑิต (โรคพืช)
คณะกรรมการที่ปรึกษา	รองศาสตราจารย์ ดร. รัชดาวรรณ ชีวังกูร อาจารย์ที่ปรึกษาหลัก ดร. มิลาน ซี ซามาลากูน อาจารย์ที่ปรึกษาร่วม

บทคัดย่อ

ใบจุดนูนดำเป็นโรคที่มีสาเหตุมาจากราวงศ์ Phyllachoraceae สามารถพบได้บ่อยในเขตร้อนและชื้น ส่วนใหญ่พบในประเทศแถบเอเชีย เช่น ประเทศไทย โดยอาการมักเกิดที่ขึ้นใบ ลำต้น และผล ในปัจจุบันมีการศึกษาที่สำคัญเกี่ยวกับราชนิดนี้เพียงไม่กี่ชิ้นเท่านั้น เนื่องจากราสาเหตุโรคดังกล่าวไม่สามารถเจริญเติบโตในอาหารเลี้ยงเชื้อได้ ส่งผลให้ข้อมูลการกระจายตัวทางภูมิศาสตร์ และข้อมูลทางอณูชีววิทยาจึงเป็นเรื่องยากสำหรับการศึกษา ซึ่งการศึกษาส่วนใหญ่รายงานว่าโรคนี้เกิดจากสกุล *Phyllachora* อย่างไรก็ตาม มีรายงานโรคใบจุดนูนดำบางส่วนเกิดจากสกุลอื่น และยังพบการแพร่ระบาดของโรคใบจุดนูนดำหลายจุดซึ่งยังไม่สามารถระบุสาเหตุได้ ในประเทศไทยต้นโพธิ์มีบทบาทสำคัญในการอนุรักษ์วัฒนธรรมและประวัติศาสตร์ อย่างไรก็ตาม ต้นโพธิ์กำลังเผชิญกับภาวะที่กลืนไม่เข้าคายไม่ออกเนื่องจากการโจมตีของเชื้อโรค โดยเฉพาะบนใบ ซึ่งทำหน้าที่เป็นจุดศูนย์กลางของความงาม ดังนั้นการศึกษาครั้งนี้จึงมีจุดประสงค์เพื่อสำรวจและเก็บรวบรวมโรคใบจุดนูนดำใบโพธิ์ในพื้นที่จังหวัดเชียงใหม่ และทำการศึกษาความสัมพันธ์ทางพันธุกรรรมของรา โดยใช้ข้อมูลทางสัณฐานวิทยา ร่วมกับข้อมูลทางอณูชีววิทยา โดยสามารถรวบรวมตัวอย่างของใบโพธิ์ที่แสดงอาการใบจุดนูนดำได้ทั้งหมด 12 ตัวอย่าง จาก 7 อำเภอ ได้แก่ แม่แจ่ม แม่วาง แม่แตง แม่ริม สันทราย หางดง และ สันป่าตอง โดยใบจุดนูนดำจะแสดงอาการแผลจุดเดี่ยวสีดำ หรือรวมกันเป็นจุดใหญ่เรียก Pseudostromata มีลักษณะยาว ไม่สม่ำเสมอ ไม่ต่อเนื่อง เบาบาง รวมตัวกัน เกลี้ยง เป็นมันเงา ในผิวหนังถึงชั้นใต้ผิวหนัง epiphyllous จากนั้นทำ การวิเคราะห์ลำดับนิวคลิโทไทด์ของรา ในตำแหน่ง ITS และ LSU พบว่า จัดจำแนกราได้จำนวน 1 สกุล คือ *Neophyllachora* ในการศึกษาครั้งนี้ได้พบ

Neophyllachora fici ซึ่งเป็นการรายงานชนิดใหม่จากประเทศไทย ซึ่งได้อธิบายลักษณะสัณฐานวิทยาและวงศ์วานวิวัฒนาการไว้ในการศึกษาครั้งนี้ด้วย

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ABSTRACT

Tar spot is a common fungal disease often found in tropical and damp areas. They mostly exist in Asian countries like Thailand, and the symptoms usually occur on leaves, stems, and fruits. Only a few significant studies about this fungus are available because of their biotrophic nature, which makes them unable to grow in culture, therefore, sequence data from fresh collections are difficult to obtain. On one hand, most studies recorded this disease to be caused by the genus *Phyllachora*. On the other hand, some Tar spot symptoms are known to be caused by other genera, and there are still several Tar spot infestations in which the causal agent is yet to identify. In Thailand, the Bodhi tree plays an important role in culture and historic preservation. However, the Bodhi tree has been facing a dilemma due to attacks of pathogens, particularly on the leaves, which serve as the center of its beauty. This study aimed to identify the causal agent of the Tar spot on Bodhi tree leaves taken from different locations around Chiang Mai province, northern Thailand, through morpho-molecular evaluation. We isolated eight leaf samples of *Ficus religiosa* taken from Chiang Mai, Thailand. Symptoms on the host appear as black, solitary to gregarious, mainly on the upper surface. Pseudostromata is elongated, irregular, discrete, sparse, coalescent, glabrous, shiny, intraepidermal to subepidermal, epiphyllous, multilocular, occasionally amphigenous, rarely covering the leaf surface. Morphologically the new species was characterized by pseudostromatic ascomata with ostiole, septate paraphyses, cylindrical to fusiform asci, and globose to elliptical ascospores with sometimes 1-2 guttules, central concave depression present mostly in the globose form, a mucilaginous sheath that is irregularly thickened and widely thickened in the lateral part. The asexual morph produces ellipsoidal and hyaline conidia. The newly

obtained sequences were positioned within *Neophyllachora* and formed a distinct clade but close to *Neophyllachora fici* with high bootstrap support in the phylogenetic analyses. In addition, both species are reported in the same host genus (*Ficus*) but with different locality (Thailand Vs. Taiwan). Further the synopsis table for *Neophyllachora* and the identification key to the genus are provided.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Tar spot is a common fungal leaf disease characterized by the formation of black stromata, which is a slightly raised, semi-circular, dark brown to black and lustrous structure (Cannon 1991). The leaf spots caused by tar spot pathogens on leaves decrease the rate of photosynthesis and alter the physiological and biochemical aspects resulting in a reduction in crop production (Ballesta et al. 2010, Karami et al. 2014). The fungal disease has a wide host range, and the size of the spots can range up to several cm across (Hsiang et al. 2008). Tar spot also affects some economically and culturally important crops such as wheatgrass (Conners 1967), job's tears (Titatarn et al. 1984), sorghum (Long et al. 1985), bodhi tree (Hsieh et al. 2003), and corn (Ruhl et al. 2016).

Members of Phyllachoraceae are mostly obligate parasites which form "tar spots" on leaves and occasionally on stems and fruits with most are host-specific (Cannon 1997). The taxa are known as minor pathogens that rarely kill the host tissues but provide pathway for the secondary infection from severe pathogens by retaining for a long time in the host tissue (Cannon 1991, Hock et al. 1992, Parbery 1996). Phyllachoraceae was established by Theissen & Sydow (1915) with *Phyllachora* as the type genus. The family accommodates more than thousand species worldwide and are included in 54 genera (Tennakoon et al. 2020, Wijayawardene et al. 2022). However, molecular data are available only for a few members because of the difficulties in obtaining cultures from fresh samples (Hyde et al. 2020).

The taxa are characterized by ascohymenial development with paraphyses, thin-walled asci, which may have an apical ring that does not stain blue in iodine (J-) and often hyaline and 1-celled ascospores. The asexual morph is coelomycetes, spermatial or disseminative (Hawksworth et al. 1983). The family was placed in different orders including Dothideales (Horst 1990), Sphaeriales (Nannfeldt 1932, Miller 1949, Müller

and von Arx 1962, Wehmeyer 1975), Xylariales (Luttrell 1951, Barr1990), Glomerellales (Chadefaud 1960, Locquin 1984), Phyllachorales (Barr 1976a, b, 1983), Polystigmatales (Eriksson 1982, Hawksworth et al. 1983), and Diaporthales (Cannon 1988). Maharachchikumbura et al. (2016) placed Phyllachoraceae in Phyllachorales with Phaeochoraceae and this classification has been accepted by further studies (Hongsanan et al. 2017, Hyde et al. 2020). Consequently, Mardones et al. (2017) and Guterres et al. (2019) added two new families namely Telimenaceae and Phaeochorellaceae to this order.

In Thailand, several studies have reported *Phyllachora* species, including *Phyllachora bambusae, P. chloridis, P. coicis, P. cynodontis,* P. *cynodonticola, P. digitariae, P. graminis, P. pterocarpi, P. repens, P. thysanolaenae* and *P. vetiveriana* (Giatgong 1980, Nuangpai et al. 1984, Lenne 1990, Athipunyakom & Likhitekaraj 2009, Dayarathne et al. 2017, Tamakaew et al. 2017). This study provides the first report of the genus *Neophyllachora* from Thailand which was also reported from the leaves of *Ficus religiosa* for the first time. We also introduce a new species to *Neophyllachora* based on the evidence from both morphology and phylogeny. The synopsis table for *Neophyllachora* with the key to the species are provided.

1.2 Research Objectives

1.2.1 To provide morpho-molecular identification of the fungi causing Tar spots on *Ficus religiosa.*

1.2.2 To assess the biogeography of the fungi causing Tar spots on *Ficus religiosa* in Chiang Mai Province.

1.3 Usefulness of the Research (Theoretical and/or applied)

1.3.1 This study aims to increase the knowledge and attention about the flourishing Tar spot infestation in *Ficus religiosa* leaves in Chiang Mai Province. The results of taxonomy and phylogeny will be published for the scientific community for the use of pathologists, taxonomists, and quarantine systems. The sequences and phylogenetic data will be submitted to databases for future studies.

CHAPTER 2

LITERATURE REVIEW

2.1 An overview of Tar spot

Tar spot is a fungal leaf disease that infects several plants. The symptom starts with small yellow spots on growing leaves, then expands into large black blotches. In addition, the pathogen produces a circular pattern of black fruiting bodies within these spots (Zacaroni et al. 2013). As the fungus grows, the spreading yellow spot slowly turns from a yellow-green to a deep, tarry black, referred to as a tar spot (https://www.hamilton.ca/home-property-and-development/property-gardens-trees/tar-spot-disease). In the winter season, the disease can cause intense defoliation in susceptible plants (Zacaroni et al. 2013). However, tar spots can be easily confused with the black saprophytic organisms that grow on dead leaf tissue. To elude this, take notice that saprophytes usually have a dusty appearance and can be rubbed off the leaf tissue while tar spot stromata cannot be rubbed off, and it is usually surrounded by a narrow tan halo (Kleczewski et al. 2019). According to Hudler et al. (1998), the disease was first reported in Ohio in the 1940s, and their study unveiled that the fungus *Rhytisma acerinum*, was responsible for the tar spot occurring on a variety of maples in Europe, where various studies about tar spot diseases were conducted (Hsiang et al. 2008). The disease has diverse host plants, including some economically important crops and trees. The causal agents are commonly from *Rhytismales* and *Phyllachorales*. The former is frequently found in forest areas or foothills of high ranges occupying a special niche mostly on forest plants, while the former prefers a cropland environment or open plain (Thaung, 2008).

2.2 Tar spot disease caused by Phyllachorales

Phyllachorales are mostly linked to angiosperms, with the following families preferentially parasitized: *Arecaceae, Fabaceae, Lauraceae, Melastomataceae,*

Moraceae, Myrtaceae, and Poaceae (Cannon 1997). Additional host families studied include *Asclepiadaceae* (Pearce et al. 2001*), Erythroxylaceae* (Cannon 1988), *Proteaceae* (Pearce et al. 2001), and *Rosaceae* (Cannon 1988).

Currently, there are at least 3 families belonging to *Phyllachorales* - *Phaeochoraceae, Phyllachoraceae* (Maharachchikumbura et al. 2016; Hongsanan et al. 2017), and *Telimenaceae* (Mardones et al. 2017). According to Maharachchikumbura et al. (2016), members of the *Phyllachoraceae* is characterized by forming leaf spots on the host that are abundant but scattered, raised, mostly rounded to oblong or elongated, sometimes parallel with leaf venation, surrounded by a light-brown necrotic region; lacking periphyses; having numerous paraphyses, branched or unbranched; 8-spored asci, persistent, cylindrical to fusiform, often present with an apical ring; ascospores fusiform to narrowly oval, hyaline, often with a mucilaginous sheath. *Phyllachoraceae* is similar *to Phaeochoraceae,* but the former species are characterized by 8-spored asci, an often-present apical ring, usually hyaline ascospores, rarely pale brown, thin and smooth-walled, while the latter is characterized by 6-8-spored asci, usually without apical structure, yellow to olivaceous ascospores or in various shades of brown, thick-walled (Maharachchikumbura et al. 2016; Mardones et al. 2017).

Phyllachora is the largest genus of *Phyllachoraceae*, and they are morphologically characterized by clypeate pseudostroma in leaf tissues, generalized infection of the entire section of the mesophyll forming leaf spots on the host, mostly rounded to oblong or elongated, surrounded by,light-brown necrotic region; perithecium globose; numerous paraphyses, branched, slightly longer than asci; asci 8-spored, persistent, cylindrical to fusiform, a short pedicellate, an apical ring often present; and ascospores 1–3 seriate, fusiform to narrowly oval, hyaline, sometimes with a gelatinous sheath (Dos Santos et al, 2016; Maharachchikumbura et al. 2016; Yang et al. 2019*). Phyllachora* species are commonly found with *Poaceae* but have been reported to infect more than 1000 plant species including *Cyperaceae, Fabaceae, Lauraceae, Moraceae, Myrtaceae, Poaceae, Proteaceae,* and *Rosaceae* (Li et al. 2022).

In a study conducted by Li et al. (2022), they found that the *Phyllachora* genus is paraphyletic since the host of *Phyllachora pomigena* remains unknown, the species formed a single clade. They further explain that in the phylogenetic analysis, the new species described are included within the *Phyllachora* genus and separated from other

taxa with a single subclade. Their hosts are *Cenchrus flaccidus* and *Chloris virgata,* both belonging to *Poaceae* (graminicolous).

2.3 Reports on Tar spot disease caused by *Phyllachora* worldwide

In 1991, black spots (caused by *Phyllachora repens)* were seen on the leaves of Bodhi trees at the National Chung Hsing University Campus in Taiwan. The diseased trees resulted in severe defoliation out of season. The obligate parasite produced a teleomorph state and spermogonia however, no anamorph was found (Hsieh et al. 2003). Although this incident is interesting, no recent studies are available after the report.

The tar spot of corn (*Phyllachora maydis*) was first confirmed in the United States in 2015. In 2018, a yield-reducing epidemic of tar spots occurred in northern Indiana and surrounding states. Following this epidemic, tar spot was detected in 172 counties across six states in the Midwest. Fields in the most severely affected regions reached 100% disease incidence and over 50% severity on the ear leaf (Kleczewski et al. 2019).

2.4 Reports on Tar spot disease caused by *Phyllachora* in Thailand

In a disease survey conducted in November 1983, tar spots (*Phyllachora coicis*) were identified from the upper and lower leaf surfaces of Job's tears (*Coix lachryma-jobi*) in the vicinity of Dong Ma Da village of Mae Suay district of Chiang Rai province. The symptoms were characterized by roughly circular raised black bodies (fungal stromata) about 1.5 mm in diameter and were estimated to have a more than 70 percent level of infection (Nuangphai 1984).

A different study by Boon-Long et al. (1987) reported the occurrence of tar spot disease in sorghum and the pathogen was identified as *Phyllachora sorghi.* Hitherto, no further studies are available after the report.

2.5 An overview of the Bodhi Tree

The Bodhi tree (*Ficus religiosa*) is a perennial tropical tree that belongs to *Moraceae*. It has grayish bark and heart-shaped leaves and can grow up to 9 feet in diameter. This large broadleaf evergreen tree is native to Southeast Asia and India and recorded from the mid-19th century as the ‘tree of knowledge (https://www.encyclopedia.com/plants-and-animals/plants/plants/bo-tree). The Bodhi

tree has other common names like the Bo tree, Peepul tree, and Sacred fig. It is culturally, spiritually, and historically significant to Buddhism. Medically, based on some historical facts, the fig tree was used to treat ailments and disorders including asthma, diabetes, diarrhea, epilepsy, gastric problems, and inflammatory, infectious, and sexual disorders (http://selectree.calpoly.edu/tree-detail/612). According to the record, the oldest and largest Bodhi tree in Thailand is located in the eastern part of the country in Wat Ton Pho Si Maha Pho, Tambon Khok Pip, Prachin Buri (https://www.tourismthailand.org/Attraction/ton-pho-si-maha-pho-the-great-bodhitree). Most Thai people, if not all, considered the Bodhi tree holy and an essential part of their culture and identity.

2.6 Reports on diseases of Bodhi Tree caused by various pathogens

According to a report by Schrader (2020), the common causal agents of Bodhi tree diseases are ascomycetes, and these pathogens can cause many small black spots on the leaf surface with different shapes of the bulge. As the disease advances, the Bodhi tree will eventually die of withered leaves. As of this time, the only way to manage the disease is by cutting off and burning the diseased leaves as soon as symptoms appear.

Like any other plant, the Bodhi tree is not exempt from different thriving diseases. Abeygunawardhane (1969) reported the leaf spot disease in the Bodhi tree, which is caused by *Glomerella cingulata*, and the brown root disease caused by *Phellinus noxius*, which was responsible for the mysterious death of individual Bodhi trees. Leaf blight disease was reported in Bodhi trees from Jaipur, India, and the causal agent was identified as *Phyllosticta* sp. (Sharma et al. 2011). Another leaf spot disease was reported from Lahore, Pakistan, caused by *Curvularia aeria* (Nayab & Akhtar 2016).

In 2007, a news report from Hindustan Times (2007), surfaced on the internet raising concern about the unknown disease of the sacred Bodhi tree in Bodh Gaya, northeastern India. The symptom includes hundreds of fresh leaves falling off daily which is quite unusual. It was reported that the cause of the incident was undernutrition and treatments were done but then no further details about the diagnosis were revealed and no further reports were given.

A study conducted by Li et al. (2022) in Zhanjiang, Guangdong, China reported the leaf spot disease on the Bodhi tree which was identified as caused by *Diaporthe tulliensis*. They describe the symptoms as circular to oval-shaped spots with pale white centers and brown-black edges surrounded by a chlorotic halo. Proper control management is needed to address this potential loss as repeated annual defoliation may weaken the tree and decrease its aesthetic value in the landscape.

2.7 Reports on diseases of *Ficus* spp. caused by *Phyllachora spp.*

Based on the recent data, there are 69 species of *Phyllachora* that are pathogenic to *Ficus* spp., but only 3 species are pathogenic to *Ficus religiosa* (USDA Fungal Databases; retrieved from https://nt.ars-grin.gov/fungaldatabases).

2.8 The genus *Neophyllachora*

In 2017, Dayarathne et al. introduced a new genus, *Neophyllachora* to accommodate the following: *Neophyllachora cerradensis, N. myrciae, N. myrciariae, N. subcircinans* and *N. trucantispora*, which are related to *Phyllachora* species but constitutes an independent strongly supported monophyletic clade within Phyllachoraceae. Some species of the aforesaid genus can badly infect some economically important crops which could lead to yield loss (Li et al. 2022).

2.9 Reports on diseases of *Ficus* spp. caused by *Neophyllachora spp.*

A study conducted by Tennakoon et. al (2021) is the first report on the occurrence of the Tar spot in *Ficus septica*, which is a promising topical herbal medicine to cure small cutaneous ulcers (Deli et al. 2022). The causal agent is named *Neophyllachora fici*. This study will also report one disease of *Ficus religiosa* caused by the genus *Neophyllachora.* Nonetheless, only a few significant studies about the aforementioned genera are available because of their biotrophic nature, which makes them unable to grow in culture, therefore, sequence data from fresh collections are difficult to obtain (Tamakaew et al. 2017).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection and disease symptoms

A survey of *Ficus religiosa* (Bodhi trees) with tar spot symptoms was carried out from September to December 2022. Twelve samples were taken from seven different locations in Chiang Mai Province, Thailand (Figure 1). Leaf samples were kept in plastic bags, labeled properly, and carried to the laboratory within 24 h of collection.

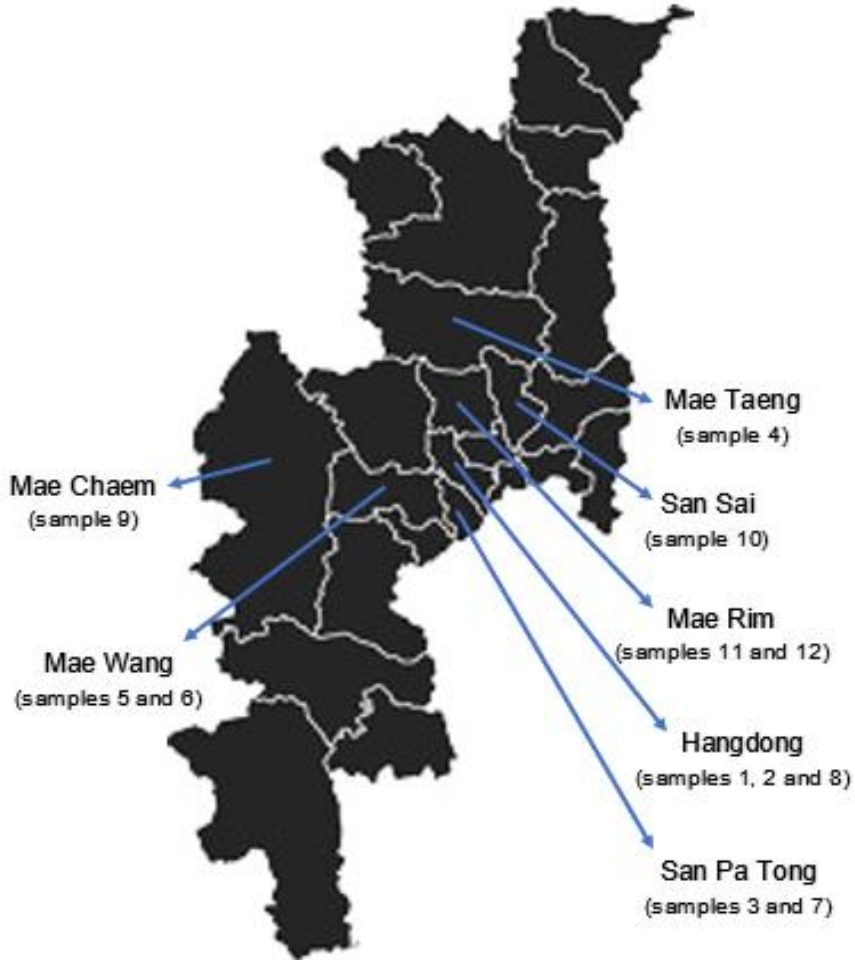

Figure 1. Chiang Mai Province map showing the location of the collected samples

3.2 Morphological studies

After being transferred to the laboratory, leaf samples were examined using a stereo microscope (Zeiss Stemi 305). Observations, photographs, measurements, and descriptions were made from squash mounts of fresh fruiting bodies and sections of the ascomata mounted in water and 95% lactic acid. Melzer's reagent was used to check the apical apparatus in the ascus. Photographs were made using a Nikon SMZ745T stereo microscope (×4.5) and ZEISS Scope A1 microscope (×40-100) and measurements were carried out using the Tarosoft (R) Image Frame Work program (Tarosoft, Bangkok, Thailand. Photoplates were prepared in Adobe Photoshop 2020 version 21.0.2 software.

3.3 Spore germination test

For the spore germination test, a small amount of the sticky-like fruiting bodies was taken from the fresh samples using a sterile scalpel and spread in a zigzag on Potato Dextrose Agar (PDA) plate. The PDA plate was then incubated at room temperature and was observed under the stereo microscope after 24 h to check for spore germination. Newly germinated spores were picked up from the incubated PDA plate using a sterile scalpel and transferred to new PDA and water agar (WA) plates. The plates were incubated at room temperature and were checked 3 times (after 24 h, 48 h, and 72 h) under the stereo microscope to see the fungal growth.

3.4 DNA extraction, PCR amplification, and sequencing

DNA was extracted from eight samples directly from ascomata using the DNA extraction kit (FAVORGEN, Ping-Tung, Taiwan), following the protocols in the manufacturer's instructions. Partial small subunit nuclear rDNA (SSU), large subunit (LSU), and internal transcribed spacer (ITS) genes were amplified using the primer pairs NS1 and NS4 (White et al. 1990), LR0R and LR5 (Vilgalys & Hester 1990) and ITS4 and ITS5 (White et al. 1990) respectively. Amplifications were performed in 25 µl of PCR mixtures containing 12.5 µl PCR Master Mix, 9.5 µl deionized water, 1 µl of DNA template, and 1 µl of each primer. The PCR thermal cycle program was performed with an initial step of 3 mins at 94°C, followed by 35 cycles of 30 sec at 94°C, 58 sec at 30°C, and 1 min at 72°C, with a final extension of 10 mins at 72°C. The amplifications were visualized by gel electrophoresis at 100V for 30 min. The PCR products were sequenced

and automatically determined in a genetic analyzer at the 1st Base Company (Kembangan, Malaysia) using the aforementioned PCR primers.

3.5 Taxon sampling

DNA sequences generated in this study were subjected to BLAST searches (NCBI) (https://www.ncbi.nlm.nih.gov) to select taxa for phylogenetic analyses. The sequences for the phylogenetic analyses were retrieved from GenBank following Tennakoon et al. (2021). The final combined alignment comprised 53 sequences including the new sequence.

3.6 Phylogenetic analyses and species identification

Multiple alignments were automatically made with MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server), using default settings (Katoh & Standley 2013) and further edited manually by BioEdit v. 7.0.5.2. The ML phylogenetic tree was generated using the RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis 2014) in the CIPRES Science Gateway platform (Miller *et al*. 2010) with 1000 separate runs. MrBayes v. 3.1.2 was used to perform Bayesian analysis (Huelsenbeck & Ronqvist 2001). GTR+I+G model was selected as the best-fit model for each gene using MrModeltest v. 2.3 (Nylander 2004) under the Akaike information criterion (AIC). Markov Chain Monte Carlo sampling (MCMC) was run for 5,000,000 generations and trees were sampled every 100th generation. The first 10% of trees that represented the burn-in phase were discarded and only the remaining 90% of trees were used for calculating posterior probabilities (PP) for the majority rule consensus tree. The resulting trees were drawn in FigTree v1.4.0 (Rambaut 2012), then edited in Microsoft PowerPoint (2013) and Adobe Photoshop CS6 version 10.0.

CHAPTER 4

RESULTS

4.1 Sample collection and disease symptoms

In this study, the leaf samples were collected from October to December 2022. In our survey of Chiang Mai Province, we found small and large black spots. We collected 12 samples (7 small black spots and 5 large spots) from 7 different locations (Figure 1.)

4.2 Morphological studies

Based on our observation, those leaf samples with large spots were found from strangler trees (Figure 2). Strangling is one of the characteristics of the tropical figs of the genus *Ficus* in the family *Moraceae*. This growth pattern upon host trees, which often results in the host's death, is common in tropical forests worldwide. Species of trees that possess the ability to strangle are called strangler figs or just stranglers (Britannica, 2019). Contrariwise, leaf samples with small spots were collected from non-strangler trees.

The symptom morphology of our collections (Figure 3) was found to fit with the generic concept of the Tar spot symptom in having the formation of black stromata, which is a slightly raised, semi-circular to irregular, dark brown to black lustrous structure as described by Hyde & Cannon (1999). However, the large spots are found to have craggy bulging structures and mostly have irregular sizes. Our samples are parasitic on leaves of *Ficus religiosa* (*Moraceae*). Symptoms on the host appear as black, solitary to gregarious, mainly on the upper surface (Figure 4). The measurements and descriptions of the sexual morph characters and the asexual spores (conidia and spermatia) are shown in Tables 1 and 2, respectively. Photographs of both sexual morph and asexual morph characters are shown in Figures 5 and 6, respectively. In addition, all samples were stained with a drop of Melzer's reagent, and the asci were confirmed to be J- as they all did not stain blue (Figure 7).

Table 1. Sexual morph characters measurements and descriptions

sexual morph characters	Measurements	descriptions
Ascomata	83–172 × 127–349 µm	perithecial, globose to subglobose, solitary or aggregates, ostiolate (conspicuous)
Peridium	21–30 thick	dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells
paraphyses	1–2 µm wide	filiform, numerous, persistent, septate, unbranched, longer than asci
Asci	59–140 × 14–25 µm ($\bar{x}$ = 88 × 20 µm, n = 30)	8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures
ascospores	8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30)	uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness

Table 2. Asexual morph characters measurements descriptions

asexual morph characters	Measurement	descriptions
conidiomata	-	visible conidiogenous cells, spermatial or conidial wide conidial locules
conidiogenous cells	-	holoblastic, covering the base of the conidiomata
conidia	2.0–4.0 × 3.5–6.0 µm ($\bar{x}$ = 3.33 × 2.28 µm, n = 50)	ellipsoidal, hyaline, rarely septate
spermatia	0.90–1.40 × 7.0–16.0 µm ($\bar{x}$ = 1.03 × 11.48 µm, n = 50)	botuliform or falciform, narrowly rounded at both ends, curved, aseptate, hyaline, smooth-walled

Figure 2. *Ficus religiosa*. a. strangler tree. b. non-stangler tree

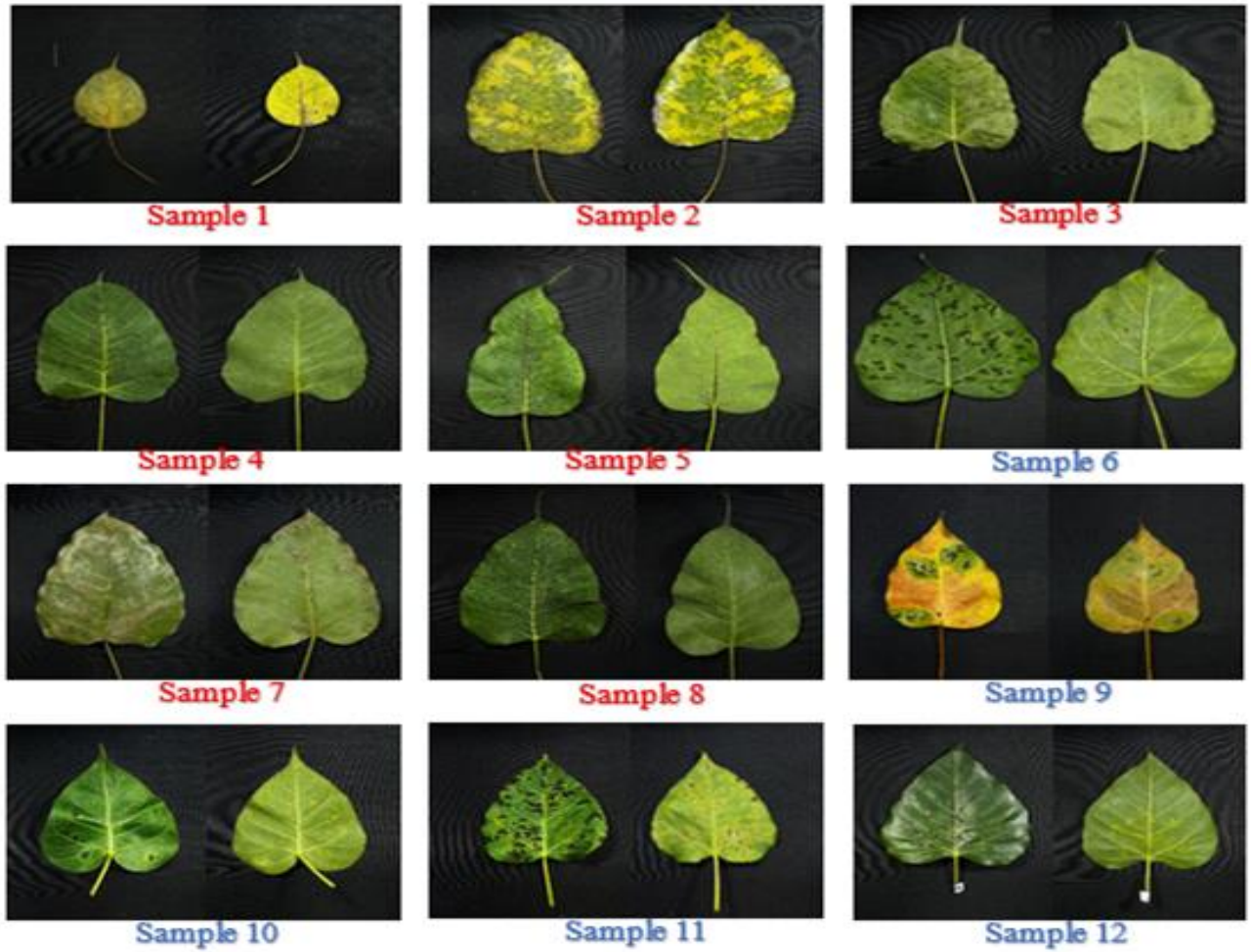

Figure 3. Symptom morphology of the 12 collected leaf samples from *Ficus religiosa*. a. large spots- samples 6 and 9-12. b. small spots- samples 1-5 and 7-8.

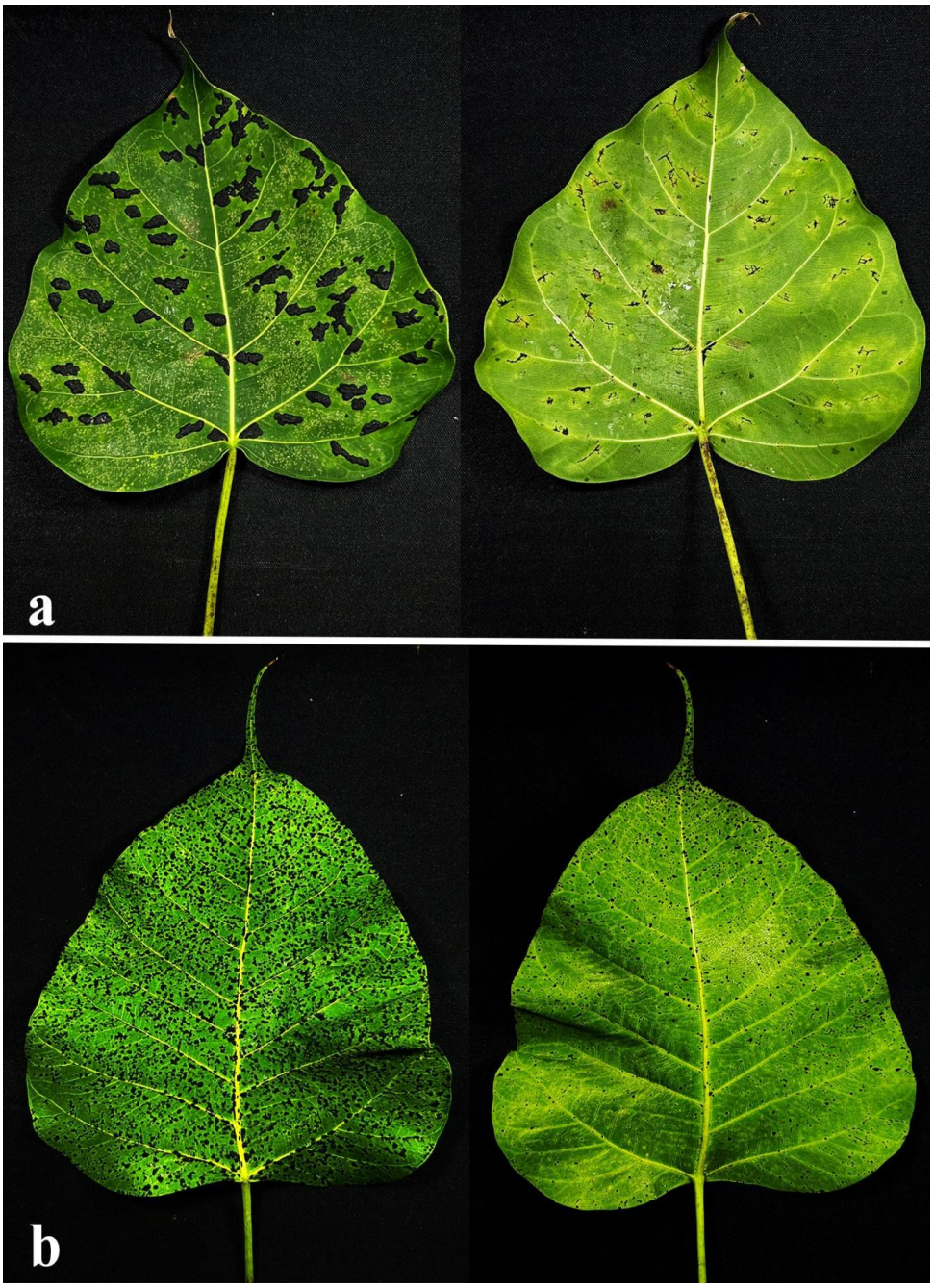

Figure 4. *Ficus religiosa* leaves. a. large spots. b. small spots

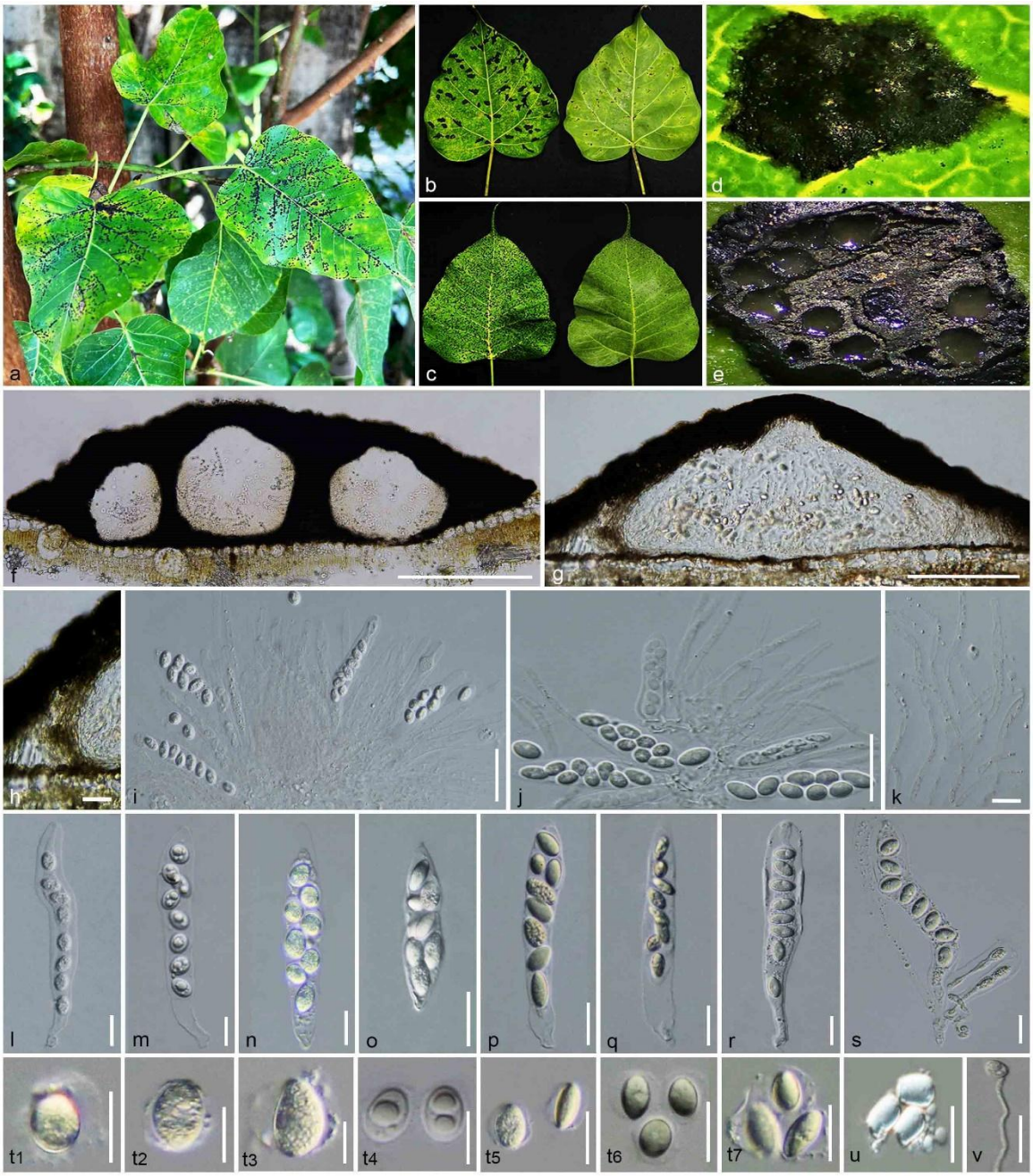

Figure 5. Sexual morph of *Neophyllachora* sp. (large and small spots). a. Tar spot on living leaves, b. Upper and lower leaves of with large spot, c. Upper and lower leaves of with small spot. D. Pseudostroma, e. Horizontal section through pseudostroma, f. Vertical section through pseudostroma, g. Vertical section through ascomata, h. Vertical section through peridium, i, j Asci and paraphyses. k. Paraphyses, l–s. Asci, t1–t7. Ascospores, u. Conidia, w. Germinated spores. Scale bars: f = 1000 µm, g = 100 µm, h = 100 µm, k = 5 µm, I, j, i–s= 50 µm. t1–t7, u, w= 10 µm.

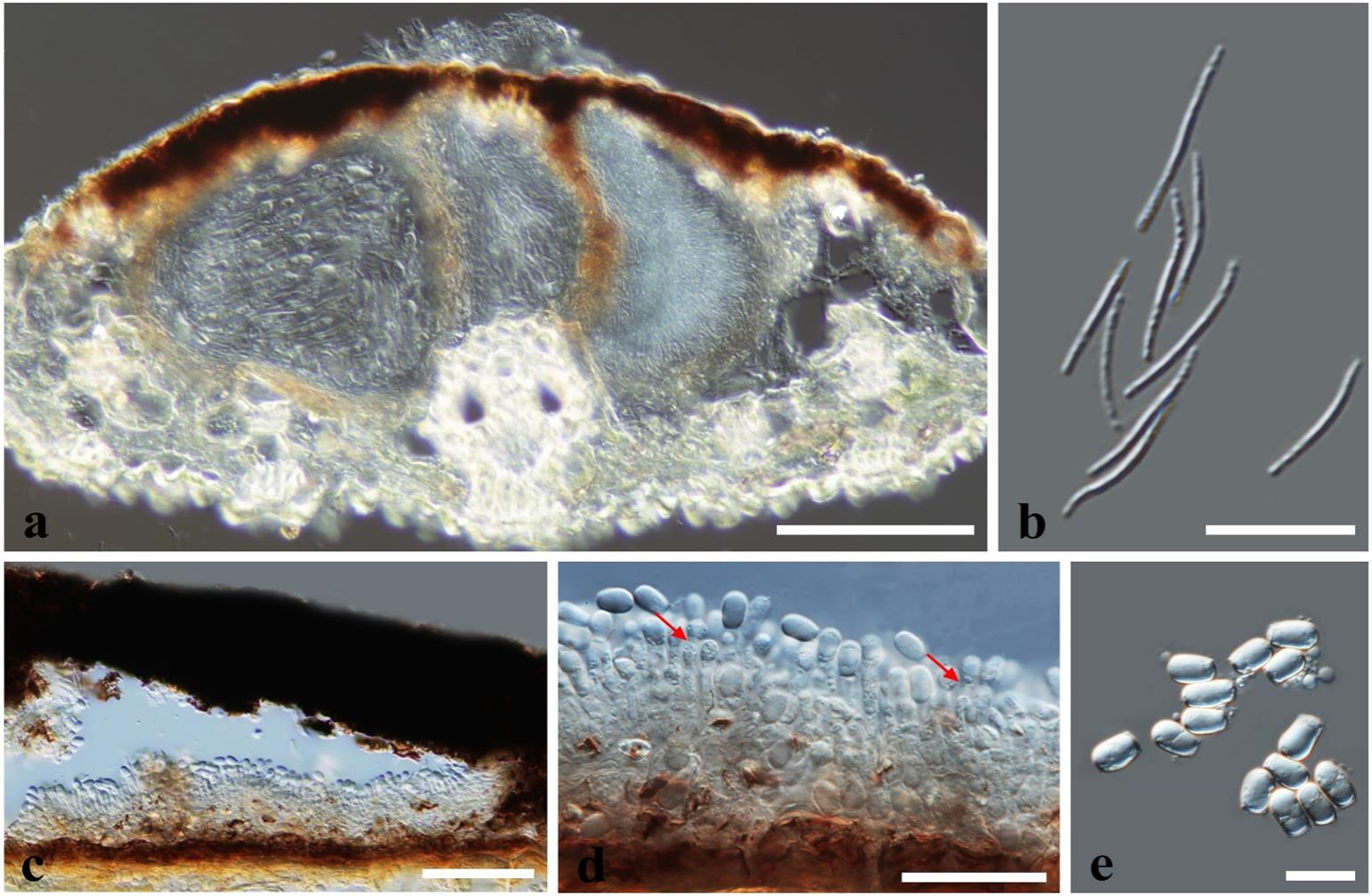

Figure 6. Asexual morph of *Neophyllachora sp.* a. spermogonia. b. spermatia. c. conidiomata. d. conidiogenous cells.

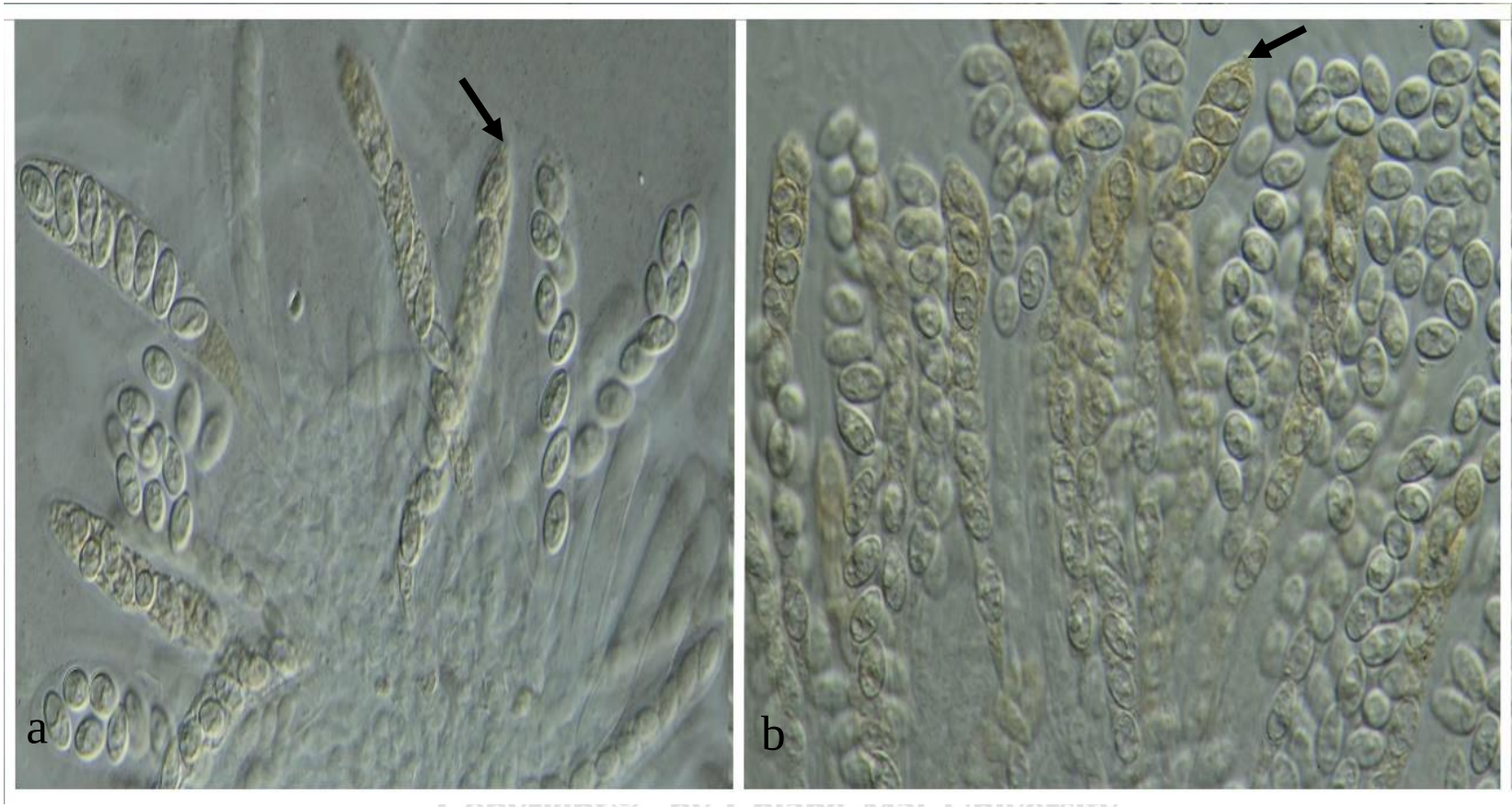

Figure 7: Asci stained with Melzer reagent. a. small spots. b. large spots

4.3 Spore germination test

Spores germinated (Figure 8) after the PDA plates were incubated for 24 h at room temperature. Then, we attempted to isolate the fungus by transferring the newly germinated spores to PDA and WA plates which were then incubated at room temperature. However, there was no sign of any fungal growth after 24h, 48h, and 72 h of incubation.

Figure 8: Germinated spores. a-b. small spots. c-d. large spots**.**

4.4 Phylogenetic analysis

The PCR amplification for SSU and LSU gene regions failed following the condition referred to by Dayararthne et al. (2017). Thus, the phylogenetic analyses were conducted only using the ITS gene region. All *Neophyllachora* species lack the LSU gene and the SSU is available only for several species including *N. cerradensis*, *N. subcircinans* and *N. truncatispora* and the phylogenetic placement of our new sequences concurred with the combined multigene analysis. The newly generated sequences were cladded together with *Neophyllachora fici* with high bootstrap support (Figure 7). The base pair comparison between *Neophyllachora fici* and the newly obtained sequences shows more than 2% differences in the ITS region. In addition, the blast hits of all the new sequences showed closest to *Neophyllachora*. The final dataset comprised 65 strains including 7 new sequences with 525 aligned characters including gaps (Table 3.)

All genera in Phyllachoraceae are well-resolved except the generic type *Phyllachora* which has recovered as polyphyletic. The best scoring RAxML tree was selected to represent the relationships among the taxa, with the final ML optimization likelihood value of -8093.468208 (Figure 9). The parameters for the GTR+I+G model of ITS were as follows: estimated base frequencies; A—0.235449, C—0.269319, G—0.261529, T—0.233702, substitution rates AC—1.107447, AG—2.360770, AT—1.392100, CG—0.585365, CT—3.555061 and GT = 1.000000. The ML and Bayesian analyses both resulted in trees with similar topologies. Bayesian posterior probabilities from MCMC were evaluated with a final average standard deviation of split frequencies of 0.002338.

Table 3. Taxa table

No.	Taxon	Strain	Genbank Accession (ITS)
1	*Camarotella costaricensis*	MM_149	KX451913
2	*Camarotella costaricensis*	MM_21	KX451900
3	*Camarotella* sp.	MM_27	KX451901
4	*Coccodiella miconiae*	ppMP1342	MF460365
5	*Coccodiella miconiicola*	CBMAP_H290A	MF460368
6	*Coccodiella* sp.	MM_165	KX451917
7	*Neophyllachora cerradensis*	UB15626	KC683454
8	*Neophyllachora cerradensis*	UB16014	KC683455
9	*Neophyllachora cerradensis*	UB21823	KC683470
10	*Neophyllachora cerradensis*	UB21908	KC683471
11	*Neophyllachora fici*	MFLU 19_2702	MW114384
12	*Neophyllachora fici*	NCYU 19_0326	MW114386
13	*Neophyllachora myrciae*	UB21292	KC683463
14	*Neophyllachora myrciae*	UB22192	KC683476
15	*Neophyllachora myrciariae*	UB21781	KC683469
16	*Neophyllachora religiosa*	CRC-H190	xxx
17	*Neophyllachora religiosa*	CRC-H191	xxx
18	*Neophyllachora religiosa*	CRC-H192	xxx
19	*Neophyllachora religiosa*	CRC-H193	xxx
20	*Neophyllachora religiosa*	CRC-H194	xxx
21	*Neophyllachora religiosa*	CRC-H195	xxx
22	*Neophyllachora religiosa*	CRC-H196	xxx
23	*Neophyllachora subcircinans*	UB21747	KC683467
24	*Neophyllachora subcircinans*	UB09748	KC683441
25	*Neophyllachora subcircinans*	UB21238	KC683461
26	*Neophyllachora subcircinans*	UB21347	KC683466

Table 3. Taxa table (continued)

No.	Taxon	Strain	Genbank Accession (ITS)
27	*Neophyllachora truncatispora*	UB14083	KC683448
28	*Phyllachora arthraxonis*	MHYAU_072	MG269749
29	*Phyllachora arundinellae*	MHYAU_108	MG269761
30	*Phyllachora capillipediicola*	MHYAU_20089	KY498084
31	*Phyllachora capillipediicola*	MHYAU_20090	KY498115
32	*Phyllachora chloridis*	MFLU 15_0173	KY594026
33	*Phyllachora chloridis-virgatae*	MHYAU_20058	KY498102
34	*Phyllachora chloridis-virgatae*	MHYAU_20137	KY498092
35	*Phyllachora cynodonticola*	MFLU 16_2978	KY594025
36	*Phyllachora cynodonticola*	MFLU 16_2977	KY594024
37	*Phyllachora cynodontis*	MHYAU_20043	KY471329
38	*Phyllachora cynodontis*	MHYAU_20042	KY471328
39	*Phyllachora flaccidudis*	IFRD9445	ON075524
40	*Phyllachora graminis*	101486	AF257111
41	*Phyllachora graminis*	DAOM_2409	HQ317550
42	*Phyllachora graminis*	MM-166_P	KX451920
43	*Phyllachora heterocladae*	MFLU 18_1221	MK305902
44	*Phyllachora imperatae*	MHYAU_014	MG269746
45	*Phyllachora indosasae*	MHYAU_125	MG195637
46	*Phyllachora isachnicola*	MHYAU_179	MH018561
47	*Phyllachora isachnicola*	MHYAU_180_P	MH018562
48	*Phyllachora jiaensis*	IFRD9448	ON075527
49	*Phyllachora keralensis*	MHYAU_20082	KY498106
50	*Phyllachora miscanthi*	MHYAU_167	MG195644
51	*Phyllachora miscanthi*	MHYAU_157	MG195643
52	*Phyllachora panicicola*	MFLU16_2979	KY594028

Table 3. Taxa table (continued)

No.	Taxon	Strain	Genbank Accession (ITS)
53	*Phyllachora pogonatheri*	MHYAU_071	MG269748
54	*Phyllachora pogonatheri*	MHYAU_070	MG269747
55	*Phyllachora sandiensis*	IFRD9446	ON075525
56	*Phyllachora sinobambusae*	MHYAU_085	MG195630
57	*Phyllachora* sp.	MHYAU_123	MG195631
58	*Phyllachora* sp.	MHYAU_158	MG195633
59	*Phyllachora sphaerocaryi*	MHYAU_178	MH018560
60	*Phyllachora sphaerocaryi*	MHYAU_217	MK614100
61	*Phyllachora virgataes*	IFRD9447	ON075526
62	*Polystigma pusillum voucher*	MM_113	KX451907
63	*Telimena bicincta*	MM_108	KX451910
64	*Telimena canafistulae*	MM_13	KX451906
65	*Telimena leeae*	TH549	KX451934

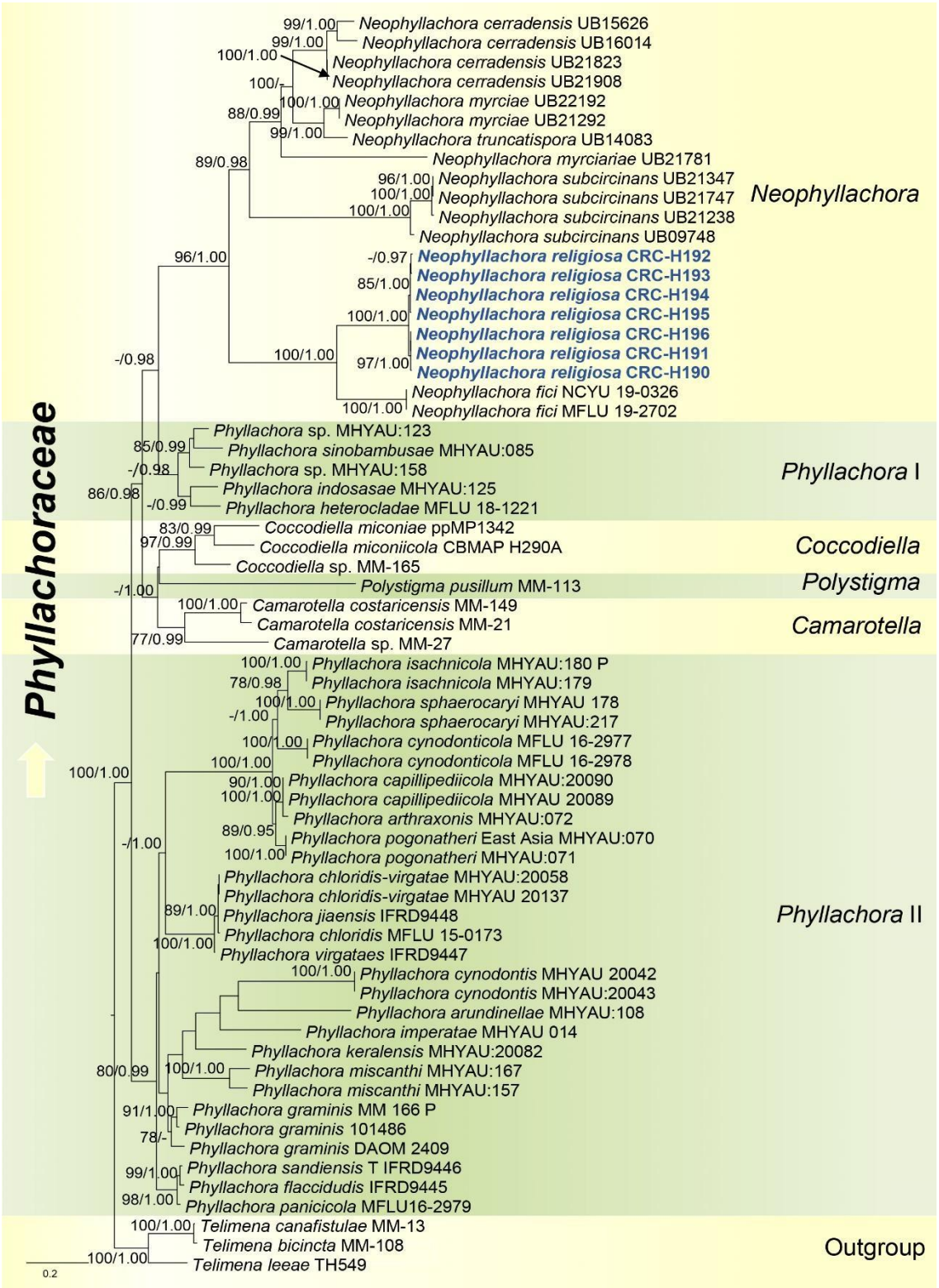

Figure 9. RAxML tree based on analyses of ITS sequence data. Bootstrap support values for ML equal or greater than 70%, and Bayesian posterior probabilities (BP) equal or greater than 0.95 are given as ML/BP above the nodes. The tree is rooted to *T. bicincta* (MM-108), *T. canafistulae* (MM-13) and *T. leeae* (TH549).

4.5 Taxonomy

Phyllachorales M.E. Barr

MycoBank number: MB 90495; Facesoffungi number: FoF 06410; Index Fungorum number: IF 90495

Notes: *Phyllachorales* was established by Barr (1983), and currently has three families, namely *Phaeochoraceae*, *Phyllachoraceae*, and *Telimenaceae* (Dayarathne et al. 2017; Mardones et al. 2017; Yang et al. 2019: Hyde et al. 2020). According to Parbery (1967) and Cannon (1991), *Phyllachorales* species are leaf- or steam-inhabiting microfungi with shiny black stromata and are morphologically characterized by deep black stromata of various shapes. This characteristic, however, is not seen in species of *Polystigma* which have brightly colored stromata. In addition, the perithecia of *Phyllachorales* are usually strongly melanized that may be superficial, erumpent, or immersed in the host tissue, have thin-walled paraphyses that frequently deliquesce, unitunicate asci of cylindrical to clavate shape, with an ascus crown and an inconspicuous apical ring not staining blue in iodine; and globose to filiform ascospores, which in most species are hyaline and 1-celled, with only a few genera including species with brown or septate ascospores.

Phyllachoraceae Theiss. & P. Syd.

MycoBank number: MB 81156; Facesoffungi number: FoF 01329; Index Fungorum number: IF 81156

Notes: *Phyllachoraceae* species have ascohymenial development with paraphyses and thin-walled asci. Some species may have an apical ring, that does not stain blue in iodine (J-) and ascospores that are often hyaline and 1-celled (Cannon 1991; Maharachchikumbura et al. 2015, 2016; Hyde et al. 2020). Members of this family are mostly reputed to be highly host-specific, and the majority of them lack sequence data (Dayarathne et al. 2017, Hyde et al. 2020). In 1915, Theissen and Sydow introduced *Phyllachoraceae* to accommodate *Phyllachora*.

Phyllachora Nitschke ex Fuckel

MycoBank number: MB 4049; Facesoffungi number: FoF 02126; Index Fungorum number: IF 4049

Type species – *Phyllachora graminis* (Pers.) Fuckel

Notes – *Phyllachora* species are named based on their host association (Cannon 1988). Their

clypeate pseudostroma in the leaf appears in various sizes from a subcuticular or intra-epidermal to a generalized infection of the entire section of the mesophyll, inducing characteristic black shiny superficial symptoms (Dos Santos et al. 2016). According to Cannon (1991), the depth of ascomata is not a valid character to distinguish genera as it can be influenced by the consistency of the host.

Neophyllachora Dayar. & K.D. Hyde

MycoBank number: MB 553633; Facesoffungi number: FoF 13499; Index Fungorum number: IF 553633

Type species – *Neophyllachora myrciae* Dayar. & K.D. Hyde

Notes: *Neophyllachora myrciae*, which was previously known as *Dothidea myrciae* (Léveillé 1846) is the type species of *Neophyllachora*. Dayarathne & Hyde (2017) introduced *Neophyllachora* to accommodate this novel species. They described the members of this genus as having sub-epidermal, intra-epidermal stromata without a deeper invasion of mesophyll, and clavate asci. At present, there are 6 *Neophyllachora* species listed in Species Fungorum (accession date: 07.02.2023). Five of them were listed in 2017, namely *N. cerradensis* (Dayarathne & Hyde), *N. myrciae* (Dayarathne & Hyde), *N. myrciariae* (Dayarathne et al.), *N. subcircinans* (Dayarathne et al.), *N. truncatispora* (Dayarathne et al.), and *N. fici* (Tennakoon et al.) was added in 2021.

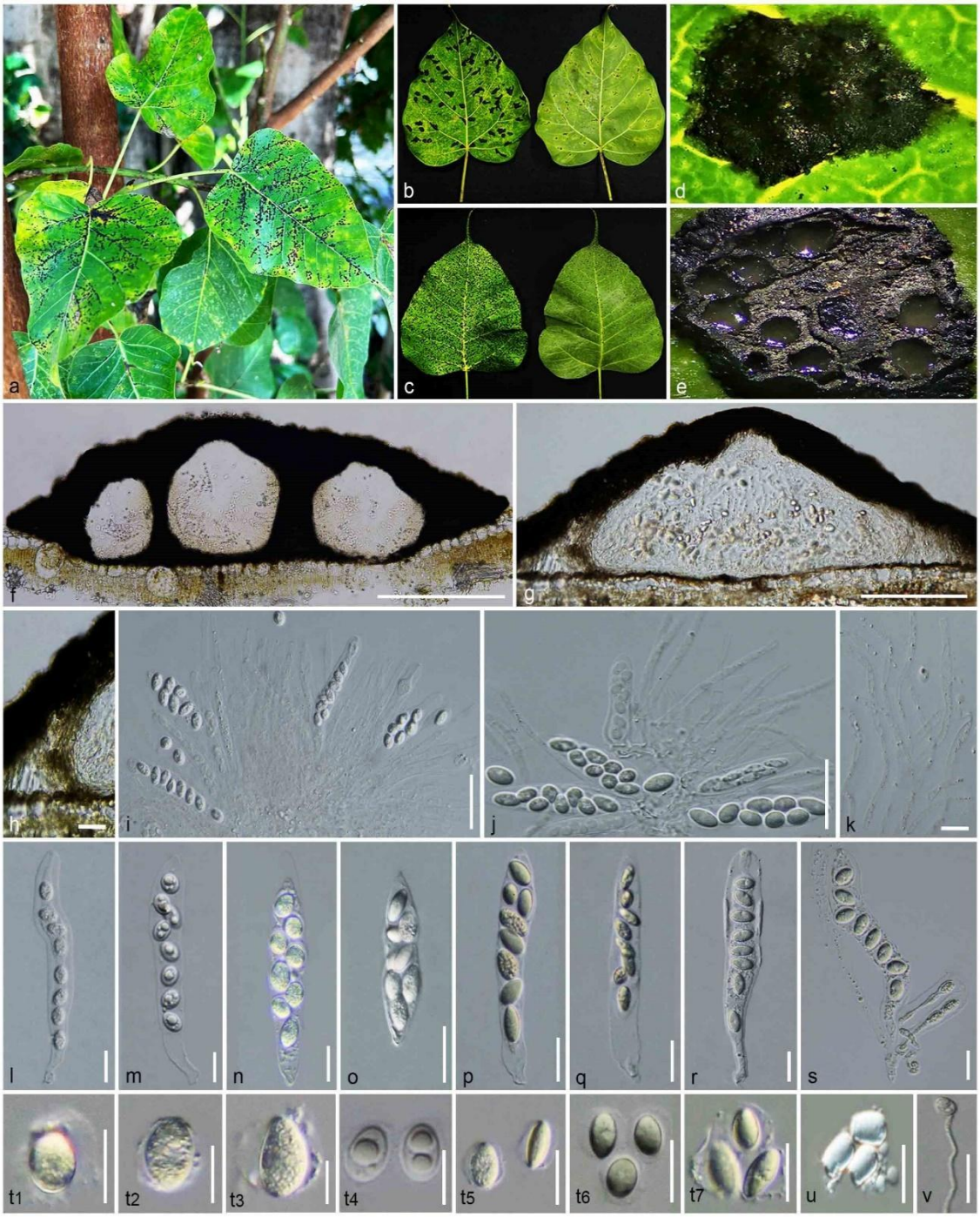

Figure 10. ***Neophyllachora*** **sp. (large and small spots)**. a. Tar spot on living leaves, b. Upper and lower leaves of with large spot, c. Upper and lower leaves of with small spot. D. Pseudostroma, e. Horizontal section through pseudostroma, f. Vertical section through pseudostroma, g. Vertical section through ascomata, h. Vertical section through peridium, i, j Asci and paraphyses. k. Paraphyses, l–s. Asci, t1–t7. Ascospores, u. Conidia, w. Germinated spores. Scale bars: f = 1000 µm, g = 100 µm, h = 100 µm, k = 5 µm, I, j, i–s= 50 µm. t1–t7, u, w= 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Mae Wang District, Wat Ampharam, Ban Kad, on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

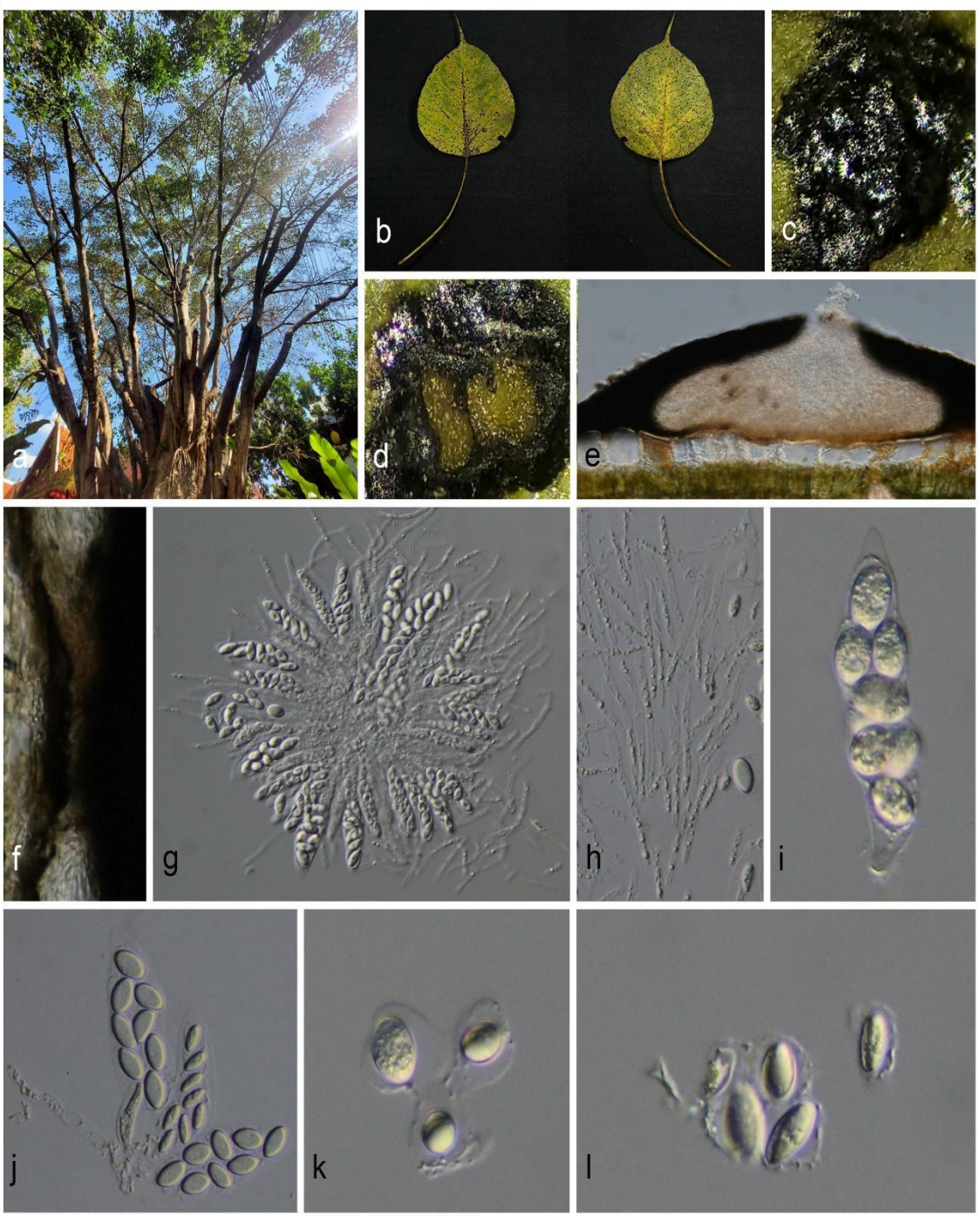

Figure 11. *Neophyllachora* sp. (sample 1). a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g, i-j = 50 µm, h = 5 µm, k-l = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Hang Dong District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

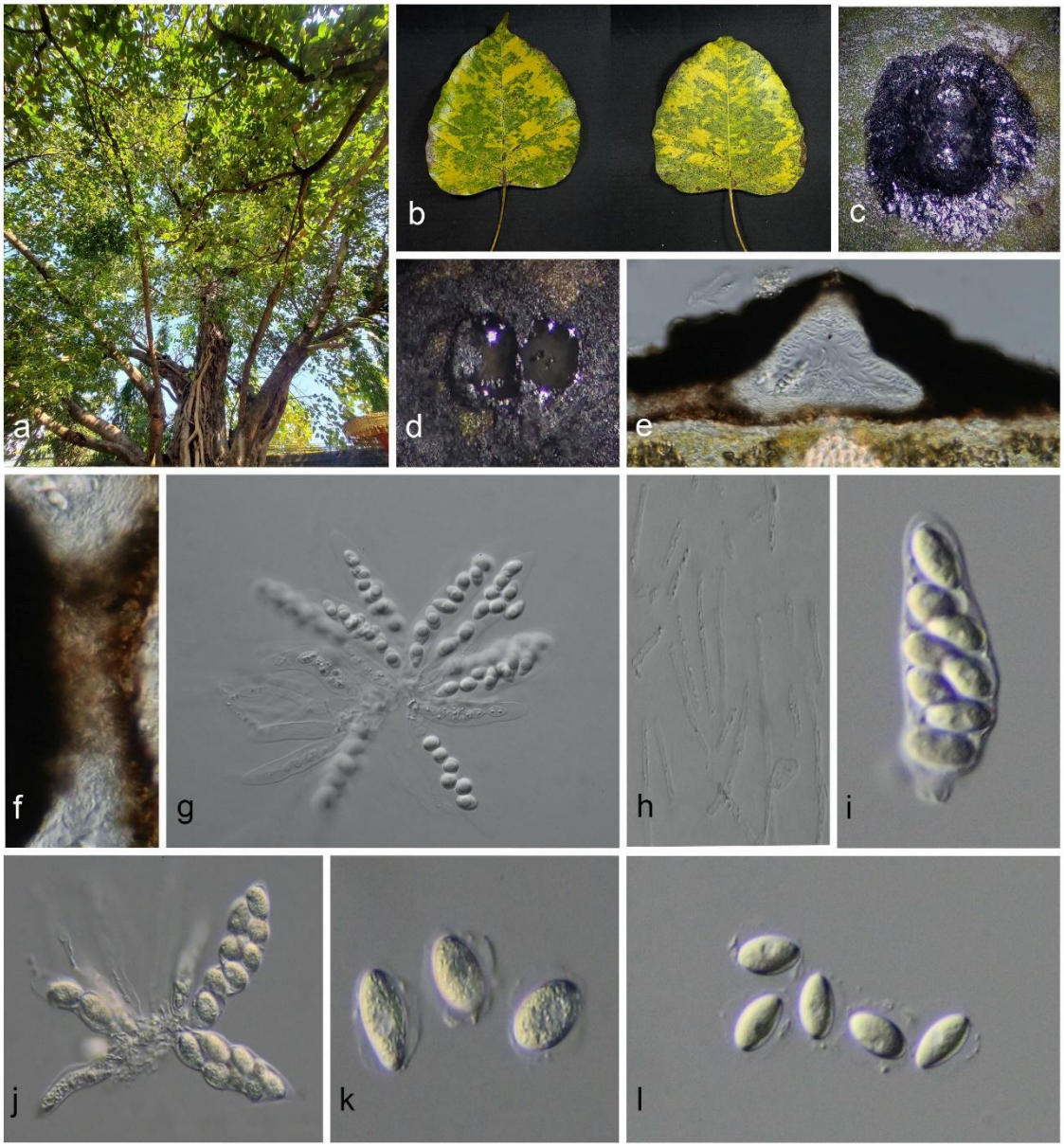

Figure 12. ***Neophyllachora*** **sp. (sample 2)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g, i-j = 50 µm, h = 5 µm, k-l = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Hang Dong District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

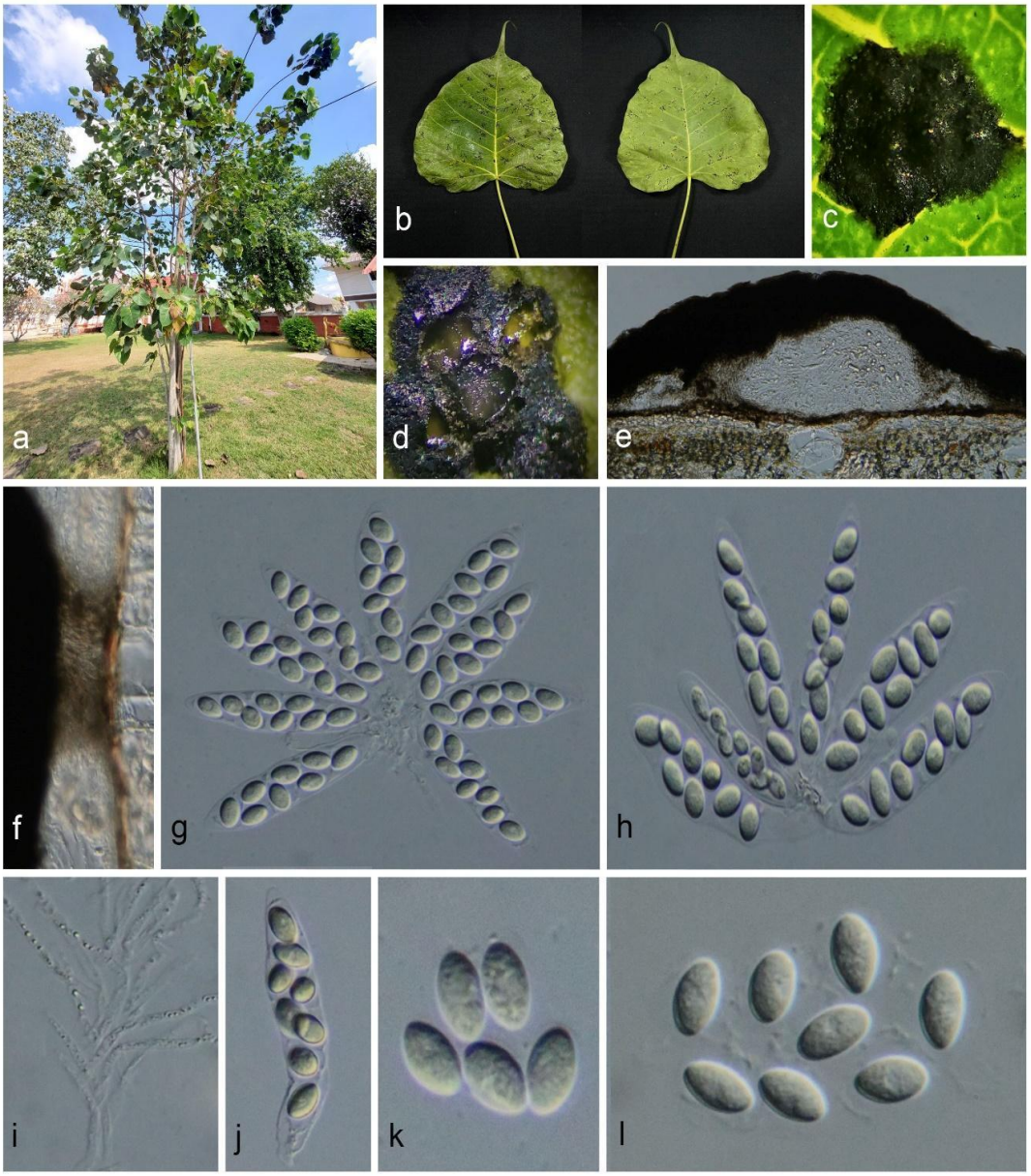

Figure 13. ***Neophyllachora*** **sp. (sample 3)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g-h, j = 50 µm, i = 5 µm, k-l = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, San Patong District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia, Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

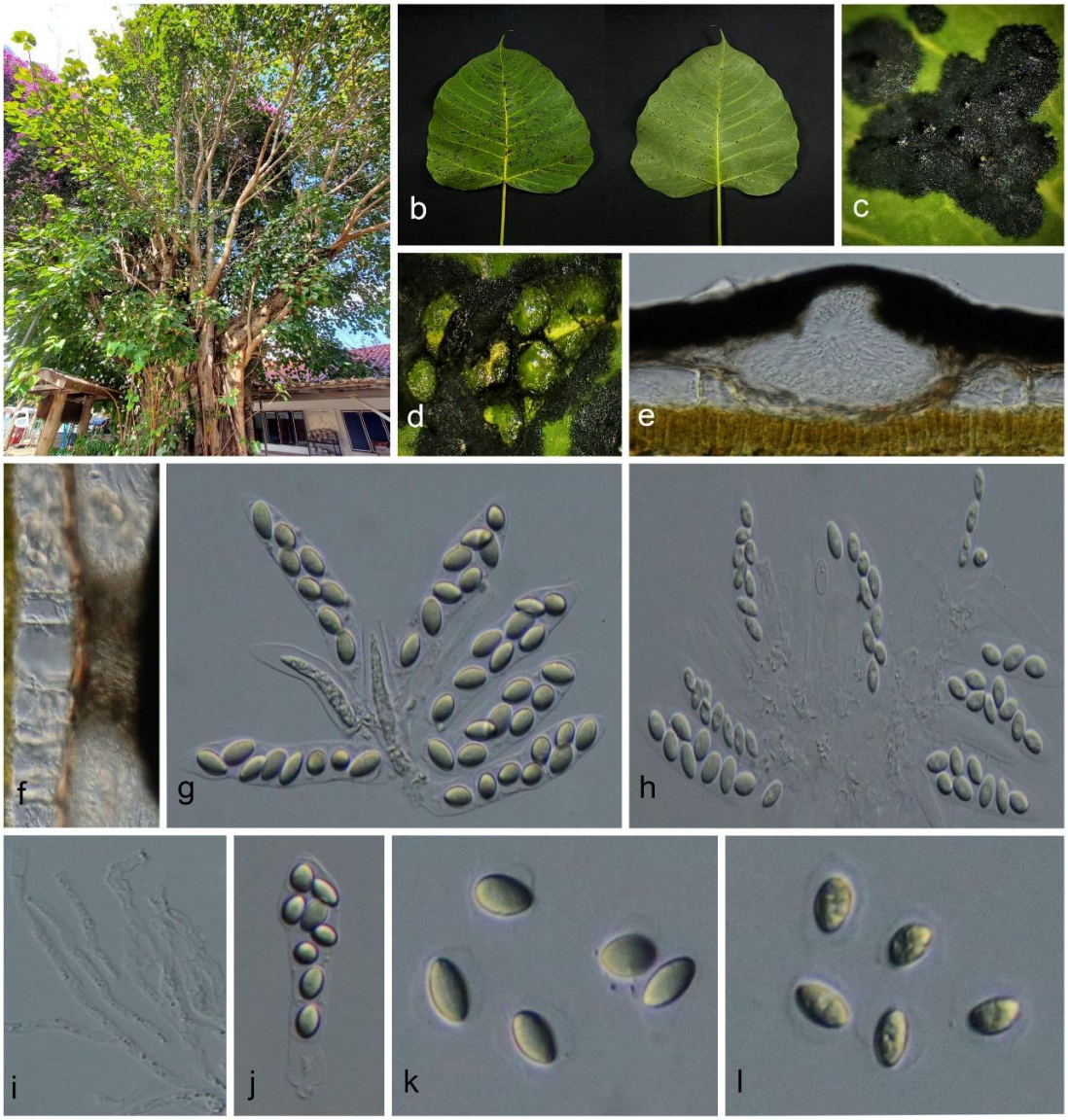

Figure 14. ***Neophyllachora*** **sp. (sample 4)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g-h, j = 50 µm, i = 5 µm, k-l = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Mae Wang District, Wat Ampharam, Ban Kad, on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

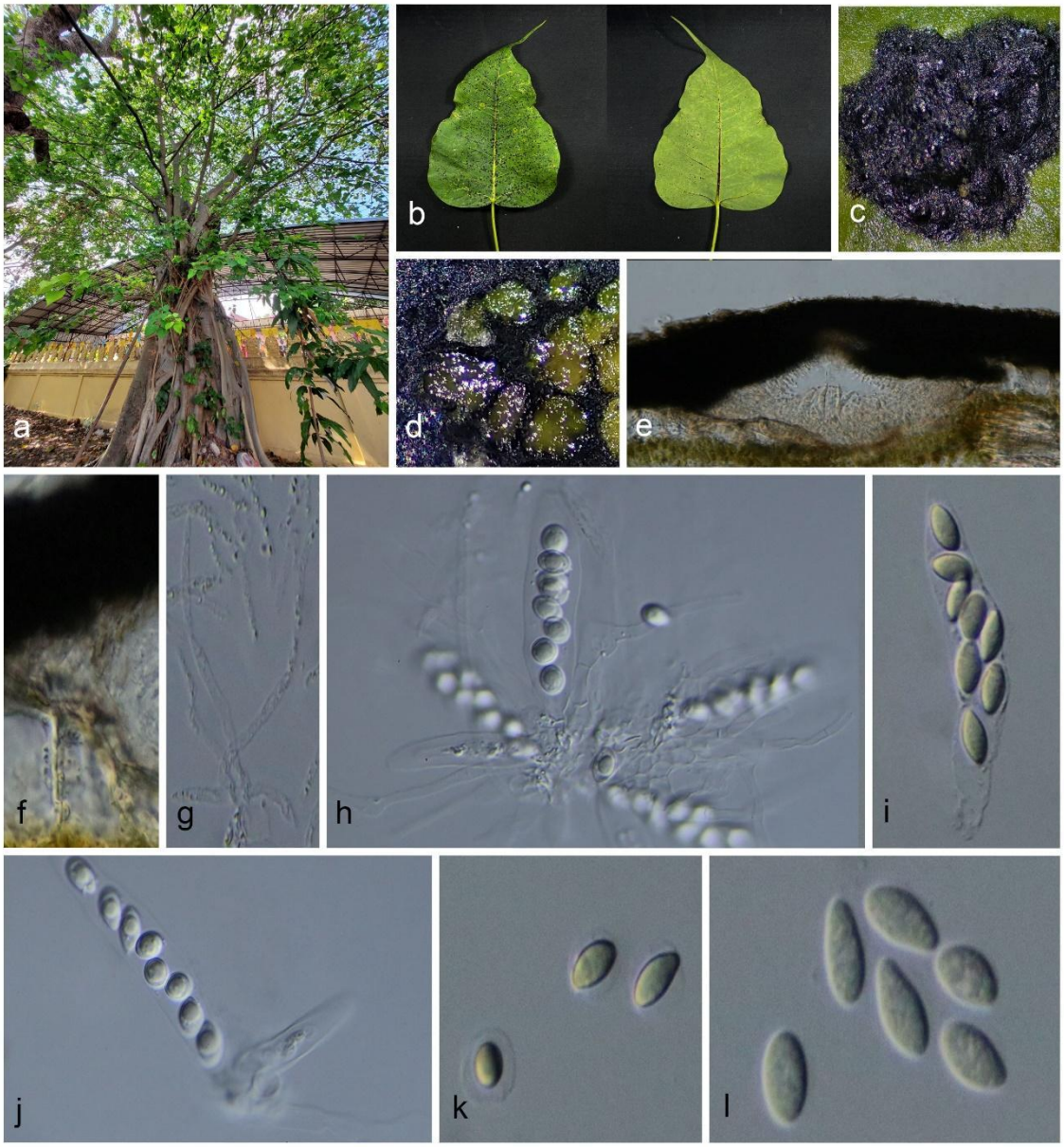

Figure 15. ***Neophyllachora*** **sp. (sample 5)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g = 5 µm, g, h-j = 50 µm k-l = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Mae Wang District, Wat Ampharam, Ban Kad, on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

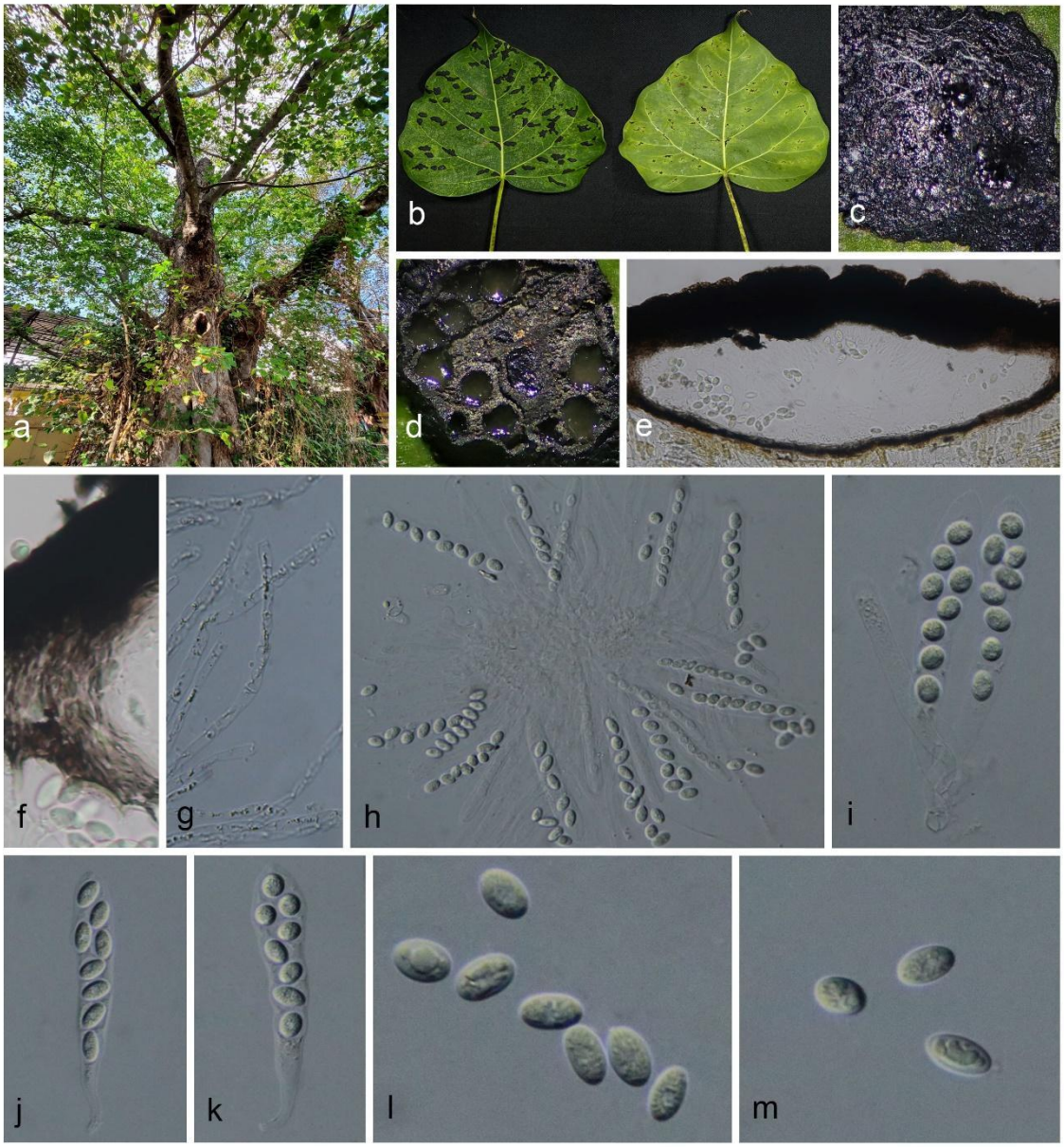

Figure 16. ***Neophyllachora*** **sp. (sample 6)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g = 5 µm, g, h-k = 50 µm, l-m = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Mae Wang District, Wat Ampharam, Ban Kad, on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

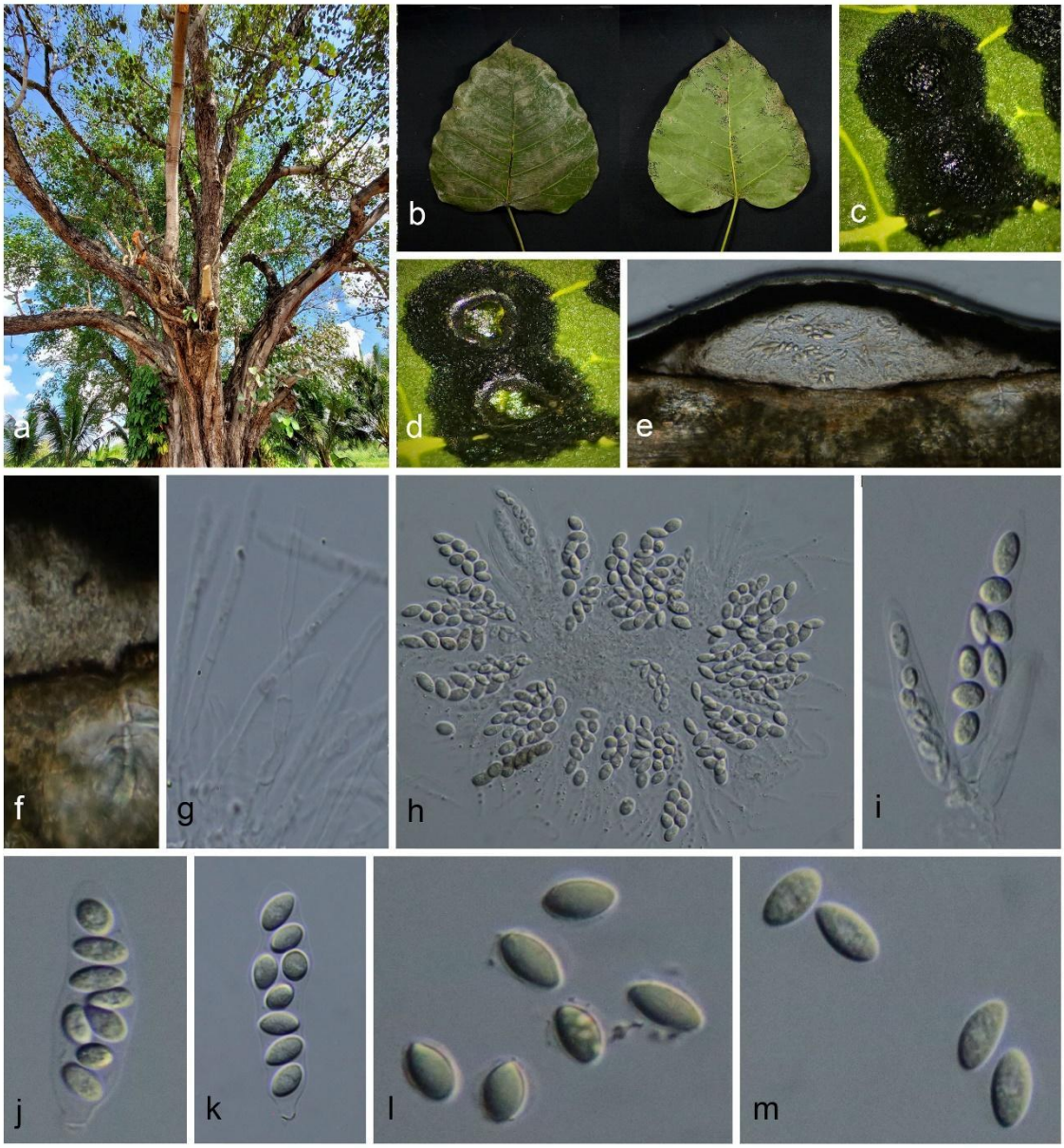

Figure 17. ***Neophyllachora*** **sp. (sample 7)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g = 5 µm, g, h-k = 50 µm, l-m = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, San Patong District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia, Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

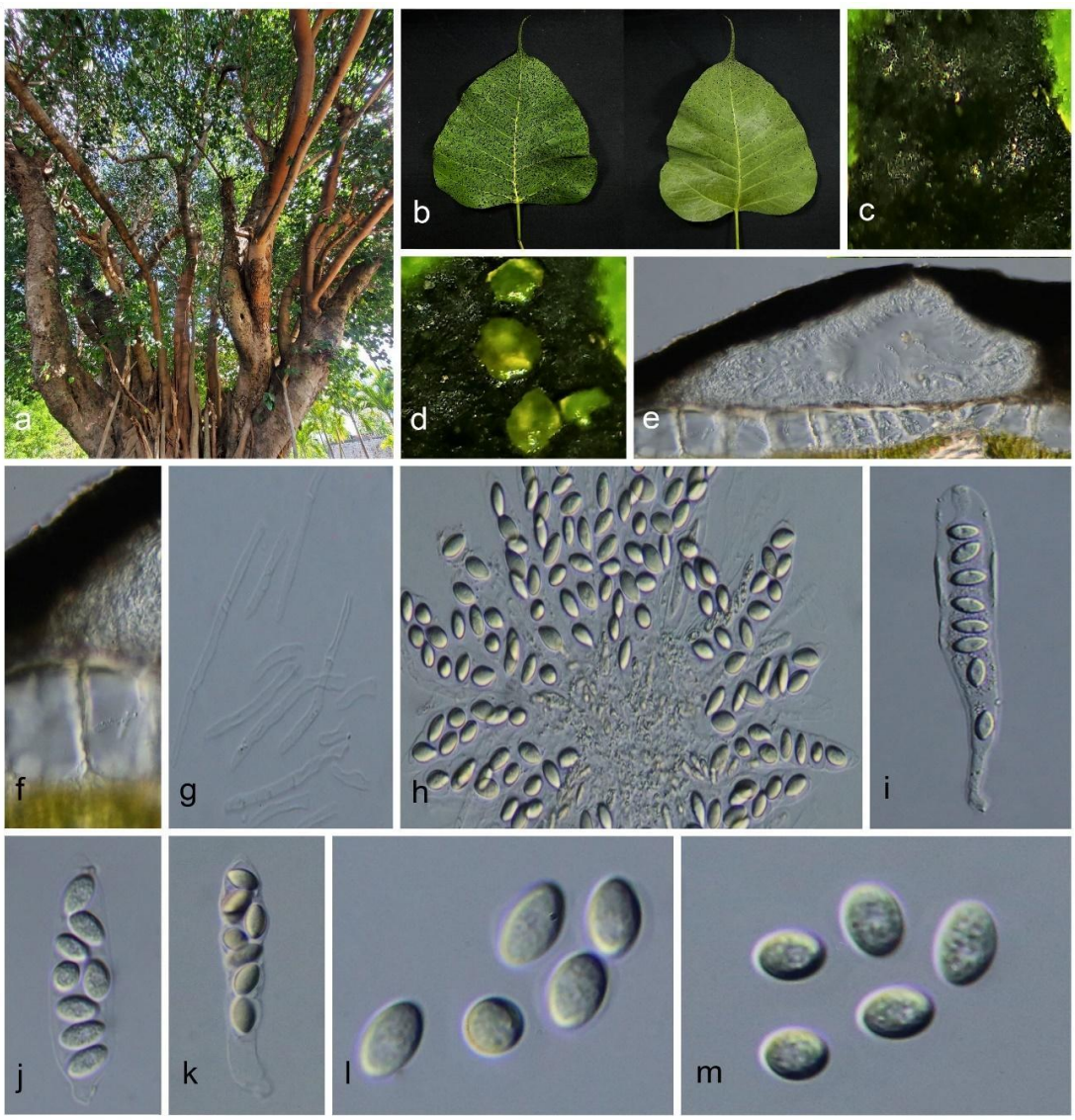

Figure 18. ***Neophyllachora*** **sp. (sample 8)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g = 5 µm, g, h-k = 50 µm, , l-m = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Hang Dong District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia, Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

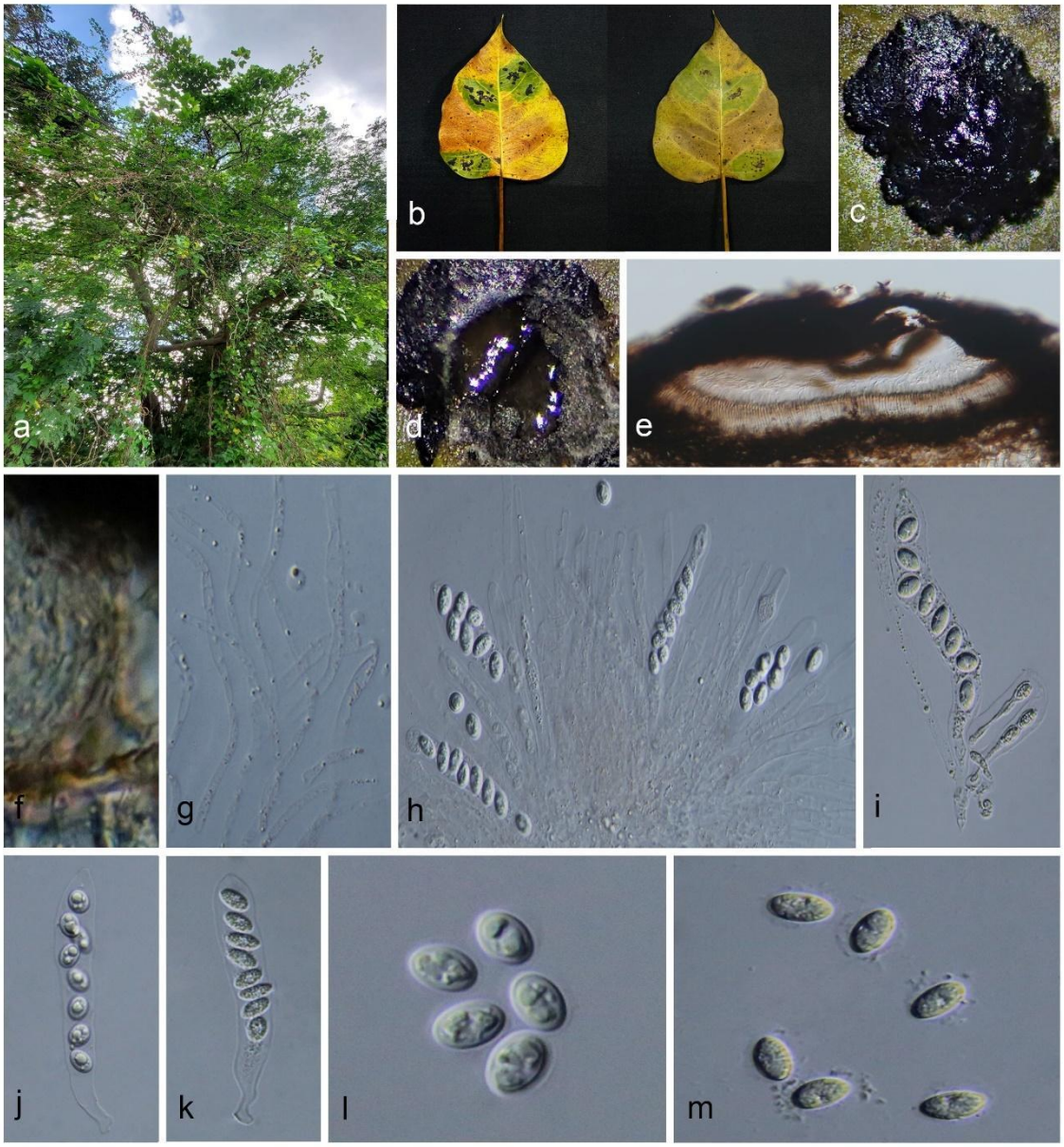

Figure 19. ***Neophyllachora*** **sp. (sample 9)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g = 5 µm, h-k = 50 µm, l-m = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness

Material examined: Thailand, Chiang Mai Province, San Sai District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia, Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

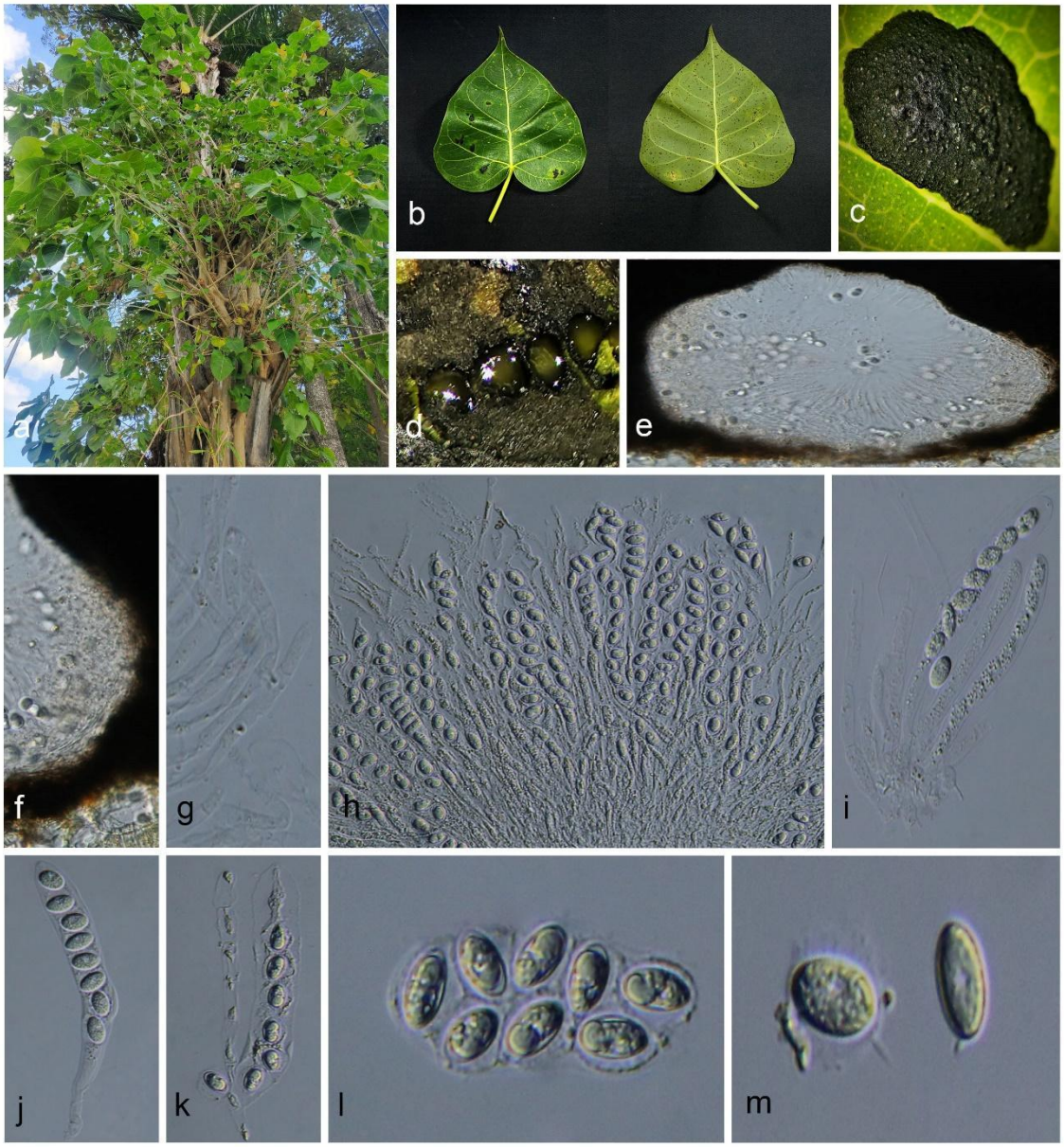

Figure 20. ***Neophyllachora*** **sp. (sample 10)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g = 5 µm, h-k = 50 µm, l-m = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, San Sai District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

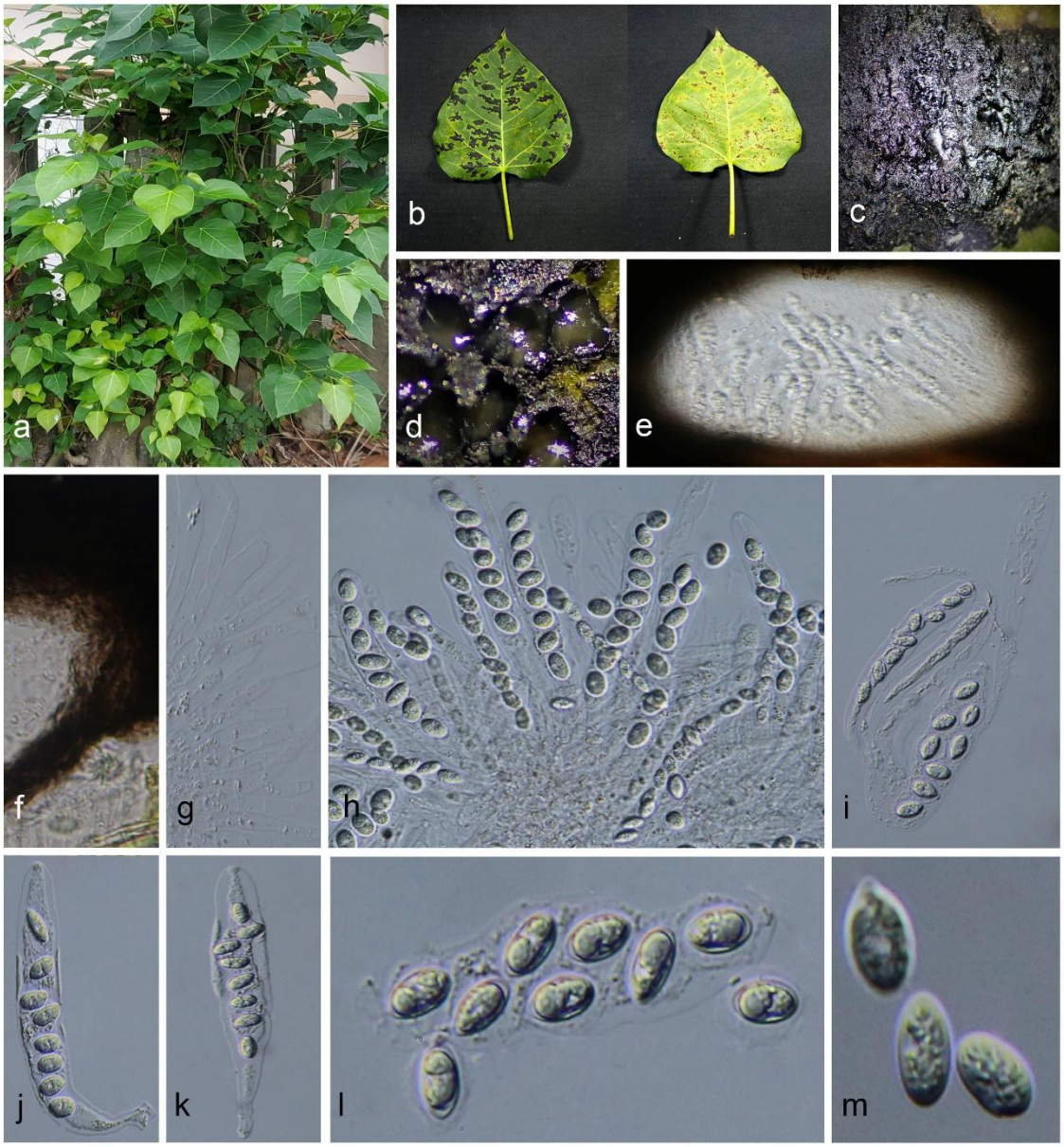

Figure 21. ***Neophyllachora*** **sp. (sample 11)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, g = 5 µm, h-k = 50 µm, , l-m = 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Mae Rim District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

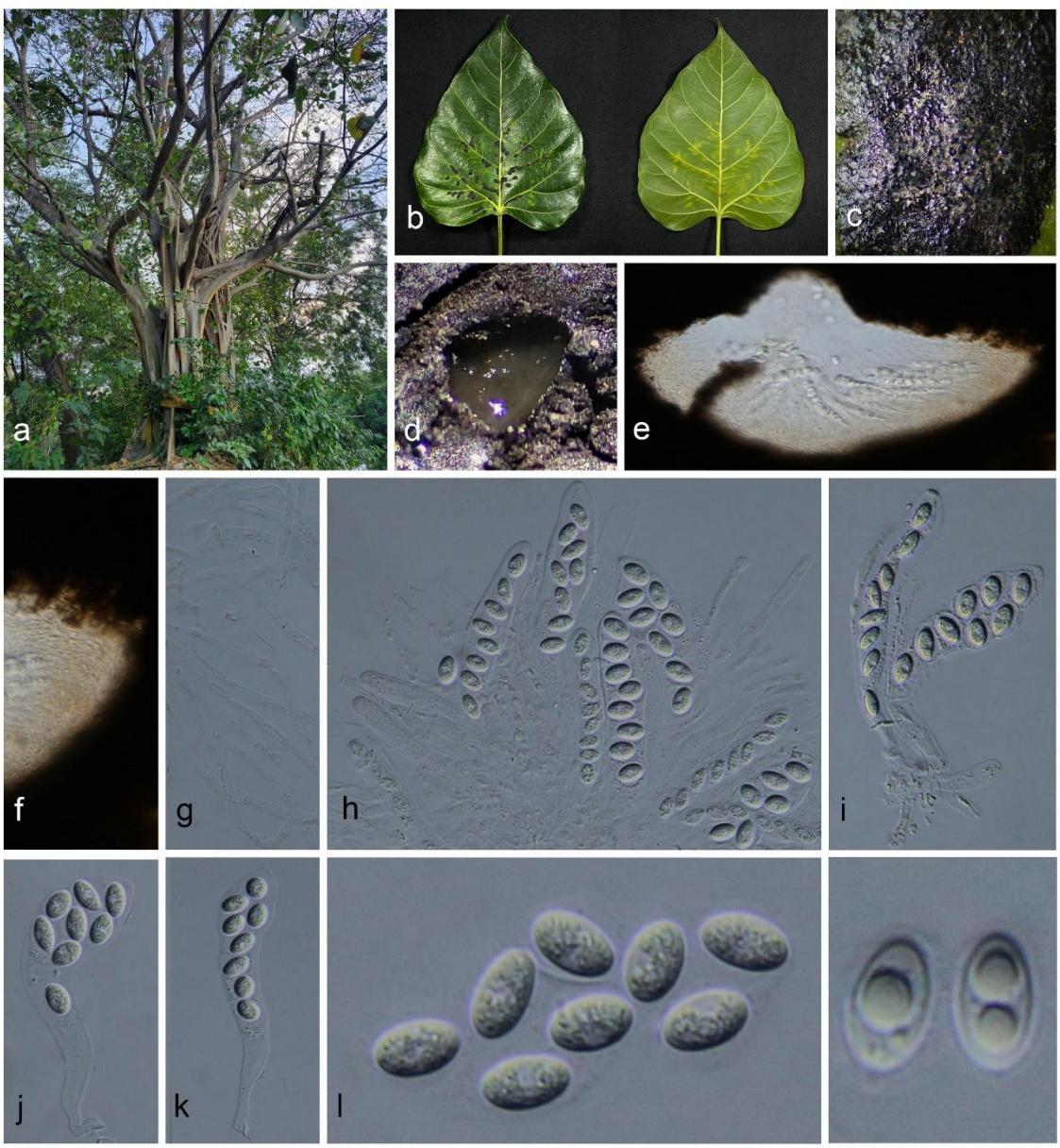

Figure 22. ***Neophyllachora*** **sp. (sample 12)**. a. Host (*Ficus religiosa)*, b. Upper and lower leaves with small spots, c. Pseudostroma, d. Horizontal section through pseudostroma, e. Vertical section through pseudostroma, f. Vertical section through peridium, g. Asci and paraphyses. h. Paraphyses, i–j. Asci, k-l. Ascospores. Scale bars: e-f = 100 µm, h = 5 µm, h-k = 50 µm, l-m= 10 µm.

Descrptions: Epiphytic on living leaves of *Ficus reliogiosa*. Tar spots appears miniscule and lustrous. *Pseudostroma* 0.1–1.5 mm diam., on upper leaves surface, black punctiform spots, intra-epidermal, scattered but sometimes gregarious, prominent, epiphyllous, moderate to highly domed. **Sexual morph**: *Ascomata* 83–172 × 127–349 µm, perithecial, globose to subglobose, solitary or aggregates, ostiolate, *Ostiole* conspicuous. *Peridium* 21–30 thick, dark brown to black, Lateral part wider than basal part, compactly arranged strongly melanized cells. *Paraphyses* 1–2 µm wide, filiform, numerous, persistent, septate, unbranched, longer than asci. *Asci* 59–140 × 14–25 ($\bar{x}$ = 88 × 20 µm, n = 30) µm, 8-spored, unitunicate, persistent, cylindrical to fusiform, short pedicellate, walls uniform in thickness but not specially thickened at the apex and without visible apical structures. *Ascospores* 8–12 × 5–9 µm ($\bar{x}$ = 13 × 7 µm, n = 30), uniseriate to biseriate, overlapping, unicellular, hyaline, globose to elliptical, 1-2 guttulates, with a central concave depression, covered by a mucilaginous sheath, irregularly thickened sheath, 1–4.5 µm thickness.

Material examined: Thailand, Chiang Mai Province, Mae Rim District on living leaves of *Ficus religiosa* (*Moraceae*), 19 November 2022.

Notes: *Neophyllachora* was originally established by Dayarathne et al. (2017) based on the phylogenetic analysis for the species that were previously placed within *Phyllachora.* The genus comprises six species with *Neophyllachora myrciae* as the type. The members are mainly reported from Brazil and Taiwan on *Myrcia*, *Myrciaria*, *Psidium* and *Ficus* species. The taxa are characterized by the formation of subepidermal, intra-epidermal stromata without a deeper invasion of the mesophyll and clavate asci and the asexual morph has been reported in several species such as *N. cerradensis*, *N. myrciae* and *N. truncatispora* in ceolomycetous states (Dos Santos et al. 2016, Dayarathne et al. 2017).

CHAPTER 5

DISCUSSION AND CONCLUSION

In our survey around Chiang Mai Province, we found small and large black spots on the leaves of Bodhi tree. We collected 12 samples (7 small black spots and 5 large black spots) from seven different locations (Figure 1). Based on our observation, those leaf samples with large spots were found from strangler trees. Strangling is one of the characteristics of the tropical figs of the genus *Ficus* in the family *Moraceae*. This growth pattern upon host trees, which often results in the host's death, is common in tropical forests worldwide (Britannica, 2019). Contrariwise, leaf samples with small spots were collected from non-strangler trees. However, the large spots are found to have craggy bulging structures and mostly have irregular sizes.

In this study, we attempted to isolate the fungus by transferring the newly germinated spores to PDA and WA plates which were then incubated at room temperature. However, there was no sign of any fungal growth after 24 h, 48 h, and 72 h of incubation. We selected 8 samples for morpho-phylogenetic studies; 4 with small black spots and 4 with large black spots. The former showed the same morphological characteristics of the black spot in having black, abundant, scattered, raised, mostly rounded to oblong or elongated, epiphyllous and shiny, superficial pseudostromata, which is sometimes parallel with the leaf venation. The latter almost have the same morphological characteristics of the black spot as the former, except that they are bulky, bristly, and bigger in size. Spermatia was produced in spermogonia and was grown with ascomata in the same pseudostromata. Spermatia has also has been reported in several species of *Phyllachoraceae* including *Parberya arxii* (P.F. Cannon) C.A. Pearce & K.D. Hyde. (Pearce and Hyde 2001), *Phyllachora maydis* Maubl. (Monteiro *et al*. 2013) and *Phyllachora heterocladae* C.L. Yang, X.L. Xu & K.D. Hyde. (Yang *et al*. 2019). Pearce and Hyde (2001) reported filiform spemartia produced in Spermatogonia in *Parberya arxii* that was originally placed in *Sphaerodothis* (Sacc. & P. Syd.) Shear and referred these structures as andromorph. Previously, Hyde and Cannon (1999) described an

anamorphic state of *Sphaerodothis arengae* (Racib.) Shear ex Theiss. & Syd., which included ellipsoidal α-conidia and filiform β-conidia. However, the filiform spermatia found in *P. arxii* differed from *S. arengae* but are similar to those found in many *Phyllachora* species, particularly those taxa on grasses. In Australian *Phyllachora* collections spermatogonia developed either within the stromatic development of the teleomorph, in small irregular shaped locules at the side or above the developing ascomata mainly on moraceous hosts (Pearce and Hyde 2006). Evidently, our species also reported from a moraceous host and the spermatial locule lies aside the developing ascomata. *Neophyllachora myrciae* also shows sexual, spermacial and conidial structures and the species was originally introduced as *Dothidea myrciae* Lév. However, the author mentioned both spermacial and conidial structures as asexual states (dos Santos et al. 2017). Nonetheless, the relationship between spermatial state and telemorphic state remains unresolved (Cannon 1991). Moreover, we are also unable to make a conclusion about the lifecycle of the new species as the sampling was done only for a particular period in both young and mature trees. Thus, additional taxon sampling with detailed study of each stage of disease symptoms are needed for further investigation.

All samples were stained with a drop of Melzer's reagent, and the asci were confirmed to be J- as they all did not stain blue. The new species shows close phylogenetic association to *N. fici* and both species are reported in the same host genus (*Ficus*) but with different locality (Thailand Vs. Taiwan). Morphologically, our new species differs from *N. fici* in the size of asci (55–185 × 11–26 vs. 90–100 × 15–19 µm) and the thickness of the peridium (15–40 vs. 20–25 µm), and mainly differs in the ascospore characteristics. Our new species possesses ascospores which covered with thick gelatinous sheath (1–4.5 µm) whereas *N. fici* lacks sheath in the ascospores. Our new species further differs from the type *N. myrciae* in the smaller pseudostromata (2–3 vs. 3–6 µm), thinner paraphyses (1.5–2.5 vs. 2.5–4.5 µm), shape of asci (fusoid vs. cylindrical to fusiform), shape of ascospores (lunate vs. globose to elliptical) and the presence of sheath in the ascospores, arrangement of ascospores (1-2 seriate vs. biseriate to multiseriate), color of ascospores (hyaline to olivaceous vs. hyaline), host (*Ficus religiosa* vs. *Myrcia* sp.) and the distribution (Thailand vs. Brazil) but shares similar characteristics in the size of ascomata, asci and coelomycetes asexual state. *N. thailandica* shares similar ascospores characteristics to *N. cerradensis* in elliptic-oblong

shape ascospores with gelatinous sheath. However, our new species differs in the size of ascospores (8–15 × 5–12 vs. 15–22 × 6–9 µm), thickened gelatinous sheath with hyaline to olivaceous color ascospores while *N. cerradensis* has thin walled and hyaline ascospores (Table 4). In summary, our new species mainly differs from the extant species in the ascospores characteristics which has thick gelatinous sheath that are irregularly thickened, irregularly guttulate at the immature stage and a large gattulate present in the maturity, and the shape ranges from globose to elliptical and a central concave depression present in the globose shaped ascospores.

Table 4. Synopsis of *Neophyllachora* species

	***Neophyllachora* species**						
Morphological characters	***N. cerradensis***	***N. fici***	***N. myrciae***	***N. myrciariae***	***N. subcircinans***	***N. truncatispora***	***N. religiosa***
Pseudostromata (diam) (mm)	2–4	2–3	3–6	0.5–1.5	1–4	2–5	2–3
Shape of Ascomata	Ampulliform to Globose	Globose to Subglobose	Globose to Ampulliform	Ampulliform	Ampulliform	Globose to Ovoid	Globose to Subglobose
Position of Ascomata	Occasionally coalescing	Epiphyllous	Occasionally coalescent	Immersed in the pseudostromata	Immersed in the pseudostromatic tissue	Immersed in pseudostroma	Epiphyllous
Peridium thickness (µm)	17–21	20–25	-	19–24	15–21	-	15–40
Size of Ascomata (µm)	260–362 × 168–264	150–300 × 200–400	205–485 × 148–207	161–432 × 126–267	329–417 × 193–275	160–200 × 100–350	150–300 × 200–400
Size of Asci (µm)	69–92 × 17–28	90–100 × 15–19	89–117 × 13–19	63–90 × 11–15	73–100 × 12–19	69–117 × 14–25	55–185 × 11–26
Shape of Asci	Fusoid	Cylindrical to Fusiform	Fusoid	Clavate-fusoid	Cylindrical	-	Cylindrical to Fusiform
Width of Paraphyses (µm)	1.6–3	1.5–2.5	2.5–4.5	2–3	2–3.5 µm	2.5–5	1.5–2.5
Septate of Paraphyses	Septate	Aseptate	Septate	Septate	Septate	Septate	Septate
Size of Ascospores (µm)	15–22 × 6–9	12–13 × 10–11	14–18 × 5–7	14–19 × 5–8	11–16 × 7–9	18–26 × 7–8	8–15 × 5–12
Shape of Ascospores	Elliptic-Oblong	Globose to Subglobose	Lunate	Elliptical	Oblong to ellipsoid	Sublunate to fusoid	Globose to elliptical
Color of Ascospores	Hyaline	Hyaline	Hyaline	Hyaline	hyaline to light olivaceous	Hyaline	Hyaline to Olivaceous
Ascospores arrangement	Biseriate	1–2 seriate	Biseriate to multi-seriate	Obliquely biseriate	Mostly uniseriate, sometimes with biseriate	-	Mostly uniseriate, sometimes with biseriate

Table 4. (continued)

***Neophyllachora* species**							
Morphological characters	***N. cerradensis***	***N. fici***	***N. myrciae***	***N. myrciariae***	***N. subcircinans***	***N. truncatispora***	***N. religiosa***
Ascospores wall	Covered by a thin gelatinous sheath	Absent	Thin- walled	Covered by a thin gelatinous sheath	Thin wall surrounded by a gelatinous sheath	Wall thickenings at both acute ends	Covered by a thick gelatinous sheath
Gattules	microgattulate cytoplasm	-	-	Irregularly guttulate	Centrally gattulate	-	Irregularly gattulate
Asexual morph	Coelomycete	Unknown	Coelomycete	Unknown	Unknown	Coelomycete	Coelomycete
Host	Leaves of *Myrcia torta*	Leaves of *Ficus septica*	*Myrcia* sp.	Leaves of *Myrciaria delicatula*	*Psidium* sp.	Leaves of *Myrcia camapuanensis*	Leaves of *Ficus religiosa*
Distribution	Brazil	Taiwan	Brazil	Brazil	Brazil	Brazil	Thailand
References	Dos Santos *et al*. 2016	Tennakoon *et al*. 2021	Dos Santos *et al*. 2016	Dos Santos *et al*. 2016	Dos Santos *et al*. 2016	Dos Santos *et al*. 2016	**This study**

Key to species of *Neophyllachora*

1. Parasitic on *Ficus*, *Myrcia*, and *Myrciaria* species……..…….2

1’. Parasitic on *Psidium* species, ascospores thin-walled, short-ellipsoidal covered with thin-walled gelatinous sheath…….……*N. subcircinans*

2. Parasitic on *Ficus*, *Myrcia* species……..…….…….…..3

2’. Parasitic on *Myrciaria* species, clavate-fusoid asci…..……...……..…. *N. myrciariae*

3. Parasitic on *Myrcia* species, fusoid asci, ascospores covered with gelatinous sheath……..4

3’. Parasitic on *Myrcia* species, lunate-reniform to half-moon shape ascospores with thick walled……..…….*N. truncatispora*

4. Elliptic-Oblong ascospores with microguttulate cytoplasm…….. *N. cerradensis*

4’. Lunate ascospores with thin-walled……..…….…… *N. myrciae*

5. Parasitic on *Ficus*, globose to ellipsoidal ascospores with gelatinous sheath ……..……..……*Neophyllachora* sp.

5’. Parasitic on *Ficus*, globose to subglobose ascospores without gelatinous sheath …….…….…….…....*N. fici*

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