Taxonomy and ecology of epifoliar fungi

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Abstract
Epifoliar fungi are poorly studied symbionts that co-inhabit the surface of living plants. They are relatively understudied and generally lack molecular data thus there is considerable taxonomic confusion in the group as early taxonomic studies were based on morphology. Many taxa are difficult to isolate for obtaining cultures and therefore molecular analysis is a limitation for biotrophic species unless sequenced directly from the fruiting bodies. Epifoliar fungi evolved from diverse ancestors and include mainly members of the Dothideomycetes, Eurotiomycetes, Lecanoromycetes, and Sordariomycetes. The classification of epifoliar fungi is challenging due to taxonomic confusion in historical classifications and insufficient molecular data. In this study, we provide a summary of major epifoliar families (Asterinaceae, Meliolaceae, Micropeltidaceae, Microthyriaceae, Parmulariaceae and Zeloasperisporiaceae). The modes of nutrition of each family are also reviewed. Character analysis of a combined LSU, SSU and rpb2 dataset shows that epifoliar fungi have different taxonomic and evolutionary relationships in Ascomycota. Epifoliar fungi are generally considered to be host-specific, but this needs to be confirmed using molecular data as morphological differences are minor. Therefore, future research should focus on addressing the drawbacks of current studies and use new molecular approaches. To obtain better insights into epifoliar fungi, a combination of taxonomic and ecological studies is needed.

Keywords – Ascomycota – Character analysis – Epiphytes – Nutrition – Symbionts

Introduction
Epifoliar fungi live on the surface of living plants and are widely distributed, but are most common in tropical and subtropical areas worldwide (Schoch et al. 2009, Wu et al. 2011, Hyde et al. 2013, Hongsanan et al. 2014, Li et al. 2016). Mycologists have defined epifoliar fungi as a community on the leaf surface sharing similar ecological niches and used the term nutrition guilds (Gilbert & Reynolds 2002, 2005). Epifoliar fungi are considered functionally commensal with their host plants, without causing disease (Anthony et al. 2002, Gilbert et al. 2007).
Some biotrophic and saprobic species cause economical damage to their hosts, especially some species in Asterinaceae, Capnodiaceae, Lembosiaceae, and Meliolaceae (Chomnunti et al. 2014, Hongsanan et al. 2016, Zeng et al. 2017, 2020, Gleason et al. 2019). Their blackened mycelium covers the leaf surfaces and reduces the exposure area for sunlight (Hongsanan et al. 2016). This phenomenon causes a reduction of yield and marketable price of economically valuable crops due to the low level of photosynthesis and the unattractive appearance (Stover 1975, Chomnunti et al. 2014, Hongsanan et al. 2015b). Some species in Schizothyriaceae produce blemishes on fruits (apples and pears) by reducing their marketable price (Williamson & Sutton 2000, Batzer et al. 2002, 2016, Gleason et al. 2019).

Epifoliar fungi are highly diverse in their mode of nutrition and have various morphological adaptations. Therefore, they require the surface of living plants according to their host preferences to complete their entire life cycle (Gilbert & Reynolds 2005, Gilbert et al. 2007). However, the competition for limited nutrient conditions and prolonged exposure to ultraviolet radiation are assumed to be some causes for sharing key ecological traits, even though they are polyphyletic (Zeng et al. 2020). Studies have addressed the evolutionary history of epifoliar fungi along with their ecological shifts between other related fungal taxa (Ismail et al. 2016). Epifoliar fungi have evolved with several ecological guilds as saprobes, obligate parasites or commensals on plants.

There has been increased interest in the morphological and ecological evolutionary analysis of epifoliar fungi. Ancestral state reconstruction is one of the methods commonly used by researchers which provide information of ancestors regarding a particular character (Ismail et al. 2016). There are few studies regarding character analysis of epifoliar fungi and most of them focused on flyspeck fungi and Capnodiales (Ismail et al. 2016, Abdollahzadeh et al. 2020, Renard et al. 2020). Outcomes of these studies help to enhance the knowledge of the evolution and adaptation of other microbial communities that exist on plant surfaces. Epifoliar fungi are also a good indicator of plant health as this fungal group is more sensitive to microclimatic changes (Gilbert et al. 1997).

In this study, we selected six epifoliar fungal families, which belong to black moulds and flyspeck group with diverse life modes which are Asterinaeae, Lembosiaceae, Microthyriaceae and Parmulariaceae in Dothideomycetes, Meliolaceae in Sordariomycetes, and Micropeltidaceae in Lecanoromycetes (Table 1). We summarize the important morphological characters of these families and also review their ecological adaptations in terms of nutrient uptake processes, with the ancestral character analysis using Bayesian MCMC analysis.

Table 1 Families and genera in this study (Treated from Hongsanan et al. 2020).

| Class            | Order    | Family   | Genera                  |
|------------------|----------|----------|-------------------------|
| Sordariomycetes  | Meliolales| Meliolaceae| Amazonia, Asteridiella, Cryptomeliola, Endomeliola, Irenopsis, Meliola, Setameliola |
| Dothideomycetes  | Asterinales| Asterinaceae| Asterina, Asterolibertia, Asterostomella, Batistinula, Cirrosia, Dothidasteromella, Echidnodella |
|                  |          |          | Halbania, Meliolaster, Parasterinopsis, Platypeltella, Prillieuxina |
| Class         | Order          | Family       | Genera                                      |
|--------------|----------------|--------------|---------------------------------------------|
|              |                | Lembosiaceae | Pycnocarpon                                 |
|              |                |              | Schenckiella                                |
|              |                |              | Trichasterina                               |
|              |                |              | Trichopeltospora                            |
|              |                |              | Uleothyrium                                 |
|              |                |              | Vizellopsis                                 |
|              |                |              | Lembosia                                    |
| Microthyriales | Microthyriaceae |              | Arnaudiella                                 |
|              |                |              | Asterinella                                 |
|              |                |              | Calothyriopsis                              |
|              |                |              | Chaetothyriothecium                         |
|              |                |              | Hamatispora                                 |
|              |                |              | Microthyrium                                |
|              |                |              | Neoanungitea                                |
|              |                |              | Paramicrothyrium                            |
|              |                |              | Pseudomicrothyrium                          |
|              |                |              | Pseudopenidiella                            |
|              |                |              | Seynesiella                                 |
|              |                |              | Tumidispora                                 |
| Microthyriales | Microthyriaceae |              | Helioccephala                               |
|              |                |              | Mitopeltis                                  |
|              |                |              | Neoscoleobasidium                           |
|              |                |              | Parazalerion                                |
|              |                |              | Thyriodictyella                             |
|              |                |              | Tothia                                      |
| Parmulariales | Parmulariaceae  |              | Aldona                                      |
|              |                |              | Aldonata                                     |
|              |                |              | Antonioniymces                              |
|              |                |              | Aulacostroma                                |
|              |                |              | Campoa                                      |
|              |                |              | Cirsiopsis                                  |
|              |                |              | Cocconia                                    |
|              |                |              | Cycloschizon                                |
|              |                |              | Cyclostomella                               |
|              |                |              | Dothidasteroma                              |
|              |                |              | Ferrarisia                                  |
|              |                |              | Hysterostomella                             |
|              |                |              | Kiehliya                                    |
|              |                |              | Mintera                                     |
|              |                |              | Pachypatella                                |
|              |                |              | Palawaniella                                |
|              |                |              | Parmularia                                  |
| Parmulariopsella | Parmulariopsell |              | Parmulariopsella                            |
|              |                |              | Parmalina                                    |
|              |                |              | Placoasterella                              |
|              |                |              | Placomasa                                   |
|              |                |              | Placostromella                              |
|              |                |              | Pleiostomellina                             |
|              |                |              | Polycyclina                                 |
|              |                |              | Polycyclus                                  |
|              |                |              | Prothyrium                                  |
|              |                |              | Pseudolembosia                              |
|              |                |              | Rhagadolobiopsis                            |
|              |                |              | Rhagadolobium                               |
|              |                |              | Rhipidocarpom                               |
Table 1 Continued.

| Class             | Order              | Family                    | Genera                  |
|-------------------|--------------------|---------------------------|-------------------------|
|                   |                    | Zeloasperisoriales        | Zeloasperisporiales     |
|                   |                    | Zeloasperisporiaceae      | Zeloasperisporium       |
| Lecanoromycetes   | Micropeltidales    | Micropeltidaceae          | Diptyopeltella          |
|                   |                    |                           | Dichtothyriella         |
|                   |                    |                           | Dichtothyria           |
|                   |                    |                           | Dichtothyrium          |
|                   |                    |                           | Haplopletheca          |
|                   |                    |                           | Micropeltis            |
|                   |                    |                           | Scolecopeltidium       |
|                   |                    |                           | Stomiothecatopsis      |

Materials & Methods

Morphological studies

Fresh and herbarium leaf specimens were observed by using a Motic SMZ 168 series microscope. Leaf sections were rehydrated in water or 5% KOH and mounted separately in ammoniacal Congo Red, Cotton Blue and Melzer’s reagent. (Senanayake et al. 2020). The micro morphologies were examined using a Nikon ECLIPSE 80i compound microscope, scanning electron microscope and photographed using a Canon 750D digital camera fitted to the microscope. Tarosoft (R) Image Framework program and Adobe Illustrator CS3 (Adobe Systems, USA) were used for figures processing and measurements.

Character state analysis

A data matrix (LSU, SSU and rpb2 sequence data) containing 182 taxa were generated in this study. Multiple sequence alignments for each gene (LSU, SSU and rpb2) were generated with MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) and manually adjusted in BioEdit v. 7.0.4 (Hall 1999) where necessary. Incomplete portions at the ends of the sequences were excluded from the analyses. The individual datasets were concatenated into a combined dataset using FaxBox (1.41) (Villesen 2007, Ekanayaka et al. 2017). Ambiguously aligned regions were excluded and gaps were treated as missing data. Maximum likelihood analysis was performed in CIPRES webportal (Miller et al. 2010) using RAxML-HPC2 Workflow on XSEDE (8.2.9) tool (Stamatakis 2006). The bootstrap analysis for each ML tree was performed with 1000 thorough bootstrap replicates with the same parameter settings using the GTR+G+I substitution model selected by MrModel Test 2.2 (Nylander 2004).

The bipartition tree file resulting from ML analysis was exported to RASP 3.2.1. Each terminal in the tree was coded for either obligate parasitic (A), saprophytic (B), lichenocolous (C) and commensal (D). BayesTraits tree was performed and visualized in RASP 3.2.1 using default settings as follows: 1,010,000 iterations for BayesTraits with a burn-in of 10,000, sampling 1000 trees and with 10 ML trees; 50,000 generations for Bayesian Binary MCMC, with 10 chains, a sampling frequency of 100, a temperature of 0.1, state frequencies fixed (JC), and among-site rate variation equal (Yu et al. 2015, Thiyagaraja et al. 2020).

Results

Character analysis of ecological niches in epifoliar fungi

Character analysis based on DNA sequence data of LSU, SSU and rpb2 shows the possible ancestral ranges of nutrition modes for Asterinales, Meliolales, Micropeltidales, Microthyriales, Parmulariales and Zeloasperisorialens (Fig. 1). The Bayesian MCC analysis suggests three possible
ancestral ranges of nutrition modes (saprophytic (B), saprophytic+parasitic (AB) and parasitic (A)) for node 2. However, the occurrence of saprophytic nutrition mode is more prominent with a 74% Bayesian support than AB (15%) and A characters (11%). The most recent common ancestor of Meliolales (Node 1) shows two possible ranges (A and AB) with the occurrence of 90% and 10% respectively. According to the literature, some Meliolinutes fossils (i.e., Meliolinutes anfractus and Meliolinutes buxi) have hyphopodiate hyphae, suggesting a parasitic nature during the Eocene to Miocene periods (Samarakoon et al. 2019). Therefore, we can assume that Meliolales has convergent evolution sharing the common ancestor of other species in Sordariomycetes. It is also assumed that host abundance, physical environment, geographic barriers to fungal dispersal, the genetic variability of the host or fungal populations may affect the geographic distribution and the ecological outcome of plant-fungal symbioses (Zeng et al. 2020).

Species of Micropeltidaceae are the only epifoliar fungal group placed in Lecanoromycetes. Zeng et al. (2019) observed that these species have a relationship with Cyanobacteria sp. This study provided evidence for the nutrition mode of Micropeltidaceae and its evolutionary position in Lecanoromycetes. The most common ancestor of Micropeltidaceae is represented by node 3, which indicates the possible ancestral nutrition mode as a parasitic (A) state with the occurrence of 90% and 10% of undetermined (F) state. Lecanoromycetes species have different types of nutrition modes which have evolved from time to time. Zeng et al. (2019) concluded that this family co-evolved with the diversification of Angiospermae in the Cretaceous period (Zeng et al. 2019). Therefore, we suggest that the ecological factors and their relationship with other environmental factors are important in the evolution of epifoliar fungal taxa.

In Dothideomycetes, Natipusillales is a freshwater cleistothecioid order that is closely related to the Zeloasperisporiaceae lineage (Hongsanan et al. 2016). This may be a fine example of the convergent evolution of morphologically different taxa in different habitats. The most common ancestor can possibly be saprophytic (node 4) with 100% occurrence. There is no clear evidence to support the Zeloasperisporiaceae as parasites. Therefore, we treat these taxa as saprophytes. Node (5) shares a saprophytic state (B) as the prominent ancestral characters in Microthyriaceae and Zeloasperisporiaceae lineages with 96% of Bayesian support and 4% of undetermined characters. Microthyriaceae consists of biotrophic species but molecular data is unavailable. Therefore, the common ancestor of these two clades (Zeloasperisporiales and Microthyriales) is dominated by a saprophytic state (node 4). The most common ancestor of Parmulariales is represented by node 6 and shows possible ancestral ranges, A, B, AB and F. The prominent A state shows 50% of occurrence, while B, AB and F show 16%, 20% and 14%, respectively. Parmulariaceae is highly diverse in their nutrition modes, and the lack of molecular data limited this analysis to only the type species. Node 7 represents the possible ancestral ranges in the common ancestor of Microthyriales, Parmulariales and Zeloasperisporiales. The possible ancestral ranges are B, A, AB and F with the occurrence of 80%, 7%, 6% and 1%, respectively. The most common ancestor of Asterinales is represented by node 8 with possible ancestral ranges as A (44%), B (26%), C (19%) and F (11%). Epiphytic species of Asterinales may have evolved as a separate lineage representing a parasitic life mode as this clade shows heterogeneity in lifestyle. The possible state of a common ancestor (Node 9) of these above 4 orders (Asterinales, Microthyriales, Parmulariales and Zeloasperisporiales) can be determined as B (82%), A (8%), AB (7%) and F (3%). Node 10 shows the determined possible characters of two root nodes (Dothideomycetes and Eurotiomycetes), as A (38%), B (35%), C (13%) and F (14%).

According to the above analysis, we can assume that the ancestral life mode of Meliolales was more likely to be parasitic. Hyphopodia-like structures in many Meliolinutes fossil records also reflect their parasitic nature. It appears that species of Zeloasperisporiales and Microthyriales have evolved from saprobic ancestors and no parasitic relationships in their lineages have been identified. Most of the epifoliar taxa in Microthyriales are assumed to be saprobic and the parasitic taxa of this order lack sequence data. The most common ancestors of Asterinales and Parmulariales are likely to be weakly parasitic as they represent only 50% parasitic nature as compared with other life modes. Species of Micropeltidiales are phylogenetically closely related to species of Cyanodermella which are placed in Ostropales. Species of Cyanodermella have a broad biological spectrum of life-
strategies and are capable of switching their life modes according to environmental conditions (Nieuwenhuijzen et al. 2016). According to our analysis, the life mode of ancestral Micropeltidales is more likely to be parasitic and they may be capable of altering their nutrition mode in a wide range of life-strategies. On the other hand, the ascomata of *Cyanodermella candida* and the pycnidia of *C. oleoligni* are blue-green which resembles the upperwall in Micropeltidales (Nieuwenhuijzen et al. 2016).

**Figure 1** – Ancestral character state reconstruction based on the nutritional modes of epifolir fungi.
Using Bayesian Binary MCMC. Pie charts at terminals show the representative character and the internal nodes represent the marginal probabilities for each alternative ancestral area. Bayesian posterior probability values are indicated above the branches.

Figure 1 – Continued.
Figure 1 – Continued.

**Morphology**

**Ascomata**

Epifoliaceous fungi generally produce special fruiting structures called thyriothecia which are small, superficial, shield-shaped and flattened structures (Hofmann 2010). Most of the thyriothecioid ascomycetes belong to Dothideomycetes, and have various ecological and morphological characteristics. Thyriothecioid ascomycetes are categorized into families based on the size, shape and cellular arrangement of the thyriothecia (Barr & Huhndorf 2001, Hofmann 2010). Hofmann (2010) described the anatomy of thyriothecia based on Asterinaceae and Microthyriaceae (Fig. 2). A thyriothecium comprises an upper, thick, darkly pigmented scutellum (Hofmann et al. 2010, Hongsanan et al. 2014). The cells of the scutellum are pseudoparenchymatous and arranged in radiating rows, branching dichotomously at the margin (Hofmann 2010). The cells vary from isodiametric to cylindrical, meandering or epidermoid (Hofmann 2010). However, the composition and development of the scutellum is similar to the discoid thalli of green algae (Neustupa 2003, Thompson & Wujek 1997). Asci usually develop below the scutellum and when asci are mature ascospores are released from the scutellum opening (Hofmann 2010). The thyriothecia of Asterinaceae are superficial, black, dimidiate, with radial hyphae and opening by breaking of lateral cells in contact with the central star-shaped or irregular fissures in the scutellum (Hongsanan et al. 2014). Species of Lembosiaceae differ from Asterinaceae in their elongated thyriothecia opening by longitudinal or X- or Y-shaped slits (Rahayu & Parbery 1991, Hofmann 2010). Thyriothecia of Microthyriaceae are superficial, flattened, with cells of the upper walls radiating in parallel arrangement from the distinct central ostiolar opening, a have poorly developed basal wall and opening by the dissolving or breaking of the oldest scutellum cells (Wu et al. 2014). Species of Zeloasperisporiaceae have poorly developed thyriothecia without ostioles and comprise ellipsoid angular cells, radiating from the center to the outer rim (Hongsanan et al. 2015a). Ascospore release from thyriothecia in Zeloasperisporiaceae is not clear. Species of Micropeltidaceae have distinct
thyriothecia characterized by flattened, blue-green upper walls with pseudoparenchymatous hyphae, and a central ostiole.

Meliolaceae and Parmulariaceae differ from other thyriothelial epifoliar families as they have ascostromatal structures. The ascostroma of Parmulariaceae are dark brown to black, shield-like and elliptical with a carbonaceous origin (Inácio & Cannon 2008, Inácio et al. 2012, Dai et al. 2018, Hyde et al. 2020). The ascostroma in Parmulariaceae opens by radiating fissures to release ascospores (Guatimosim et al. 2015, Dai et al. 2018), while Meliolaceae has superficial globose to subglobose ascomata covered with setae or appendages (Hongsanan et al. 2015b, Zeng et al. 2020). In Meliolaceae, the rudimentary ostiole of the ascomata may dissolve, to compensate for turgor, and the ascospores are released in a gelatinous mass (Nayar et al. 1998). Thyriothecium and ascomata variations of epifoliar fungi are illustrated in Fig. 2a, b.

Figure 2 – Anatomy of a thyriothecium.

Hamathecium

The hamathecium of epifoliar fungi is poorly studied and rarely mentioned in previous descriptions (Hofmann 2010). The interascal tissue of Asterinales (Asterinaceae, Lembosiaceae and Aulographaceae) are usually absent, unclear or have disintegrated during the development of asci (Eriksson 1994, Hofmann 2010). However, some genera of Asterinaceae have distinct hyaline to brown, branched and septate, distinct pseudoparaphyses (Fig. 3). Pseudoparaphyses are often present in the hamathecium of Microthyriaceae but sometimes they can deliquesce and are lacking (Wu et al. 2011). The hamathecia of Parmulariaceae usually comprise hyaline, septate pseudoparaphyses with brown apices (Dai et al. 2018). In Micropeltidaceae, the hamathecium commonly consists of sterile filaments of paraphyses (Zeng et al. 2019). These filaments can be hyaline and rarely colored, simple or branched, tubular or not, with or without septa, shorter or longer than the asci and sometimes tapering at the apex (Batista 1959, Zeng et al. 2019). The presence or absence of paraphyses is an important character in the generic segregation of Micropeltidaceae (Wu et al. 2011,
Zeng et al. 2019, Hyde et al. 2020). Pseudoparaphyses are absent in Zeloasperisporiaceae (Hongsanan et al. 2015a). Members of the Meliolaceae have a hamathecium with evanescent paraphyses (Hongsanan et al. 2014).

Figure 2a – Thyriothecium variations in epifoliar fungi. a–c Asterinaceae. d, e Lembosiaceae. f, g Micropeltidaceae. h Zeloasperisporiaceae. i Parmulariaceae. j–l Microthyriaceae. Scale bar: a–e = 100 µm, f, g = 150 µm, h, k, l = 50 µm, i = 1 mm, j = 25 µm.
Asci

The asci of thyriothecioid ascomycetes are usually bitunicate comprising a thin ectotunica and a thickened endotunica in the apical part of the ascus (Hofmann 2010). Sometimes, a distinct ocular chamber can be seen in the ascal apex; however, apical rings or zonations are absent (Eriksson 1981). The morphological development of asci and dehiscence inside thyriotheccia varies among the different families of thyriothecioid ascomycetes (Hofmann 2010). Asci of Asterinaceae and Lembosiaceae vary in shape from globose to subglobose, oblong, cylindrical, clavate and ovoid and usually contain eight spores with or without a pedicel (Hongsanan et al. 2014). Some asci have a thick, hyaline apical region, lacking a distinct ocular chamber (Hongsanan et al. 2014). Micropeltidaceae and Microthyriaceae have elongated and cylindrical, 4–8-spored asci, without a distinct ocular chamber (Hofmann 2010, Zeng et al. 2019). Species of Parmulariaceae have 8-spored, thick-walled, bitunicate, cylindrical, short-pedicellate asci with a distinct ocular chamber (Guatimosim et al. 2015, Dai et al. 2018). Species in Meliolaceae have unitunicate asci with 2–3-ascosporous (Mibey & Hawksworth 1997, Hosagoudar & Riju 2013, Hongsanan et al. 2015b). The asci of Zeloasperisporiaceae are globose to ovoid or clavate, bitunicate, 8-spored, fissitunicate, apedicellate with an apical ocular chamber (Hongsanan et al. 2015a). The asci in each family are shown in Fig. 4.
Figure 3 – Variations in the morphology of the hamathecium in epifoliar fungi. a, b Micropeltidaceae. c, g Microthyriaceae. d Parmulariaceae (stained in Cotton Blue). e Meliolaceae. f Asterinaceae. Scale bars: b, c = 10 μm, f = 50 μm, d, e, g = 20 μm.

Ascospores

The majority of Asterinaceae and Lembosiaceae species have globose ascospores, but range from cylindrical, 2-celled, hyaline when young, becoming brown at maturity (Hofmann et al. 2010). Ascospores of Micropeltidaceae are often uni- or multi-cellular, constricted or not, hyaline, rarely chlorinate or brown and filiform spores may have a length to width ratio of 10:1 according to Batista (1959) and Batista & Bezerra (1964). The ascospores of Meliolaceae are uniform in shape, thick-walled, cylindrical to oblong and fusiform or obovoid with 3–4-septa (Hongsanan et al. 2015b, Zeng et al. 2020). The ascospores of Zeloasperisporiaceae are 1-septate, obovoid to clavate, hyaline,
sometimes with a sheath and are slightly constricted at the septum (Wu et al. 2011, Crous et al. 2015). Microthyriaceae usually have 1-septate, hyaline or brown ascospores, sometimes bearing fine appendages or cilia at one end (Wu et al. 2011, Hongsanan et al. 2020). Species of Parmulariaceae have hyaline to dark brown, oblong to ellipsoid, 1-septate, smooth- to verrucose-walled ascospores (Dai et al. 2018). The ascospores of each family are shown in Fig. 5.

Figure 4 – Variations in the morphology of asci in epifoliar fungi. a, k Lembosiaceae. b, c Asterinaceae. d, e Meliolaceae. f, i Zeloasperisporiaceae. g Microthyriaceae. h Micropeltidaceae. j, l Parmulariaceae. Scale bars: a–c, e–h = 20 μm, d, i–l = 10 μm.
Figure 5 – Variations in the morphology of ascospores. a, b Lembosiaceae. c Asterinaceae. d, e Micropeltidaceae. e Pyriform, brown and unequally 1-septate (Parmulariaceae). f Parmulariaceae. g, h Zeloasperisporiaceae. i, j, m Meliolaceae. k Microthyriaceae. Scale bars: a–c = 20 µm, g, h = 5 µm, d–f, i–m = 10 µm.

Asexual morphs
Asexual morphs of epifoliar fungi have been recorded for some genera in Asterinaceae, Micropeltidaceae, Microthyriaceae and Zeloasperisporiaceae (Fig. 6). They have similar ecological niches, such as distribution patterns and host specificity (Hofmann et al. 2010). In Asterinaceae, coelomycetous states form pycnothyria with branched, hyaline conidiophores; monoblastic or percurrent, hyaline to brown conidiogenous cells and pale brown to brown conidia (Hongsanan et al.)
Phialides on hyphae are considered as the asexual morph of Meliolaceae; however, this structure and its function are poorly known (Hongsanan et al. 2015b). The asexual morph of Zeloasperisporiaceae has 1-septate, fusiform to obclavate or cylindrical, pale brown to brown conidia, which develop from a conidiogenous cell arising from dark hyphae (Hongsanan et al. 2015a). The asexual morphs of Microthyriaceae are almost unknown, only Ramaley (1999) reported a hyphomycetous state for Microthyrium guadalupensis that was obtained by cultivation experiments (Hofmann 2010).

Figure 6 – Asexual morphs of epifoliar fungi. a Pycnothyria (Asterinaceae). b Conidia (Asterinaceae). c Phialides (red arrows) (Meliolaceae). d Crowded conidia on conidiogenous cells. e Conidia (red arrow) on conidiogenous cell (black arrow) with sympodial proliferation (Zeloasperisporiaceae). Scale bars: a = 20 µm, b = 10 µm, c–e = 25 µm.
Outline of epifoliar fungi in Ascomycota with family descriptions

Epifoliar fungi are currently distributed in four major classes Dothideomycetes, Eurotiomycetes, Lecanoromycetes and Sordariomycetes (Hofmann 2010, Hongsanan et al. 2016, Zeng et al. 2019). Thus, we follow the classification in Hongsanan et al. (2020), Wijayawardene et al. (2020) and Hyde et al. (2020).

Dothideomycetes

Dothideomycetes are one of the largest classes in Ascomycota (Hongsanan et al. 2020, Hyde et al. 2020). This class comprises highly diversified taxa, mainly characterized by bitunicate asci and usually with fissitunicate dehiscence (Hongsanan et al. 2020). Species of this class are mainly saprobes, with many asexual morphs comprising important plant pathogens. Species are endophytic, epiphytic, fungicolous, lichenized, or lichenicolous taxa (Hongsanan et al. 2020, Hyde et al. 2020). The major groups of foliar epiphytes in this class are Asterinales, Microthyriales, Parmulariales and Zeloasperisporiales.

Asterinales (Asterinaceae and Lembosiaceae)

Taxonomic overview

Asterinaceae and Lembosiaceae are biotrophic epifoliar families, appearing as black colonies on the surface of living leaves (Kirk et al. 2001, Hofmann 2010, Hosagoudar et al. 2013a). Species of Asterinaceae are characterized by orbicular, flattened thyriothecia opening by either star-like fissures or by a lysigenous pore (Hofmann 2010, Hongsanan et al. 2014). However, members of Lembosiaceae can be identified by orbicular, dark, flattened thyriothecia with central dehiscent X- or Y-shaped opening (Hongsanan et al. 2014, Dai et al. 2018). The thyriothecia of Asterinaceae and Lembosiaceae usually comprise asci which are globose to oval, or clavate, with ellipsoid, conglobose and initially hyaline ascospores becoming brown to dark brown when mature (Hosagoudar et al. 2013b, Hongsanan et al. 2014).

Ecology

Plant-parasitic members of Asterinaceae and Lembosiaceae are mostly associated with the surface of living leaves, rarely with living stems or other plant organs (Hofmann et al. 2010). They parasitize a broad range of living leaves of Angiosperms in tropical and subtropical regions (Kirk et al. 2001). However, plant-parasitic members of Asterinaceae and Lembosiaceae are almost absent in the temperate northern hemisphere (Toro 1952, Hofmann 2010). Most of the species are obligate biotrophs dependent on a living host plant and considered host-specific (Hofmann 2010, Hongsanan et al. 2014). Their morphology is usually quite similar, and it has yet to be established whether these taxa are species, genus or family-specific (Hongsanan et al. 2014). The aspect of host specificity has never been carefully studied (Hofmann et al. 2010, Hosagoudar & Abraham 2000). Therefore, studies focusing on host specificity are needed in these genera.

There is no record of serious damages being caused to host plants by this group, but they can severely decrease growth. However, infection of Asterina congesta on sandal trees increased the production of cyclic compounds, such as proline, indicating high-stress levels in the host (Hosagoudar et al. 1997).

Mode of nutrition

Appressoria are major structures for obtaining nutrients of Asterinaceae (Hofmann 2010, Chethana et al. 2021a) (Fig. 7). These appressoria usually form from laterally or intercalary arranged surface mycelia and germinated conidia or ascospores (Hofmann 2010). Arrangement of appressoria can be opposite, alternate and intercalary on hyphae and the cell shape can be oval, globose, pyriform or cylindrical (Fig. 8) (Hosagoudar 2012, Chethana et al. 2021b). The shape and position of appressoria are important characters for species and generic segregation combined with other characters of thyriothecia, asci and ascospores (Bezerra 2004, Hofmann & Piepenbring 2006,
Hofmann et al. 2010, Hosagoudar 2012). However, species and generic segregation based on the morphology of appressoria has not been proven using molecular approaches (Hofmann & Piepenbring 2014, Hongsanan et al. 2014). Hansford (1946) mentioned that the taxa of Asterinaceae and Lembosiaceae were ectoparasitic as they form haustoria in epidermal cells for uptake of nutrients (Hofmann & Piepenbring 2014).

![Image](image_url)

**Figure 7** – Anatomy of Appresorial structure. a Appressoria (red arrows). b Dark brown hyphae (black arrow). Scale bar: 10 µm.

![Image](image_url)

**Figure 8** – Morphological diversity of appressoria in Asterinaceae. a, b, e Lobed appressoria on mycelium. c, d Stalked appressoria on mycelium. f 2-celled appressoria on mycelium. g Surface mycelia with ovate appressoria. Scale bars: a–g = 10 µm.
The infection structures which are known as primary appressoria develop on hyphae (Hofmann 2010), penetrate the plant cuticle and develop into intracellular haustoria or compact hypostromata (Hofmann & Piepenbring 2008). Intracellular haustoria normally infect a single host cell, while hypostromata can invade a large area of host cells (Hofmann & Piepenbring 2014). Hansford (1946) observed that the surface mycelium of *Echinodes natalensis* forms small, dark swellings in the stomata cavities. These swellings develop into hyaline, unbranched hyphae to form coralloid haustoria in mesophyll cells. Hansford (1948) illustrated parasitism by *Asterina* and *Lembosia* species (Figs 9, 10). *Asterina dissiliense* has few appressoria connected to the main external hyphae and later develop as internal hyaline hyphae. They penetrate the cuticle and epidermal cells and form a thin layer around the upper ends of the palisade cells (Hansford 1946). Hansford (1948) observed coralloid haustoria developing in the upper epidermis and sometimes this penetration extended to one or two adjacent cell layers. He also described the parasitism of *Echidnodes harunganae* and observed small reddish-brown intercellular knots filled with orange-brown granules beneath the mycelium. However, no observations of haustoria were recorded (Hansford 1948).

**Figure 9** – Transverse section of hyphal development in plant tissues (Redrawn from Hansford 1946). a Haustoria (black arrow) and mycelia (red arrow) in the older part of the colony. b, d Appressoria (red arrow) and mycelium (black arrow). c Internal hyphae (red arrow) originating from terminal cells of external hyphae (black arrow). Scale bars: a–c = 20 μm, d = 10 μm.
Figure 10 – Vertical section of *Asterina* sp. and *Lembosia* sp. (Redrawn from Doidge 1942). a Internal hyphae (red arrow) developing from external hyphae (black arrow) within the epidermal cells to palisade mesophyll cells. b Collaroid haustoria (red arrow) developing inside epidermal cells. c Extensive internal mycelium (red arrow) developing beneath epidermis and mesophyll cells. Scale bars: a–c = 20 µm.

In this study, vertical hand sections of species in Asterinaceae were prepared (Fig. 11). Dark brown swellings were observed inside the stomata cavities which develop to form coralloid haustoria inside the mesophyll cells (Fig. 11a). Haustoria were not observed in some vertical sections of *Asterina*. However, a direct connection between internal mycelium and the appressoria of external mycelia was unclear. Fig. 11b (stained in Congo Red) shows vertical sections of *Lembosia* sp. in
which collaroid haustoria were observed inside epidermis cells. These collaroid haustoria are also common in Parmulariaceae which show the ecological connection of these families.

**Figure 11** – a Dark swellings inside stomata cavity (1) and haustoria (2) developing inside epidermis cells (Asterinaceae). b Collaroid haustoria developed inside epidermis cells (red arrows). Scale bars: a, b = 20 µm.

We also tried to observe the direct penetration mechanism of appressoria inside the host leaf tissue. Scrapped portions of mycelia were used for microscopic observations. Fig. 12 shows the section of mycelia through plant tissues and appressoria always develop towards the stomata. Stomata are considered an important organ for gas exchange between plant cells and the environment (Doidge 1942, Toro 1952, Hofmann et al. 2010). Therefore, we can assume that there is a relationship between the appressoria and stomata and this connection may facilitate appressoria to gain energy for absorption processes. Sometimes, these hyphae develop inside the stomata and other thin hyaline hyphae also develop from the main mycelium. These thin hyaline hyphae can be seen through the tissue but it is hard to detect any connection to haustoria. We observed kidney-shaped structures (Fig. 13) which have also been described as haustoria by Hofmann (2010). These organs are formed inside mesophyll cells and were connected with appressoria by hyaline hyphae. These haustoria may act as intermediate structures for transferring nutrients from plant tissues to the fungal cells. The infection structures are usually concentrated around the stomata. These observations probably illustrate a strong connection between the nutrient absorption process and the stomata.

**Parmulariales**

**Taxonomic overview**

Parmulariaceae is considered a polyphyletic group and comprises 35 genera with distinguishable characters (Guatimosim et al. 2015, Hongsanan et al. 2020, Wijayawardene et al. 2020). Parmulariaceae has been placed in different orders such as Dothideales, Dothiorales, Hemisphaeriales, Hysteriales and Microthyriales by different mycologists (Müller & von Arx 1962,
Ainsworth 1971, Luttrell 1973, Barr 1979, Hawksworth et al. 1983, 1995). Lumbsch & Huhndorf (2010) placed Parmulariaceae in Dothideomycetes family *incertae sedis*. Hyde et al. (2013) transferred this family to Asterinales, while Guatimosim et al. (2015) confirmed its placement in Asterinales based on sequence data of *Parmularia styrica*. However, Liu et al. (2017) placed *Parmularia styrica* in Asterinaceae *sensu lato* and revealed its different lineage from Asterinaceae *sensu stricto*. Dai et al. (2018) re-examined the type species and other selected taxa of Asterinales and established Parmulariales (Dai et al. 2018, Hongsanan et al. 2020). Parmulariaceae differs from Asterinaceae in having ascostromata with multi-locules, pseudoparaphyses, ellipsoidal to obclavate asci and usually lacking appressoria (Inácio & Cannon 2008, Inácio et al. 2012, Hongsanan et al. 2020).

**Figure 12** – Vertical and transverse sections of mycelium with appressoria. a, b Appressoria (red arrows) developing within the stomata (black arrow) cells. c, d Internal hyphae (red arrows) originating from external mycelium. Scale bars: a, b = 20 µm c, d = 10 µm.
Figure 13 – a, b Surface appearance of mycelium (b = Redrawn from Hansford 1946). c Haustoria stained in Cotton Blue (red arrows). d Hyaline hyphae (red arrow) connecting haustoria to mycelium. Scale bars: a = 50 µm, b–d = 10 µm.

Ecology

Members of Parmulariaceae have been recorded from more than 50 host families (Inácio et al. 2012, Dai et al. 2020). However, host specificity in Parmulariaceae has not been proven (Hofmann 2010, Guatimosim et al. 2015, Dai et al. 2018).

Mode of nutrition

Species of Parmulariaceae usually form a hypostroma (internal stroma) which originates from hyphae underneath the cuticle, the epidermis, or in the mesophyll (Guatimosim et al. 2015). However, von Arx & Müller (1975) observed haustoria in Pseudolembosia (Inácio & Cannon 2003). Aulacostroma species have hyaline, intracuticular mycelium (Fig. 14, black arrow), which originated from brown superficial mycelial bulbil-like structures (red arrow) in mesophyll cells resembling appressoria (Fig. 14g) (Luttrell & Muthappa 1974, Inácio et al. 2012). Inácio et al. (2012) described Antoniomyces which penetrates the internal stroma using peg-like structures. Inácio & Cannon (2003) observed distinct haustoria (red arrow) linking the internal stroma and the epidermal cells without external mycelium in Viegasella pulchella. Mintera reticulata differs from other members in Parmulariaceae in having well-developed external mycelium with appressoria (Inácio & Cannon 2003). After primary penetration by appressoria, the external mycelium forms collaroid haustoria (red arrow) inside the mesophyll cells (Inácio & Cannon 2003) (Fig. 14c). Sivanesan & Sinha (1989) introduced Aldonata and mentioned that their penetrations of epidermis or plant tissues were rarely observed. Inácio (2005) described Aldonata with brown to dark brown clypeus-like structures.
forming from the lower walls of the ascoma and developing inside the epidermal cells. We also observed brown to dark collaroid haustoria (red arrow) structures in Polycyclina rhytismoides, Cyclostomella disciformis and Aldona stella-nigra, but haustoria were not observed (Fig. 15). Internal hyphae were observed developing from the lower ascoma wall to palisade mesophyll cells. Inácio (2003) described Hysterostomella with subcuticular internal stromata when host cells are incorporated by the infective mycelium. These structures originated after the penetration of epidermis cells by peg-like or tubular hyphal filaments (Inácio 2003, Inácio & Cannon 2008). There is no evidence of infection structures other than the internal mycelia (red arrows) developing into mesophyll cells in Cocconia, Cycloschizon and Ferrarisia species (Fig. 14d, f, e). Guatimosim et al. (2014) observed collaroid haustoria (red arrows) inside the epidermal cells, and peg-like columns (black arrows) connecting to the host through the stomata in Rhagadolobiopsis sp. (Fig. 14a). Peg-like structures (red arrows) developed in the epidermal cells in Palawaniella sp. (Hansford 1947) (Fig. 14h).

Microthyriales

Taxonomic overview

The members of Microthyriaceae are epifoliar taxa appearing as small, inconspicuous, black spots on host leaves (Wu et al. 2011, Hongsanan et al. 2014). Microthyriaceae was established by Saccardo (1883) with Microthyrium as the type genus. Theissen (1913) included this family in Hemisphaeriales. Arnaud (1918) established Microthyriales to accommodate two families, Microthyriaceae and Microtheliopsidaceae. von Arx & Müller (1975) and Barr (1987) placed this family in Dothideales and Melanomatales respectively. Kirk et al. (2008) revised Microthyriales including Aulographaceae, Microthyriaceae and Leptopeltidaceae. Lumbsch & Huhndorf (2010) suggested that Microthyriaceae should be placed in Dothideomycetes as families incertae cedis due to their lack of molecular data. Currently, Microthyriaceae is placed in Microthyriales in Dothideomycetes (Hongsanan et al. 2020). Microthyriaceae is characterized by superficial, flattened thyrthrothecia, the upper wall with radial and parallel cuboid or angular cells with a prominent central opening and with setae. Asci are bitunicate, fusiform or obclavate to cylindrical-clavate or fissitunicate and ascospores are two-celled, hyaline to brown, often with ciliate appendages (Kirk et al. 2008, Wu et al. 2011, Hyde et al. 2013, Hongsanan et al. 2020).

Ecology

The ecological role of species in the family is not clear. Taxa of this family colonize living leaves and release ascospores from fallen decaying leaves. It is also not clear if the species are host generalists or host-specific. These species are commonly found superficially or subcuticular on dead or decaying leaves and stems (Wu et al. 2014).

Mode of nutrition

Appressoria or haustoria have not been reported in many genera of Microthyriaceae. Wu et al. (2011) isolated several species of Microthyriaceae and suggested that they have saprobic tendencies (Wu et al. 2011). Ryan (1926) suggested that the ascomata of this family probably develop from cells of the mycelium, hyphopodia, short lateral hyphal branch or a nodulate cell. Wu et al. (2011) observed superficial mycelia with irregular hyphopodia-like branches which penetrate the host epidermal cells in Calothyriopsis conferta (Müller & von Arx 1962). However, no infection structure was observed in our sections of Microthyrium sp. (Fig. 16).

Zeloasperisporiaceae

Taxonomical overview

Castañeda et al. (1996) introduced Zeloasperisporium typified by Zeloasperisporium hyphopodioides. This genus was first placed in Venturiaceae based on sequence data of the type
species by Crous et al. (2007). Crous et al. (2015) introduced Zeloasperisporiaceae to accommodate Neomicrothyrium and Zeloasperisporium (Hongsanan et al. 2015a, Jayasiri et al. 2018). Hongsanan et al. (2015a) introduced Zeloasperisporiales and synonymized Neomicrothyrium under Zeloasperisporum based on identical morphological characters in both sexual morphs. Species of this order are characterized by superficial, circular, flattened, brown to dark brown thyriothecia without ostioles (Hongsanan et al. 2015a). Asci are 8-spored, bitunicate, fissitunicate, globose to ovoid or clavate, apedicellate, and ascospores are obovoid to clavate and 1-septate (Wu et al. 2011, Crous et al. 2015, Hongsanan et al. 2015a).

Ecology
Species of Zeloasperisporiaceae can be found on dead and living leaves and seed pods during cold periods (December to February) in tropical regions (Hongsanan et al. 2015a, Jayasiri et al. 2018). They have been recorded from different host families such as Anacardiaceae, Apocynaceae, Fabaceae, Moraceae, and Myrtaceae (Index Fungorum 2022). Castañeda et al. (1996) isolated Zeloasperisporium hyphopodioides from the air.

Mode of nutrition
Castañeda et al. (1996) found that the asexual morph of this family has appressorium-like structures and described them as inflated hyphopodia, which are pale olive, slightly warted to lobed at the apex. We observed similar pale olive structures on the hyphae other than micronematous conidiogenous cells (Fig. 17) (red arrows) described by Crous et al. (2007) and Hongsanan et al. (2015a). Fig. 17c clearly shows that these structures (black arrow) are pale olive and relatively smaller than micronematous conidiogenous cells (red arrows). Therefore, we assume that these structures may be the inflated hyphopodia described by Castañeda et al. (1996) that are involved in nutrient uptake. The process of obtaining nutrients by sexual morphs is not clear (Hongsanan et al. 2015a). However, the structure at the margins of thyriothecia which are believed to be the organ to attach to the host surface provides a clue to their penetration process (Hongsanan et al. 2015a). We also sectioned Zeloasperisporium species to observe any attachment or absorption organ staining with Cotton Blue (Fig. 18). We could not observe any infectious structures in the epidermis or mesophyll cells.

Lecanoromycetes
Lecanoromycetes is considered the third-largest class of fungi after Agaricomycetes and Dothideomycetes. It includes the majority of lichenized fungi (Kirk et al. 2008).

Micropeltidaceae

Taxonomical overview
Micropeltis was introduced by Montagne (1842). Saccardo (1883) included it in Microthyriaceae based on morphological characters; superficial, black, dimidiate, flattened, membranous or carbonaceous perithecia. However, Höhnel (1910) introduced Hemisphaeriales to accommodate Hemisphaeriaceae and Microthyriaceae. Arnaud (1918) included these two families in Microthyriales and Clements & Shear (1931) synonymized Hemisphaeriaceae under Micropeltidaceae. Batista (1959) provided a monograph that officially established Micropeltidaceae as a distinct family. Kirk et al. (2008), Lumbsch & Huhndorf (2010) and Hyde et al. (2013) accepted 27, 24 and 12 genera in Micropeltidaceae, respectively. Hongsanan & Hyde (2017) transferred this family to Lecanoromycetes. Zeng et al. (2019) established Micropeltidales using phylogenetic analyses based on LSU, SSU, 5.8S, tef and rpb2 sequence data. Species of Micropeltidaceae are characterized by superficial, flattened, bluish-green upper walls with pseudoparenchymatous hyphae and a central ostiole. Asci are clavate to cylindrical or elongate which inclined towards the center of ascomata and ascospores are hyaline with uni or multi transverse septa (Zeng et al. 2019).
Figure 14 – Vertical transverse sections showing haustoria of genera in Parmulariaceae. a Coralloid haustoria of *Rhagadolobiopsis* sp. in host epidermis cells (Redrawn from Guatimosim et al. 2014). b Coralloid haustoria of *Viegasella pulchella* in mesophyll cells (Redrawn from Inácio & Cannon...
c Coralloid haustoria in the epidermal cells of Mintera reticulata (red arrow). d, e Internal mycelia developing in mesophyll cells of Cocconia sp. and Ferrarisia sp. (Redrawn from Hansford 1942). f Formation of internal hyphae from the lower cell wall and developing into mesophyll cells of Cycloschizon sp. (Redrawn from Hansford 1947). g Collaroid haustoria inside mesophyll cells of Aulacostroma sp. (Redrawn from Luttrell & Muthappa 1974). h Penetration of epidermis cells via peg-like structures of Palawaniella sp. (Redrawn from Hansford 1947). Scale bars: a–e = 20 µm, f–h = 25 µm.

Figure 15 – Vertical transverse sections of Polycyclina rhytismoides\textsuperscript{ab} (CUP-VZ-003029) and Aldona stella-nigra\textsuperscript{cd} (MFU 14–0011). a, b, d Brown to dark collaroid haustoria developing from lower cells of ascomata (red arrows). c Internal mycelium developing from lower cells of ascomata. Scale bars: a, b = 50 µm, c, d = 20 µm.

Figure 16 – a, b Vertical section of Microthyrium microscopicum (MFLU 09–0653). Scale bar: a, b = 20 µm.
Figure 17 – Zeloasperisporium wrightiae (asexual morph) (Figures from Hongsanan et al. 2015a). a–c Micronematous conidiogenous cells (red arrows). c, d Pale olive appressorium-like structures (black arrows). Scale bars: a–c = 10 µm, d = 5 µm.

Ecology
Species of Micropeltidaceae can be found in tropical regions (Batista 1959). However, these species are not only limited to tropical regions, and are also found in the subtropics, temperate zones with mild climates and relatively high humidity (Johnson & Sutton 2000, Cooley et al. 2004). These epifoliar taxa are recorded from a wide range of plant families and are not considered host-specific (Batista 1959, Acosta 1995, Reynolds & Gilbert 2005, 2006). Species of Micropeltidaceae are also
considered as a kind of flyspeck fungi (Zeng et al. 2019). These flyspeck taxa often have epiphytically growth on living leaves, stems and fruits (Hofmann & Piepenbring 2006).

Figure 18 – a, b Vertical section of Zeloasperisporium wrightiae (MFLU 15–1309). c Vertical section stained in Cotton Blue. Scale bars: a–c = 50 µm.

Mode of nutrition

Zeng et al. (2019) stated that the reason for the bluish-green upper walls in this family is due to the presence of cyanobacteria communities. This provides a clue of their nutrition mode as symbionts (Zeng et al. 2019). Furthermore, they may take nutrients from plant exudates or other nutrients available on the leaf surface (Batista 1956, 1959, Batzer et al. 2008). Commensal species usually do not penetrate the host and have a broad distribution range of host plants (Belding et al. 2000, Williamson & Sutton 2000, Reynolds & Gilbert 2006).
Species of Micropeltidaceae are also similar to other sooty blotch and flyspeck fungi (SBFS) which do not appear to infect a host substrate (Zeng et al. 2019). However, they use mineral, organic substances resulting from transpiration residues and epidermal secretions (e.g., potassium, calcium salts and phosphatides) (Zeng et al. 2019). On the other hand, they help to exchange gases between host plants and the environment (Batista 1959). We sectioned *Micropeltis* sp. (Fig. 19) and no nutrient absorption structures were observed. These species have no tight attachment to the leaf surface, as they easily detach even from the touch of a needle.

**Figure 19** – Vertical section of *Micropeltis goniothalamicola* (MFLU 19–1212). a, b Without staining. c, e Stained with Cotton Blue. d Stained with Melzer’s reagent. f Stained with Congo Red. Scale bars: a–f = 20 µm.

**Sordariomycetes**

Sordariomycetes is considered the second-largest class in Ascomycetes. Species of this class are characterized by non-lichenized, flask-shaped fruiting bodies or less frequently cleistothecial ascomata and unitunicate asci (Zhang et al. 2006, Maharachchikumbura et al. 2016, Hyde et al. 2020). Taxa of this class are highly diverse and have a wide range of ecological niches (Hyde et al. 2019, 2020).

**Meliolaceae**

**Taxonomical overview**

Meliolales comprises two families: Armatellaceae and Meliolaceae (Hongsanan et al. 2015b, Hyde et al. 2020, Zeng et al. 2020). They are black mildews which grow on the surface of leaves, petioles, twigs and rarely on fruits (Hongsanan et al. 2015b, Zeng et al. 2017, 2020). Species of
Meliolaceae have an ecto, endo or ectoendophytic brown mycelium with two-celled appressoria and unicellular phialides representing the asexual morph (Hongsanan et al. 2015b). Meliolaceae have globose perithecia, with mycelial setae and some genera have perithecial setae or appendages (Hosagoudar & Thomas 2013). The monotypic genus *Armatella* represents *Armatellaceae* (Hosagoudar 2003), while *Amazonia, Asteridiella, Cryptomeliola, Endomeliola, Irenopsis* and *Meliola* are accepted in Meliolaceae (Hongsanan et al. 2015b, Zeng et al. 2020). Zeng et al. (2020) described Meliolaceae as comprising four generic level clades *Asteridiella, Irenopsis, Meliola* and *Meliola*-like. However, these clades partially coincide with the morphological characters of presence and position of appendages which are used in generic delineation in Meliolaceae (Zeng et al. 2020).

**Ecology**

Meliolaceae is a biotrophic family which grows in tropical and sub-tropical regions and is less common in cold climates (Hansford 1961, Goos & Andersons 1974, Rodríguez Justavino et al. 2015, Hongsanan et al. 2015b). Species of Meliolaceae are considered to be host-specific, thus species have been introduced based on their host (Hansford 1961, Rodríguez Justavino & Piepenbring 2007). Their hosts are limited to indigenous plants which rarely belong to more than one family of phanerogams (Hansford 1961). Zeng et al. (2017) provided an updated checklist for Meliolaceae with their recorded host families. However, a recent analysis by Zeng et al. (2020) mentioned that there is no significant evolutionary trend between morphological characters and their hosts. Host switching between closely related plants in the same genus has not been observed, but occurs in host species from different genera in the same family (e.g., between *Acacia* and *Pueraria* in Fabaceae) (Zeng et al. 2020).

The habitat of Meliolaceae is living leaves of shrubs in open areas and high canopies of forest trees (Hansford 1961, Hosagoudar 2003). Dispersal of ascospores of these species is unclear and assumed to occur by rain splash, insects or air (Nayar et al. 1998). These species can survive even on non-living leaves and initiate the colonization of new leaves in the next season (Nayar et al. 1998). The abundance of Meliolaceae is high in the cool-season and they can survive in the burnt forests leaving only the perennial rootstocks (Nayar et al. 1998). Goos (1978) experimentally showed that species of *Meliola argentina* occur in areas of lowest elevation and with equal or more than 250 cm/yr rainfall. Wellman (1972) stated that shade from sun and wind is also favorable for the growth of black mildews. He concluded that the ideal condition for black mildew is a moist tropical climate (Goos 1978).

Several mycologists attempted to observe the germination of *Meliola* species under laboratory conditions and in the field. Hansford (1961) described that he “attempted repeatedly to germinate the ascospores of several species of *Meliola* and *Asteridiella*, both in the laboratory. But these attempts were always unsuccessful”. Thite (1975) observed germination of *M. jasminicola* in hanging drops. Moreover, he attempted to grow *M. argentina* and *M. palmicola* in agar media and observed 4–10% of germination (Goos 1978). However, further growth of the germ tube was not observed under laboratory conditions and may imply the necessity of host contact for complete germination (Goos 1978).

**Mode of nutrition**

Few studies have focused on analyzing the parasitic nature of Meliolaceae. Luttrell (1989) studied the parasitic interaction between *Meliola floridensis* and *Persea borbonia* (Lauraceae). Mueller et al. (1991) used ultrastructural details to study the interaction between *Meliola sandwicensis* and the host *Kadua acuminate* (Rodríguez Justavino et al. 2014). Species of this order can take nutrients from host plants using hyphopodia without causing significant pathogenic damage, and this process results in nutrient deficiency of host plants in chlorophyll, starch, sugar, protein and amino acid (Hosagoudar et al. 1997, Old et al. 2003, Rodríguez Justavino & Piepenbring 2007). However, the hyphal cover of this species can reduce photosynthesis, increase the temperature and respiration process causing a significant effect on crops, reducing yield and their quality (Wellman 1972, Anthony et al. 2002, Gilbert et al. 2007).
Hyphopodia are considered to be specific structures to obtain nutrients from the host plant. They can be distinguished by having two cells, a short basal stalk cell bearing a single capitate hyphopodium (Figs 20, 23) (Hongsanan et al. 2015b). The arrangement and shape (Fig. 21) of hyphopodia are important in identifying species in this family (Hongsanan et al. 2015b). There are two kinds of hyphopodia which are capitate and mucronate (Gaillard 1891). Capitate hyphopodia are characterized by a short stalk cell, bearing single hyphopodia and mucronate hyphopodia with a single cell (Fig. 20a) (Hansford 1961). Colonization starts with a mature ascospore having a primitive stalk cell and develops as a mature hyphopodium (Hongsanan et al. 2015b). Hyphopodia form an apoplastic complex that produces a penetration pore and develops haustoria for uptake of nutrients from the cytoplasmic membrane and epidermal cells (Fig. 24) (Rodríguez Justavino et al. 2014, Hongsanan et al. 2015b).

Figure 20 – Types of hyphopodia. a Capitate hyphopodia. b Mucronate hyphopodia (red arrows). Scale bars: a, b = 10 µm.
Berkeley (1857) opined on the parasitic nature of the genus *Meliola* and stated that leaf lesions could occur due to mites on the leaf surface. Gaillard (1892) supported Berkeley’s (1857) idea and examined leaf sections and found that *Meliolaceae* are completely superficial without direct connection with the plant tissue. However, Ward (1883) stated that the thinner spots of the upper wall of hyphae are in contact with the epidermis of plant tissue. Arnaud (1918), Doidge (1921) and Maire (1908) have observed the presence of haustoria in 16 *Meliola* species and in two of these the haustoria penetrate to subepidermal cells in the same manner as those of *Asterina*. Graff (1932) also observed that the hyphae are within the epidermis where the cell wall of the hyphae is relatively thin. He suggested that hyphae of *Meliola* may penetrate the leaf tissue. Furthermore, Graff (1932) observed hydrolyzation of the heavily cutinized wall of hyphae in contact with the host and may be evidence of a chemical reaction when penetrating the host.

Butin & Speer (1978) observed peg-like structures developing from surface mycelium into epidermal cells in *Asteridiella araucariae* (Fig. 22).

**Major challenges of studying epifoliar fungi**

Epifoliar fungi are a poorly known group due to several aspects of their morphology to phylogeny. The phylogenetic placement of some epifoliar fungi is unclear due to the lack of
molecular data as well as the difficulties in obtaining sequence data from herbarium specimens (Guatimosim et al. 2015). On the other hand, some type specimens are unavailable and the availability of some species is ambiguous or scattered worldwide herbaria (Reynolds & Gilbert 2005). Therefore, it is necessary to designate epitypes and obtain sequence data to resolve their phylogenetic placements (Dai et al. 2018). Most species of epifoliar fungi cannot be cultured in artificial media because of their biotrophic nature (Hofmann 2010). Although some sooty blotch or flyspeck species can be cultured, they rarely sporulate and are easily contaminated by other fungi (Batzer et al. 2005, Yang et al. 2010, Gleason et al. 2011, Zeng et al. 2019). Furthermore, obtaining enough fruiting structures of the same fungus is a challenge due to their small size, and sometimes they appear in mixtures with other epiphyllous fungi (Renard et al. 2020). For instance, Hofmann (2010) and Firmino (2016) found that species of *Asterina* grow together with *Microthyrium* species (Renard et al. 2020). Therefore, it is a challenge to distinguish targeted species based on their external morphology. The hyphomycetous states of these fungi need to be confirmed by molecular data (Hyde et al. 2013, Hongsanan et al. 2014).

![Figure 22](image) – Hyphae penetrating from surface mycelium into the mesophyll cells of *Asteridiella araucariae* (Redrawn from Butin & Speer 1978).
The taxonomy of some epifoliar fungi is confusing due to inadequate morphological variations and repeated descriptions in species delineation (Reynolds & Gilbert 2006). Some epifoliar species have been introduced based on different host plant species with minor morphological variations. The monograph of Meliolales by Hansford (1961) is one example of species delineation solely based on the recorded host (Reynolds & Gilbert 2005). There are some species descriptions without any illustrations and many historical epifoliar fungal species are introduced based only on a single collection (Reynolds & Gilbert 2005). For instance, Batista (1959) introduced many species using a single collection in his monograph of Micropeltidaceae (Reynolds & Gilbert 2005). Therefore, new collections and future studies are needed to clarify their taxonomic placement and species delineation.

Discussion

Epifoliar fungi are a poorly studied group of symbionts that co-inhabit the surface of living plants (Zeng et al. 2020). Sooty moulds are an important epifoliar group which exhibit a diverse spectrum of morphology, with life-modes including saprobes, pathogens and mycoparasites, and lichenised and rock-inhabiting taxa (Crous et al. 2009, Schoch et al. 2009, Schoch & Grube 2015). Epiphytic sooty moulds have a close association with insects and derive nutrients from the honeydew produced by these animals (Hughes 1976, Crous et al. 2009, Chomnunti et al. 2014). The diversity and composition of sooty mould communities depend on the area, climatic conditions and are more common during the rainy seasons (Batista & Ciferri 1963, Flessa et al. 2021). Currently, more than 1000 sooty mould species have been documented and many more remain to be discovered. It has been experimentally proven that some sooty mould species are capable of producing secondary metabolites, such as methiosetin (Herath et al. 2012). However, the number of compounds is remarkably low due to the lack of studies and knowledge of epifoliar fungi.
According to our analysis, the ancestors of epifoliar fungi are not limited to a specific life mode and they are intermixed with different life modes throughout the Ascomycota lineages. Zeng et al. (2019) concluded that co-divergence of Micropeltidaceae occurred simultaneously with their host plants. Therefore, we can assume that some environmental changes may also strongly affect species diversification of epifoliar fungi. Thus, evolutionary knowledge will enhance the understanding of taxonomic ranking, ecology and evolutionary history and will aid future studies.

DNA-based identification is commonly used in current taxonomic studies replacing traditional morphological approaches due to its accuracy, speed and lower costs (Comtet et al. 2015). However, traditional DNA barcoding is not applicable for tiny fungal colonies which are morphologically inseparable on the host surface and mixed together with other fungal species. Therefore,
environmental DNA (eDNA) metabarcoding is more convenient and this approach has been claimed as a promising tool to estimate biodiversity in terrestrial, freshwater and marine habitats (Comtet et al. 2015, Tedersoo et al. 2018, 2021). On the other hand, some fungal taxa are un culturable and hardly form visible sexual structures (Tedersoo et al. 2018). Therefore, molecular-based techniques have become important in the identification of taxa and their ecological shifts in their richness and diversity according to environmental gradients (Persoh 2015, Balint et al. 2016, Tedersoo & Nilsson 2016). Furthermore, these molecular methods provide a wider understanding of phylogeny-based relationship between fungi while altering the morphology-based classification system (Hibbett et al. 2007, Wijayawardene et al. 2018). There is little environmental data for epifoliar fungi in Sequence Read Archive (SRA) databases such as in National Center for Biotechnology Information (NCBI), the European Bioinformatics Institute (EBI) and the DNA Database of Japan (DDBJ).

Fossil fungi provide useful information concerning the past habitats, their hosts and paleoenvironmental conditions. Some epiphyllous fossils have well-preserved hyphopodia, which can be utilized to understand the biological life history of fungi (Taylor et al. 2015). Furthermore, studying epiphyllous fossil fungi may be important indicators for palaeoenvironmental studies (Saxena et al. 2021). Recent fossil calibrations for epifoliar fungi have been undertaken by Hongsanan et al. (2016) and Samarakoon et al. (2019). There are 24 fossil records of Asterinaceae and 42 Dyadosporites species (Vishnu et al. 2017, Saxena et al. 2021). Asterina eocenica is a well-preserved fossil and was used to estimate the minimum age as 54 Mya (Dilcher 1965, Samarakoon et al. 2019). The minimum age of 145 Mya in Microthyriales was estimated using Microthyriacites fossils described from Argentina, Australia, India, New Zealand, and Russia (Samarakoon et al. 2019). However, the minimum age of Microthyrium is estimated as 55 Mya based on Asterothyrites fossils (Samarakoon et al. 2019). Saxena et al. (2021) described one plant-micro- fossil record of Caldesites nigerianus in Microthyriales. Fossils of ten Meliolinites and 46 Multicellites species have been described (Species Fungorum 2022, Saxena et al. 2021). Hongsanan et al. (2016) estimated the crown of Meliola with the minimum age of 35 Mya. Samarakoon et al. (2019) suggested 54 Mya as the minimum age of the crown node for Meliola. Ženg et al. (2019) estimated the divergence time of the Micropeltidaceae crown group at 130 (165–104) Mya.

Modeling studies for climate-driven global fungal extinctions for epifoliar fungi are very few due to their cryptic nature with difficulties in distinguishing between them in mixed populations on the substrate (Lughadha et al. 2020). However, some studies have shown that a number of species are in decline with increasing temperature, which could eventually cause extinctions (Lughadha et al. 2020). Many epifoliar fungi are obligate biotrophs of plants and the extinction of plants will also significantly impact the number of epifoliar taxa (May et al. 2019). Several experiments have documented how climate change impacts plant communities in boreal regions (Panetta et al. 2018, Reich et al. 2015) with significant loss of fungi detected in 2010 as compared to 1960 in the Alps (Diez et al. 2020). Obligate symbiotic fungal-like epifoliar taxa are assumed to experience a two-stage extinction. First, the extinction of these fungi may occur due to habitat loss or other direct impacts, while the second can occur with the extinction of host plants due to climate change or other factors (Lughadha et al. 2020). Future studies with careful models are needed for predicting the risk of extinction based on climate change and other possible scenarios.

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