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Sequencing of the type species of *Arthopyrenia* places Arthopyreniaceae as a synonym of Trypetheliaceae

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Abstract

Arthopyrenia sensu lato comprises lichenicolous, lichenized and non-lichenized saprotrophic species; however, the lifestyle of several taxa as either lichenized or saprotrophic remains unclear. The systematic position of the genus was so far unresolved: while sequenced species appeared in different clades within Dothideomycetes, the type species, *A. cerasi*, had no molecular data so far. In lieu of sequence data, the family Arthopyreniaceae was assigned to Pleosporales, whereas tropical, lichenized species were reclassified in *Constrictolumina* and *Macroconstrictolumina*, shown to belong in Trypetheliaceae (Trypetheliales). In this study, the generic type, *A. cerasi*, has been sequenced for the first time. Maximum likelihood and Bayesian phylogenetic analyses using mtSSU and nuLSU sequences recovered *Arthopyrenia sensu stricto* as an early diverging lineage within Trypetheliaceae, separate from *Constrictolumina* and *Macroconstrictolumina* but in the same clade as the temperate, non-lichenized *Julella fallaciosa*. Therefore, Arthopyreniaceae is here synonymized under Trypetheliaceae and the taxonomic placement of its type species is discussed based on morphological and phylogenetic evidence. Our phylogenetic results further support the

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polyphyly of non-lichenized, temperate species of *Arthopyrenia* and *Julella sensu lato*. Consequently, *Julella fallaciosa* is transferred to *Arthopyrenia* and its close relationship with *A. cerasi* is discussed. We also conducted ancestor character state analysis to reconstruct lifestyle changes within Trypetheliales using Bayes Traits and Bayesian Binary MCMC approaches.

Keywords – ancestral character state analyses – *Julella* – lifestyles – non-lichens – phylogeny – taxonomy

Introduction

Dothideomycetes is the largest class in Ascomycota (Hyde et al. 2013, Hongsanan et al. 2020a, b). It includes mostly saprotrophic or parasitic fungi but also lichenized lineages that evolved independently relative to other large lichenized classes, such as Arthoniomycetes and Lecanoromycetes (Lutzoni et al. 2001, Gueidan et al. 2008, Nelsen et al. 2009, 2011, Schoch et al. 2009). Several lichenized lineages in Dothideomycetes also include non-lichenized saprotrophic taxa, which either appear as early diverging lineages or have been derived through loss of lichenization. These potentially secondarily saprotrophic fungi are primarily associated with Arthopyreniaceae, Lichenotheliaceae, Monoblastiaceae, Mycoporaceae, Trypetheliaceae and Xanthopyreniaceae (Coppins & Aptroot 2008, Hyde et al. 2013, Aptroot et al. 2016, Pérez-Ortega et al. 2016, Hongsanan et al. 2020b).

Arthopyreniaceae was established by Watson (1929) to encompass lichenized fungi with trentepohlioid photobiont and branched and anastomosing paraphyses. Watson (1929) originally included 13 genera within the family; however, over time only *Arthopyrenia* remained in this family, while other genera were transferred to different families, orders and classes or were subsumed under synonymy of genera in other lineages (Hyde et al. 2013, 2016, Lücking et al. 2017). In the most recent classification, Arthopyreniaceae included only two genera, *viz. Arthopyrenia* and *Mycomicrothelia*, and was assigned to Pleosporales (Hyde et al. 2013, Wijayawardene et al. 2017, 2018, 2020, Hongsanan et al. 2020a). This classification was, however, tentative, since the type species of both genera have not yet been sequenced (Liu et al. 2014, Hyde et al. 2016). Phylogenetic studies placed many species previously classified in *Arthopyrenia* or *Mycomicrothelia* within Trypetheliales (Nelsen et al. 2009, 2011, 2014, Aptroot & Lücking 2016, Hyde et al. 2016, Hongsanan et al. 2020b). *Arthopyrenia* needs critical study with regard to its placement and delimitation, for which the phylogenetic placement of the type species is essential (Hyde et al. 2013, Nelsen et al. 2011, 2014).

Species of Arthopyreniaceae occur in terrestrial habitats and are distributed mainly in tropical and temperate regions, known from both sexual and asexual states (Coppins 1988, Hyde et al. 2013). *Arthopyrenia* itself was introduced by Massalongo (1852) for an assemblage of lichenized, lichenicolous and non-lichenized fungi (Zahlbruckner 1921). It was restudied in detail by Harris (1973, 1975, 1995), Tucker & Harris (1980) and Coppins (1988). Index Fungorum (2021) shows nearly 880 species epithets under this genus name. However, many species have been transferred to Monoblastiaceae, Naetrocymbaceae, Porinaceae, Strigulaceae and Trypetheliaceae and the taxonomy of some species remains uncertain (Coppins 1988). In a more limited sense, *Arthopyrenia* presently comprises 53 species (Species Fungorum 2021), which are chiefly known from temperate regions (Harris & Tripp 2013, Hyde et al. 2013). The taxa are characterized mainly by hamathecium characteristics, such as densely arranged paraphysoids developing from both ends and a K+ sordid-green perithecial wall (Hyde et al. 2013, Hongsanan et al. 2020a). Many *Mycomicrothelia* species have been transferred to *Bogoriella* and *Pseudobogoriella* (Aptroot & Lücking 2016, Hongsanan et al. 2020b) and only nine species are currently retained in *Mycomicrothelia* (Species Fungorum 2021).

In this study, we aim to establish the phylogenetic position of *Arthopyrenia sensu stricto* by sequencing of the type species, *A. cerasi* and thereby reassess its relationship with the already sequenced *Arthopyrenia* species and with sequenced species of the genus *Julella*. We also provide detailed morphological descriptions and molecular data for *Alloarthopyrenia italica* and

Pseudopyrenula endoxanthoides, two confirmed members of Trypetheliaceae, based on new material. Ancestor character state analysis was performed to reconstruct lifestyle changes in Trypetheliales using Bayes Traits and Bayesian Binary MCMC approaches.

Material & Methods

Phenotypic analyses

Fresh material of *Arthopyrenia cerasi* and *Arthopyrenia italica* was collected in Europe, whereas *Pseudopyrenula endoxanthoides* was collected in Thailand. Specimens were examined using a Motic SMZ 168 dissecting microscope. Hand sections of the ascomata were mounted in water, 5% KOH and Lugol's solution to examine micro-morphological characteristics. Macro-morphological structures were observed with a stereo microscope (Motic SMZ-168) and photographed with Zeiss discovery v8 stereomicroscope (Carl Zeiss, Jena, Germany). Micro-morphological details were studied using a Nikon ECLIPSE 80i compound microscope fitted with a Canon 550D digital camera. For *Arthopyrenia cerasi*, macroscopic photographs were made with a Keyence VHX-5000 Digital Microscope and a VH-Z20R/W/T lens, while microscopic photographs were prepared using an Olympus BX51 compound microscope, fitted with an Olympus SC50 digital camera.

All microscopic measurements were done in water-mounted slides and made with Tarosoft Image Frame Work (09.0.7). Images used for figures were processed with Adobe Photoshop CS6 Extended 10.0 software (Adobe Systems, USA). The material of *Arthopyrenia cerasi* is deposited in the Royal Botanic Garden Edinburgh (E) and in the Meise Botanic Garden (BR). The material of *Arthopyrenia italica* and *Pseudopyrenula endoxanthoides* is deposited in the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand. Index Fungorum and Faces of Fungi numbers were registered following Index Fungorum (2021) and Jayasiri et al. (2015).

DNA extraction, PCR amplification and sequencing

DNA isolation of *Arthopyrenia cerasi* was carried out from hand-made sections of ascomata by the direct PCR method as described in Ertz et al. (2015, 2018). For the other taxa, an E.Z.N.A. ® Forensic DAT (D3591 – 01, Omega Bio – Tek) DNA extraction kit was used to extract DNA from fruiting structures by following the manufacturer's instructions. DNA samples that were intended for use as a template for PCR were stored at 4^{o}C for use in regular work and duplicated at -20^{o}C for long-term storage. DNA sequence data were obtained from partial sequences of ribosomal and mitochondrial coding genes to generate following gene markers: Mitochondrial small subunit spacers (12S, mtSSU) and large subunit nuclear rDNA (28S, LSU) and amplified with primer pairs mrSSU1 and mrSSU3R (Zoller et al. 1999) and LR0R and LR5 (Vilgalys & Hester 1990) respectively. The PCR amplification was performed following Ertz et al. (2018) for *A. cerasi* and Thiyagaraja et al. (2021) for other taxa using a final volume of 25 µl, comprised of 2.0 µl of DNA template, 1 µl of each forward and reverse primers, 12.5 µl of Taq PCR Super Mix (mixture of Easy Taq TM DNA Polymerase, dNTPs, obtained buffer (Beijing Trans Gen Biotech Co., Chaoyang District, Beijing, PR China)) and 8.5 µl of sterilized water. PCR products were examined on 1% agarose electrophoresis gels and stained with ethidium bromide and sent for sequencing to Macrogen® for *Arthopyrenia cerasi* and to Tsingke (Yunnan Province, P.R. China) for the other taxa. New nucleotide sequence data acquired were deposited in GenBank (Table 1). Alignments and phylogenetic trees were submitted to TreeBASE under submission number 28222.

Taxon sampling

The BLAST search engine of the National Centre for Biotechnology Information (NCBI) was used for the preliminary identification of newly generated DNA sequences (https://www.ncbi.nlm.nih.gov). After confirming that all newly generated sequences represented Trypetheliaceae, selected sequences of Trypetheliales (including Polycoccaceae) were retrieved from GenBank. Initially, phylogenetic analyses were conducted for Trypetheliales and their close

relatives following Hongsanan et al. (2020b). Outgroup taxa were selected from representatives of Dothideales, Capnodiales and Myriangiales (Hongsanan et al. 2020b). The final combined alignment comprised 118 terminals (Table 1), 83 of which had mtSSU and 109 of which had nuLSU sequence data.

Phylogenetic analyses and species recognition

Phylogenetic analyses of both single marker and concatenated data were performed under maximum likelihood and Bayesian inference. Sequences of the mtSSU and the nuLSU were aligned separately using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/ server/index.html, Katoh et al. 2019). Terminal ends of sequences and ambiguous regions were trimmed manually using BioEdit v. 7.0.5.2 (Hall 1999). The phylogenetic web tool "ALTER" (Glez-Peña et al. 2010) was used to convert sequence alignment from FASTA to PHYLIP for RAxML analysis and from FASTA to NEXUS format for Bayesian analysis. Best models for the BI approach was established independently for each locus using MrModeltest v.2.2 (Nylander 2004). The ML tree was generated using the RAxML-HPC2 on XSEDE (8.2.8) (Stamatakis 2014) on 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 & Ronquist 2001). Markov Chain Monte Carlo sampling (MCMC), was run for 50 000 000 generations and trees were sampled every 100th generations. 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. No conflict was detected between individual markers and so the final analyses were performed on the concatenated data set. Resulting trees were drawn in FigTree v1.4.0 (Rambaut 2014), then copied to Microsoft PowerPoint 2013 and converted to jpeg files using Adobe Photoshop CS6 Extended 10.0 (Adobe Systems. U.S.A.).

Ancestral character state analyses

Ancestral character state analyses were carried out to reconstruct the evolutionary relationship of lifestyle changes in Trypetheliales. The following states were established: lichenized, non-lichenized saprotrophic, borderline lichenized (i.e. weakly lichenized, with a whitish, thallus like area but few photobiont cells only), lichenicolous, and plant-pathogenic. The platform Reconstruct Ancestral State in Phylogenies (RASP 3.2.1) was used to construct ancestral character analyses, using the two approaches of Bayes Traits and Bayesian Binary MCMC based on the ML tree (Yu et al. 2015, 2019). Both approaches were performed and visualized in RASP 3.2.1 using settings as follows: 1 010 000 iterations for Bayes Traits 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 sample frequency of 100, a temperature of 0.1, state frequencies fixed (JC), and among-site rate variation equal. The trees were edited using Microsoft PowerPoint 2013 and converted to jpeg files using Adobe Photoshop CS6 Extended 10.0 (Adobe Systems. U.S.A.).

Table 1 Taxa names, strain numbers and GenBank accession numbers of the taxa used for phylogenetic analyses. The newly generated sequences are indicated in black boldface

Taxa	Strain	GenBank Accessions	
		mtSSU	nuLSU
Alloarthopyrenia italica	MFLU 15-0399	KX655555	KX655550
Alloarthopyrenia italica	**MFLU 17-1689**	**MZ221609**	–
Aptrootia elatior	MPN560B	KM453821	KM453754
Aptrootia robusta	MPN235B	KM453822	KM453755
Aptrootia terricola	F 17211	DQ328995	–
Architrypethelium lauropaluanum	MPN48	KX215566	KX215605
Architrypethelium nitens	MPN257	KM453823	KM453757
Architrypethelium uberinum	MPN489	–	KM453758
Arthopyrenia cerasi	**Coppins 25807 (BR)**	**MZ221617**	–

Table 1 Continued.

Taxa	Strain	GenBank Accessions	
		mtSSU	nuLSU
Arthopyrenia fallaciosa	MPN141	JN887411	JN887399
Arthopyrenia fallaciosa	MPN547	JN887412	JN887400
Arthopyrenia salicis	CBS 368.94	AY538345	AY538339
Astrothelium aeneum	MPN302	–	KX215606
Astrothelium aff. *crassum*	MPN335	KM453827	KM453761
Astrothelium confusum	Nelsen 4004a (F)	GU327685	GU327710
Astrothelium croceum	Nelsen 211D (F)	KX215567	KX215611
Astrothelium leucoconicum	MPN42	KM453830	KM453764
Astrothelium macrocarpum	MPN260	KM453829	KM453763
Astrothelium macrocarpum	NSR6	AB759879	LC127402
Astrothelium neglectum	TAK8	LC128025	LC127410
Astrothelium neovariolosum	KY777	LC128023	LC127408
Astrothelium nitidiusculum	MPN704	KM453868	KM453804
Astrothelium norisianum	MPN52C	KM453848	KM453783
Astrothelium perspersum	AFTOL2099	GU561848	FJ267701
Astrothelium siamense	KRB139	LC128021	LC127406
Astrothelium subcatervarium	Nelsen 4009a (F)	GU327707	GU327729
Astrothelium variolosum	MPN41	KX215585	KX215662
Bathelium lineare	MPN741	KM453839	KM453774
Bathelium porinosporum	MPN744	KX215586	KX215665
Bathelium porinosporum	MPN747	KX215587	KX215667
Bathelium tuberculosum	MPN112	–	KX215668
Bathelium tuberculosum	MPN113	–	KX215669
Bathelium tuberculosum	MPN81	KM453842	KM453777
Bogoriella oleosa	MPN700	KM453857	KM453794
Bogoriella oleosa	Nelsen 4007a (F)	GU327697	GU327721
Capnodium coffeicola	MFLUCC 15-0206	–	KU358920
Chaetothyriothecium elegans	CPC 21375	–	NG 058861
Constrictolumina cinchonae	Lücking 29583	JN872349	JN872351
Constrictolumina cinchonae	MPN417	KM453825	KM453759
Constrictolumina planorbis	MPN330	–	KX215670
Constrictolumina planorbis	MPN331	–	KX215671
Constrictolumina planorbis	MPN332	–	KX215672
Dictyomeridium proponens	MPN359	JN887415	JN887403
Dothidea eucalypti	CBS:143417	–	MG386106
Dothidea sambuci	DAOM 231303	AY544739	AY544681
Elsinoe centrolobii	CBS 222.50	–	NG 069000
Elsinoe lepagei	CBS 225.50	–	KX887004
Elsinoe phaseoli	CBS 165.31	–	DQ678095
Hortaea werneckii	CBS 708.76	GU561844	GU301818
Macroconstrictolumina malaccitula	MPN574	KM453824	–
Marcelaria cumingii	MPN552	KM453854	KM453789
Marcelaria cumingii	UBN137	LC034284	–
Marcelaria cumingii	RAMK:027993	LC223105	LC223104
Marcelaria purpurina	MPN323A	KM453855	KM453790
Natipusilla decorospora	ILL:AF236-1	–	NG 060263
Natipusilla limonensis	ILL:AF286-1	–	NG 060264
Natipusilla naponensis	ILL:AF217-1	–	NG 060265
Neomicrothyrium siamense	IFRDCC 2194	–	JQ036228
Nigrovothelium bullatum	MPN114	KX215589	KX215673
Nigrovothelium bullatum	MPN579	KX215590	KX215674
Nigrovothelium bullatum	MPN82	KX215591	KX215675
Nigrovothelium tropicum	MPN44	KX215592	KX215679
Nigrovothelium tropicum	MPN561	KX215593	KX215680
Nigrovothelium tropicum	MPN658	KX215594	–

Table 1 Continued.

Taxa	Strain	GenBank Accessions	
		mtSSU	nuLSU
Phaeotrichum benjaminii	CBS 541.72	AY538349	NG_057709
Polycoccum pulvinatum	Ertz 18114 (BR)	–	KT383806
Polycoccum vermicularium	Diederich 17545	–	KT383808
Polymeridium albocinereum	MPN439	KM453858	KM453795
Polymeridium catapastum	MPN358	KM453859	JN887402
Polymeridium subcinereum	CBS 130779	KC592287	–
Polypyrenula sexlocularis	RMG058	MK503260	MK503261
Polypyrenula sexlocularis	RMG057	MK503257	MK503258
Pseudobogoriella hemisphaeria	Lücking 28641 (F)	GU327695	GU327719
Pseudobogoriella miculiformis	Lücking 28637 (F)	GU327696	GU327720
Pseudobogoriella minutula	MPN567	KM453856	–
Pseudopyrenula aff. *subgregaria*	MPN288	–	KX215682
Pseudopyrenula diluta	MPN362	KM453861	KM453797
Pseudopyrenula diluta	MPN697	KM453862	KM453798
Pseudopyrenula endoxanthoides	Lücking 24079 (F)	GU327699	GU327724
Pseudopyrenula endoxanthoides	MPN573	KX215595	–
Pseudopyrenula endoxanthoides	**MFLU 20-0542**	**MZ221618**	**MZ206304**
Pseudopyrenula subgregaria	MPN391	KM453863	KM453799
Pseudopyrenula subgregaria	MPN568	KX215597	KX215684
Pseudopyrenula subnudata	MPN293	KM453865	KM453801
Roussoella nitidula	MFLUCC 11-0182	–	KJ474843
Roussoella nitidula	MFLUCC 11-0634	–	KJ474842
Roussoella solani	CBS 141288	–	MH878207
Roussoellopsis tosaensis	KT 1659	–	AB524625
Sympoventuria capensis	CPC 12839	–	MK810809
Sympoventuria capensis	CPC 12840	–	MK810810
Teratosphaeria hortaea	CBS 124156	–	MH874881
Teratosphaeria tinarooa	CBS 124583	–	MH874910
Trichodelitschia bisporula	CBS 262.69	–	MH871039
Trypethelium eluteriae	Lumbsch 19701a (F)	KM453874	GU327726
Trypethelium eluteriae	MPN563	KX215599	KX215686
Trypethelium foveolatum	MPN351	KM453881	KM453816
Trypethelium inamoenum	MPN228	KM453875	KM453810
Trypethelium platyleucostomum	MPN349	KM453870	KM453806
Trypethelium platyleucostomum	MPN350	KX215602	KX215688
Trypethelium platystomum	TSL35	AB759868	–
Trypethelium rubroplatystomum	MPN54	KM453871	KM453807
Trypethelium rubroplatystomum	MPN64	–	KX215689
Trypethelium rubroplatystomum	MPN65C	KX215603	KX215690
Trypethelium sprengelii	MPN200B	–	KX215691
Trypethelium sprengelii	MPN382	KM453867	KM453803
Trypethelium subeluteriae	MPN49C	KM453882	KM453818
Trypethelium subeluteriae	MPN748	KX215604	KX215693
Tumidispora shoreae	MFLUCC 14-0574	–	KT314074
Venturia albae	CBS 471.61	–	MK810840
Venturia chlorospora	CBS 466.61	–	MK810844
Venturia inaequalis	CBS 535.76	–	EU035460
Viridothelium tricolor	MPN399	KM453844	KM453779
Viridothelium tricolor	MPN646	KM453845	KM453780
Viridothelium virens	RAMK:030224	LC223103	LC223102
Viridothelium virens	AFTOL-ID 1774	KT232227	–
Zeloasperisporium eucalyptorum	CBS 124809	–	NG 057835
Zeloasperisporium pterocarpi	MFLUCC 17-0910	–	MH763755
Zeloasperisporium searsiae	CPC 25880	–	NG 059617

Results

Phylogenetic analyses

The final alignment comprised 1775 nucleotide positions (mtSSU: 891, nuLSU: 884). The single gene tree topologies and the combined tree topology were compared manually and were found to be congruent. The best scoring ML tree was selected to represent the relationships among the taxa, with the final ML optimization likelihood value of –22431.292969 (Fig. 1). The parameters for the GTR+I+G model of combined mtSSU and nuLSU were as follows: estimated base frequencies; A = 0.305349, C = 0.167827, G = 0.247710, T = 0.279114, substitution rates; AC = 1.088265, AG = 2.892696, AT = 2.180779, CG = 1.068550, CT = 5.897541 and GT = 1.0, proportion of invariable sites I = 0.268570; gamma distribution shape parameter $\alpha = 0.588111$. The ML and Bayesian analyses both resulted in trees with similar topologies. Bayesian posterior probabilities from MCMC were evaluated with final average standard deviation of split frequencies = 0.001817.

The overall phylogeny matched that of previous studies, including the sister group relationship of Polycoccaceae and Trypetheliaceae and the large, supported, fully lichenized clade ranging from *Pseudopyrenula* to *Astrothelium* (Fig. 1). The early diverging lineages representing *Alloarthopyrenia*, *Bogoriella*, *Constrictolumina*, *Macroconstrictolumina*, *Polypyrenula*, and *Pseudobogoriella* (Fig. 1). *Arthopyrenia cerasi* clustered with strong support with *Julella fallaciosa* within Trypetheliaceae, whereas *A. salicis* fell within Roussoellaceae (Pleosporales). The newly generated sequences of *Allarthopyrenia italica* and *Pseudopyrenula endoxanthoides* fell with their counterparts representing the same species (Fig. 1).

Ancestral character state analyses

The supported subclade representing Trypetheliaceae *sensu stricto*, starting with the genus *Pseudopyrenula*, exhibits an exclusively lichenized lifestyle, whereas the early diverging clades (Trypetheliaceae *sensu lato*) show frequent lifestyle switches, including lichenized, non-lichenized saprotrophic, lichenicolous, and borderline lichenized taxa. The sister family, Polycoccaceae, is entirely lichenicolous. Borderline lichens appear in various clades, associated with or nested within either lichenized and saprotrophic lineages. The lichenized *Macroconstrictolumina malaccitula* formed a sister clade with the borderline lichenized genus *Bogoriella*. The saprotrophic *Arthopyrenia cerasi* was strongly supported as sister to the saprotrophic *Julella*. The borderline lichenized *Polypyrenula sexlocularis*, was associated with the saprotrophic genus *Alloarthopyrenia*, while *Bogoriella* and *Pseudobogoriella* included weakly (borderline) to more distinctly lichenized taxa. Bayesian MCMC analysis reconstructed the ancestor of Trypetheliaceae as likely lichenized, while Bayes Traits reconstructed the same node as ambiguous and also the basal nodes of the remaining early diverging lineages as ambiguous (Fig. 2). Indeed, while Trypetheliaceae as a whole were reconstructed as de novo lichenized, the reconstruction of gains and losses of lichenization in the early diverging lineages of the family was ambiguous. However, there was a single transition towards stable lichenization associated with a more complex thallus anatomy in the large clade ranging from *Pseudopyrenula* to *Astrothelium*, supported by both analyses (Fig. 2).

Taxonomy

Trypetheliaceae Eschw., Syst. Lich.: 17 (1824)
Type genus: *Trypethelium* Spreng., Anleit. Kennt. Gew. 3: 350 (1804)
≡ Arthopyreniaceae Walt. Watson, New Phytol. 28: 107 (1929), syn. nov,
Type genus: *Arthopyrenia* A. Massal.

Arthopyrenia A. Massal., Ric. Auton. Lich. Crost. (Verona): 165 (1852)
Type: *Arthopyrenia cerasi* (Schrad.) A. Massal., Ric. Auton. Lich. Crost. (Verona): 167 (1852)

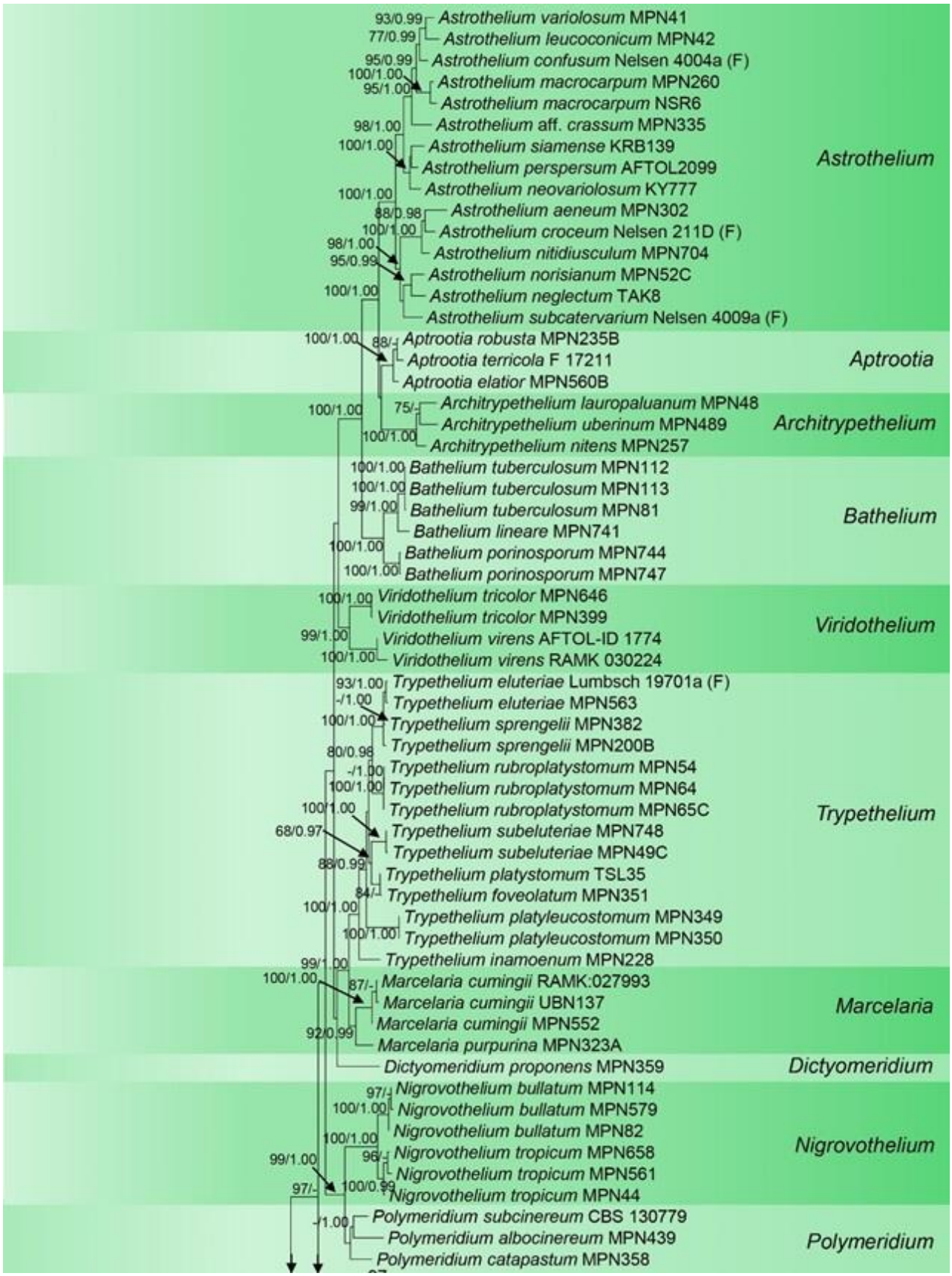

Figure 1 – RAxML tree based on analyses of combined mtSSU and nuLSU partial sequence data. Bootstrap support values for ML equal to or greater than 75%, and Bayesian posterior probabilities (BP) equal to or greater than 0.95 are given as ML/BP above the nodes. The newly generated strains and taxa used in this study are displayed in blue boldface.

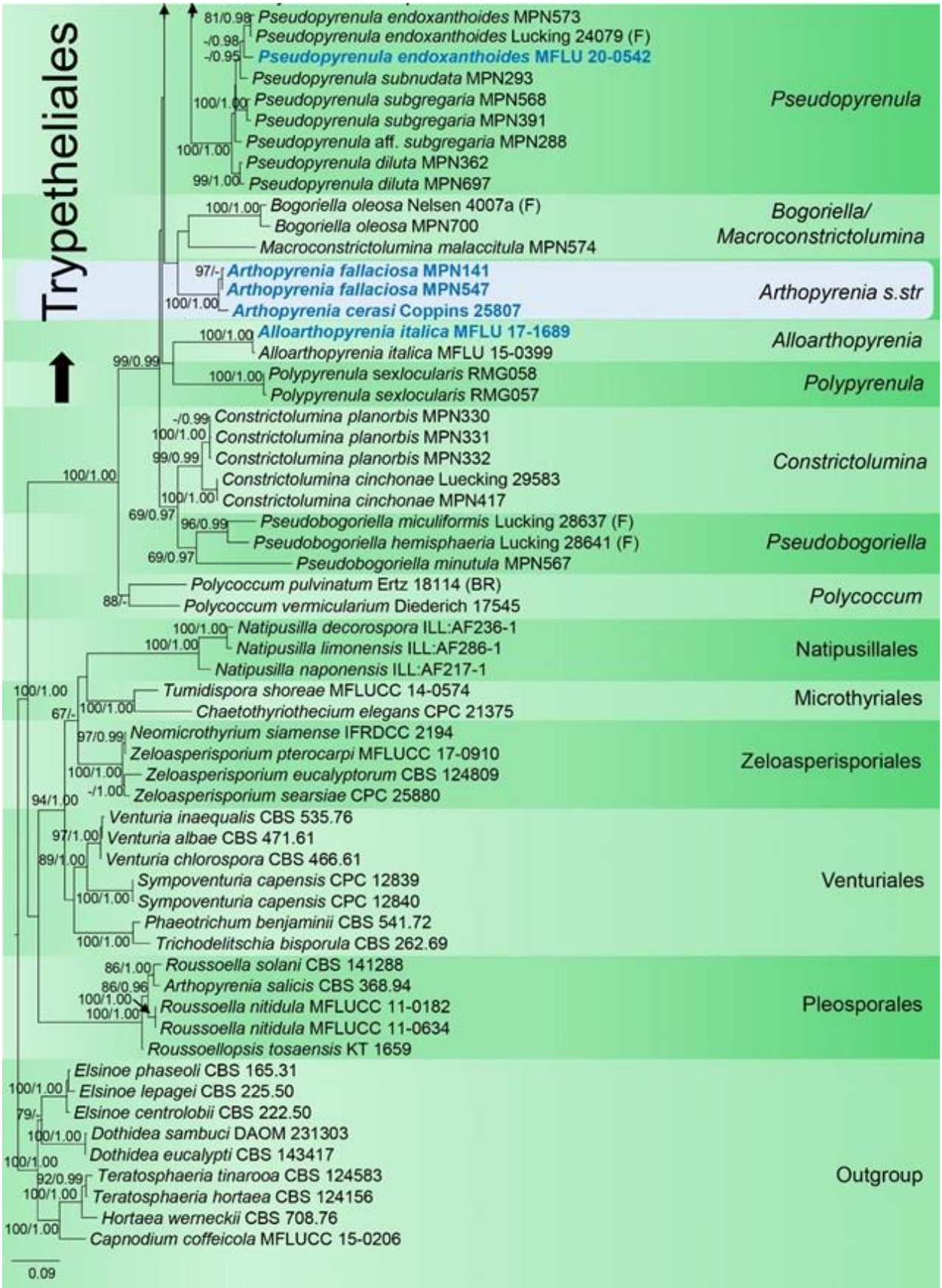

Figure 1 – Continued.

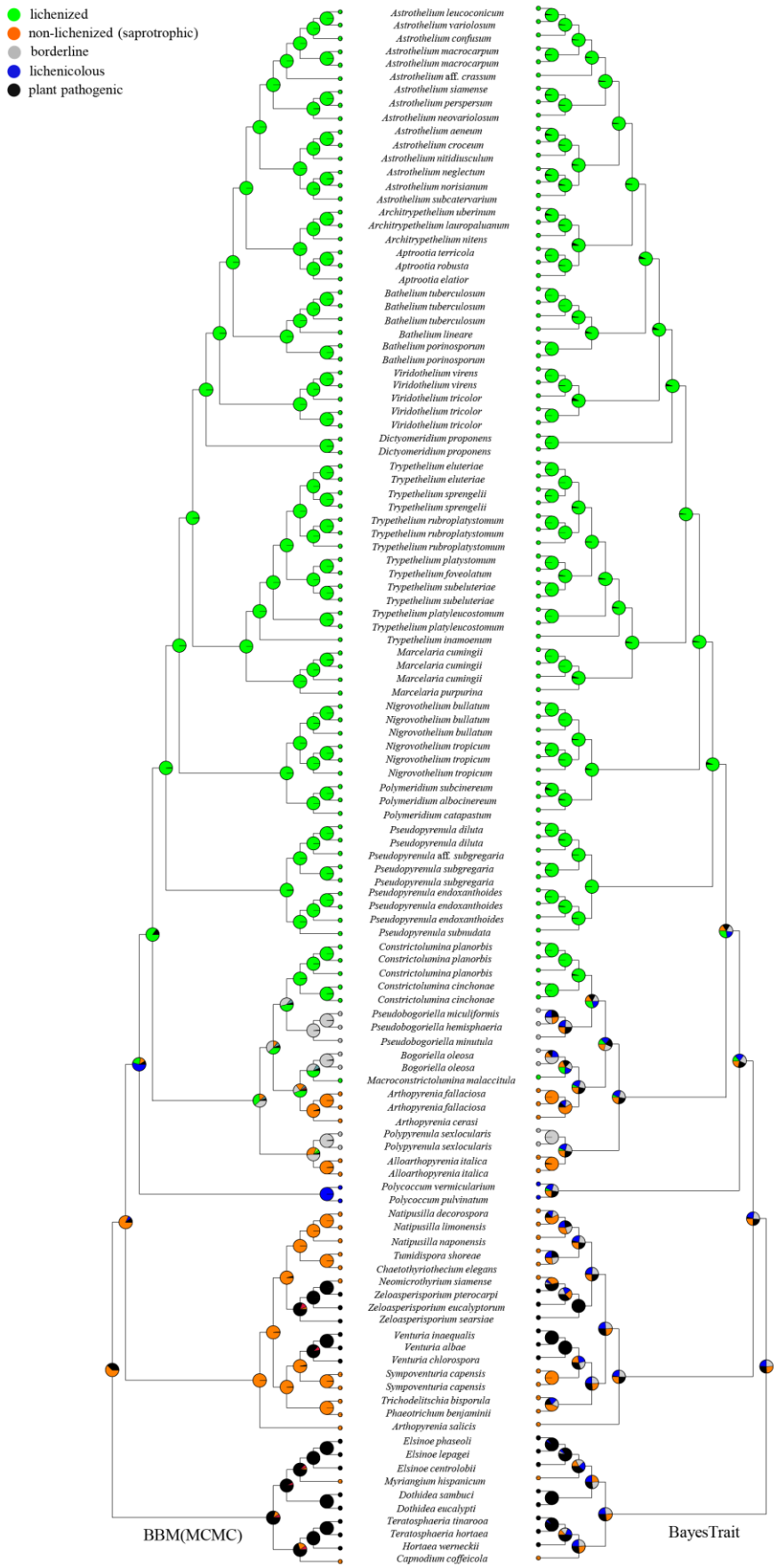

Figure 2 – Ancestral character state analyses using Bayesian MCMC (left) and Bayes Traits (right). Color symbols indicates: green – lichenized, orange – non-lichenized saprotrophic, grey – borderline lichen, blue – lichenicolous, black – plant pathogens

Notes – *Arthopyrenia* was introduced by Massalongo (1852), originally including 13 species but without indicating a type species. Among these, only *Arthopyrenia analepta*, *A. cerasi*, and *A. salicis* have remained in this genus over time, while the remaining species have been transferred to different genera and families. Massalongo (1854) himself transferred two *Arthopyrenia* species to the newly established *Acrocordia*. *Arthopyrenia* was first lectotypified by Fink (1910) with *A. rhyponta*, which subsequently became a member of *Naetrocymbe*. When the generic name *Arthopyrenia* was conserved, *A. cerasi* was chosen as the conserved type (Gams 1999). The taxonomy of *Arthopyrenia* was studied by Jatta (1911), Vainio (1921), Harris (1973, 1975), Tucker & Harris (1980) and Coppins (1988). Harris (1975) established Naetrocymbaceae to accommodate the single genus *Naetrocymbe*, transferring a few non-lichenized species to that genus and retaining *Arthopyrenia*, *Julella* and *Mycomicrothelia* within Arthopyreniaceae. *Naetrocymbe* species were thereby characterized by short-celled paraphyses with refractive bodies near the septa, obpyriform asci with a distinctive apical region, lacking a nasse, and short, rod-shaped microconidia. Aptroot (1998, 2002) emphasized the branched pseudoparaphyses as an important character. Nonetheless, many authors accepted Harris's classification (Eriksson et al. 2003, Nelsen et al. 2009, 2011, 2014, Hyde et al. 2013, Wijayawardene et al. 2017, 2018, 2020, Hongsanan et al. 2020a). Harris (1995) pointed out the close relationship between *Julella* and *Arthopyrenia*, the only difference being ascospore septation, a notion supported by other authors (Aptroot et al. 2008, Nelsen et al. 2011). The first sequenced non-lichenized species thus far retained within *Arthopyrenia*, *A. salicis*, was subsequently resolved within Pleosporales (Pinnoi et al. 2007, Zhang et al. 2008, Sar et al. 2009, Nelsen et al. 2011, 2014, Liu et al. 2014). In contrast, tropical lichenized species nested within Trypetheliales, rendering *Arthopyrenia* polyphyletic (Nelsen et al. 2011). Our results now show that even non-lichenized species of *Arthopyrenia* form a polyphyletic assembly and that *Arthopyrenia sensu stricto* is to be placed within Trypetheliaceae.

Arthopyrenia cerasi (Schrad.) A. Massal., Ric. auton. lich. crost. (Verona): 167 (1852) Fig. 3

Index Fungorum number: IF 377023; Facesoffungi number: FoF 09867

Description (adapted from Coppins & Orange 2009 and Hyde et al. 2013 and including assessment of sequenced material). *Thallus* inconspicuous or slightly bleaching the bark, endophloeodal. *Photobiont* absent. Sexual morph: *Ascomata* perithecial, c. 300–500 µm diam., black, rounded to often ellipsoid, somewhat adnate, ostiolate. *Ostiole* distinct, centrally located. *Involucrellum* dark brown, K+ greenish. *Exciple* light brown. *Pseudoparaphyses* slender, anastomosing, 1.5–2 µm. *Asci* 80–85 µm, 8-spored, bitunicate, cylindrical-clavate. *Ascospores* 17–22 × 5–7 µm, irregularly biseriate, hyaline, clavate to obovoid, rounded at the apex when mature, constricted at each septum, 3-septate, with a gelatinous sheath c. 2 µm thick in K. Asexual morph: *Pycnidia* 80–120 µm, with either macro- or microconidia. *Macroconidia* 11–13 × 2–3 µm, oblong, hyaline, 3-septate. *Microconidia* 9–14 × 0.8 µm.

Material examined – Great Britain, Scotland, VC82, East Lothian, Stenton, Cow Cleugh Burn, Grid NT617718, 160 m elev., on *Corylus* in small valley woodland, 18 v 2020, B.J. & A.M. Coppins 25807 (BR, E).

Notes – *Arthopyrenia cerasi* is characterized by a non-lichenized thallus, perithecial ascomata with a brown, K+ greenish involucrellum, anastomosing pseudoparaphyses, cylindrical asci with hyaline 3-septate ascospores and conidiomata producing frequently 3-septate macroconidia (Coppins 1988). This species often grows on *Corylus* spp. and is distributed in Europe (Coppins & Orange 2009).

Arthopyrenia fallaciosa (Stizenb. ex Arnold) Thiyagaraja, Ertz, Lücking, Coppins and K.D. Hyde comb. nov.

Index Fungorum number: IF 558410; Facesoffungi number: FoF 09868

Basionym: *Polyblastia fallaciosa* Stizenb. ex Arnold, Flora, Regensburg 46: 604 (1863).

Obligate synonyms: *Pyrenula fallaciosa* (Stizenb. ex Arnold) Willey, Enum. Lich. New Bedford: 39 (1892); *Verrucaria fallaciosa* (Stizenb. ex Arnold) Nyl., Lich. Envir. Paris: 127

(1896); *Mycoglaena fallaciosa* (Stizenb. ex Arnold) Vain., Acta Soc. Fauna Flora fenn. 49(no. 2): 166 (1921); *Polyblastiopsis fallaciosa* (Stizenb. ex Arnold) Zahlbr., Cat. Lich. Univers. 1: 348 (1922); *Julella fallaciosa* (Stizenb. ex Arnold) R.C. Harris, in Egan, Bryologist 90(2): 163 (1987).

Notes – Our updated phylogeny resulted in a close relationship between *Arthopyrenia cerasi* and *Julella fallaciosa*, to the point that the latter is to be placed within *Arthopyrenia*. This is supported by the fact that, apart from the transversely septate vs. muriform ascospores, *J. fallaciosa* and related species are morphologically and anatomically very similar to *Arthopyrenia sensu stricto* and including these in *Arthopyrenia* has been suggested even prior to molecular studies (Harris 1995). Lücking et al. (in Hongsanan et al. 2020b) provided a discussion on the taxonomy of *Julella*, pointing out that at least two groups can be distinguished: species related to the type, *J. buxi*, and species related to *J. lactea*, for which the genus name *Polyblastiopsis* is available. *Julella fallaciosa* arguably belongs to the latter group, which implies that *Polyblastiopsis* would be a synonym of *Arthopyrenia*. However, more species currently classified within *Julella* need to be sequenced to clarify this. Given that *Polyblastiopsis* is younger than *Arthopyrenia*, those species clustering with *A. cerasi* can be safely combined into the latter genus.

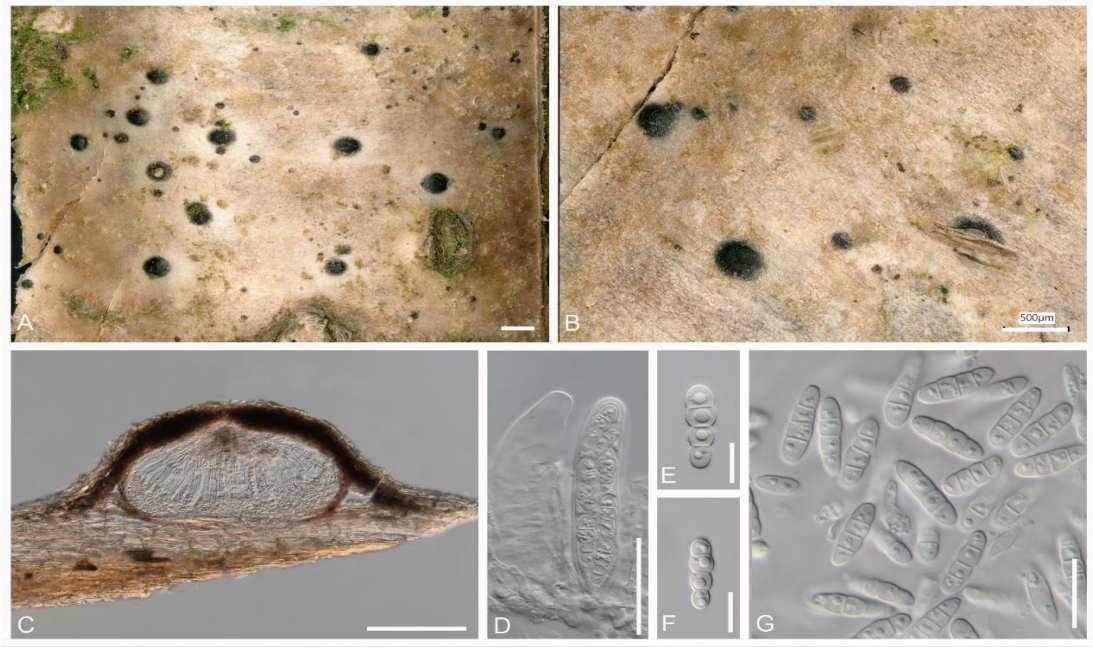

Figure 3 – *Arthopyrenia cerasi* (Coppins 25807). A, B Ascomata and conidiomata on bark. C Cross section of ascoma in water. D Asci in water. E, F Ascospores in water. G Macroconidia in water. Scale bars: B = 500 µm, C = 100 µm, D = 30 µm, E–G = 10 µm

Alloarthopyrenia italica Phukhams., Camporesi, Ariyaw. & K.D. Hyde, Fungal Diversity 80: 135 (2016) Fig. 4

= *Arthopyrenia cinereopruinosa* auct., non Schaerer (1836).

Index Fungorum number: IF 552237, Facesoffungi number: FoF 02380

Non-lichenized on bark. *Thallus* inconspicuous, whitish grey, pruinose, corticolous, crustose, epiphloeodal. *Prothallus* absent. *Photobiont* not detected. Sexual morph: *Ascomata* perithecial, approximately 135–165 µm high × 300–375 µm diam., black, circular to ellipsoidal, slightly erumpent, somewhat adnate, ostiolate. *Ostiole* distinct, centrally located, filled with periphyses. *Involucrellum* dark brown, K+ slightly greenish. *Exciple* 40–70 µm, light brown. *Hamathecium* 75–110 µm high × 195–210 µm diam. *Pseudoparaphyses* robust, ± distantly branched, numerous and anastomosed. *Asci* 55–65 × 13–20 µm ($\bar{x}$ = 60 × 16.5 µm, n = 40), 8-spored, bitunicate, cylindrical, tholus thickened, ocular chamber up to 2–3 µm, apically rounded, poorly developed stipe, inversely funnel-shaped ocular chamber. *Ascospores* 18–22 (24) × 8–12 µm ($\bar{x}$ = 20 × 10 µm, n = 40), multi-

seriate, hyaline, clavate to obovoid, 1-septate, rounded at the apex when mature, strongly constricted at the septa, upper cell wider than lower cell, gelatinous sheath distinct, 1–3 µm thick. Asexual morph: unknown.

Material examined – Italy, Province of Forlì-Cesena, near Monte Mirabello – Predappio,16 September 2017, on living *Fraxinus ornus*, Erio Camporesi (MFLU 17-1689).

Chemistry – Thallus I-, Ascomatal gel I-, K-. Asci I-, K-. Ascospores I-, K-.

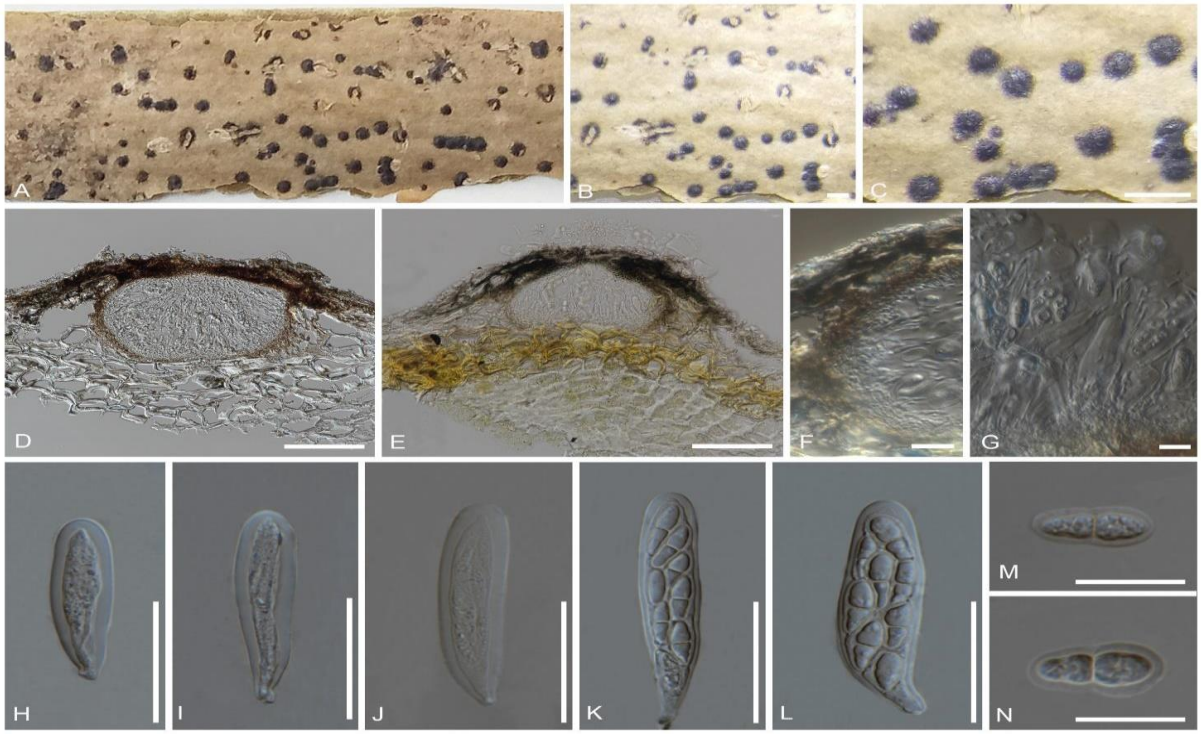

Figure 4 – *Alloarthopyrenia italica* (MFLU 17-1689). A– C, Ascomata on bark. D Cross section of ascoma in water. E Cross section of ascoma in 5% KOH. F Peridium. G Pseudoparaphyses. H–L Asci in tap water. M, N Ascospores in tap water. Scale bars: B–C = 500 µm, D, E = 100 µm, F = 50 µm, G = 10 µm, H–L, = 30 µm, M, N = 20 µm.

Notes – *Alloarthopyrenia* was introduced to accommodate a single species, *A. italica* (Hyde et al. 2016). The species was collected from living bark of branches of *Fraxinus ornus* in Italy (Hyde et al. 2016). The species shares morphological characteristics with other non-lichenized *Arthopyrenia* species (Hyde et al. 2016). Here we provide additional data for *Alloarthopyrenia italica* from material that was also collected in Italy. The resemblance of this species to *Arthopyrenia cinereopruinosa* (Schaer.) A. Massal. is striking and a more detailed comparison of these two taxa is in order. *Alloarthopyrenia italica* was originally separated from *Arthopyrenia cinereopruinosa* based on larger ascomata and 3-septate ascospores. The presumed type material of *A. cinereopruinosa* (basionym: *Verrucaria cinereopruinosa*) in H (Switzerland, Schaerer s.n., H-NYL 6684) has a leprose thallus and ascomata with 3-septate ascospores that somewhat resemble *Chrysothrix caesia* [https://plants.jstor.org/stable/viewer/10.5555/al.ap.specimen.h9507588]. This material cannot be original, as it was only published in Schaerer's exsiccate, *Enum. Critic. Lich. Europ.*, as number 243, in 1850 [https://www.biodiversitylibrary.org/item/109124#page/287/mode/1up], when the species was validly established 14 years earlier (Schaerer 1836). In the latter protologue, Schaerer (1836) listed four specimens, all as names but not representing the types of these names. Three of these were associated with the name *Verrucaria cinereopruinosa* and one with the variety *galactina*, based on *Arthonia punctiformis* var. *galactina*. The first specimen, originally identified as *Verrucaria stigmatella* by Schlechtendahl, is deposited in G and available on JSTOR

[https://plants.jstor.org/stable/viewer/10.5555/al.ap.specimen.g00290379]. *Verrucaria stigmatella* was described as non-leprose thallus and perithecia that are almost pseudostromatic, with clusters of up to 10 small perithecia, whereas typical *A. cinereopruinosa* as currently circumscribed has single to sometimes grouped, larger perithecia not in such clusters. It does not correspond to the modern concept of *A. cinereopruinosa* but rather corresponds to *Naetrocymbe punctiformis*. It therefore appears that what has usually been regarded *A. cinereopruinosa* (e.g. Harris 1975, Coppins 1988) [https://fungi.myspecies.info/all-fungi/*arthopyrenia-cinereopruinosa*] is not conspecific with the type of that name but corresponds to what is now named *Alloarthopyrenia italica*.

Pseudopyrenula endoxanthoides Vain., Hedwigia 46: 180 (1907) Fig. 5

Index Fungorum number: IF 402510; Facesoffungi number: FoF 09869

Lichenized on bark. *Thallus* present, whitish, non-pruinose, corticolous, crustose, epiphloeodal. *Prothallus* present. *Photobiont* trentepohlioid. Sexual morph: *Ascomata* perithecial, 245–315 µm high × 375–500 µm diam., black, rounded to ellipsoid, slightly erumpent, somewhat adnate, ostiolate. *Ostiole* distinct, centrally located. *Involucrellum* 20–45 µm thick, light brown, no color change in KOH. *Exciple* 15–40 µm, dark brown. *Hamathecium* 180–230 µm high × 245–295 µm diam, occasionally yellow. *Pseudoparaphyses* robust, ± distantly branched, numerous and anastomosed. *Asci* 65–75 × 10–15 µm ($\bar{x}$ = 70 × 12.5 µm, n = 40), 8-spored, bitunicate, cylindrical, tholus thickened, ocular chamber inversely funnel-shaped, up to 4–5 µm, apically rounded, with a well-developed stipe. *Ascospores* 17–20 × 4–10 µm ($\bar{x}$ = 18.5 × 7 µm, n = 40), irregularly biseriate, hyaline to pale yellow, clavate to obovoid, rounded at the apex when mature, 1–3-septate, gelatinous sheath distinct, 1–2 µm thick. Asexual morph: unknown.

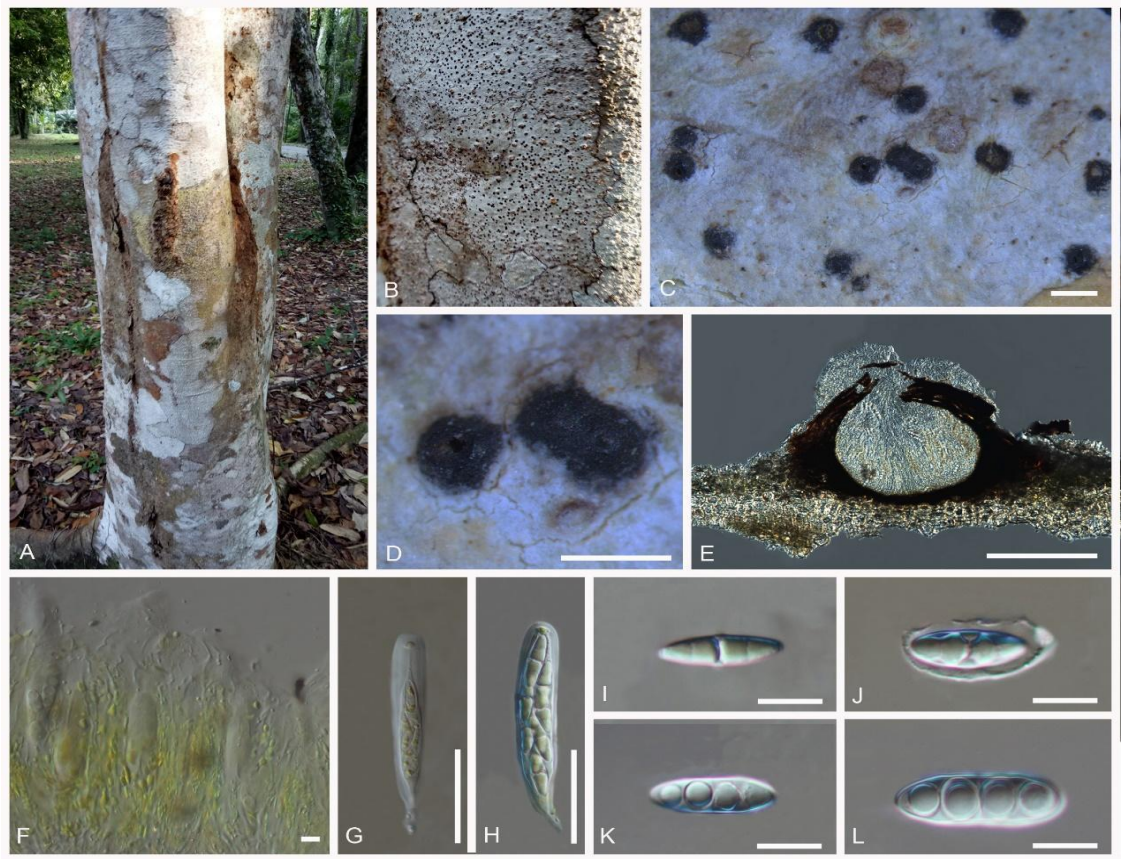

Figure 5 – *Pseudopyrenula endoxanthoides* (MFLU 20-0542). A–D Ascomata on bark. E Cross section of ascoma in water. F Pseudoparaphyses. G, H Asci in tap water. I–L Ascospores in tap water. Scale bars: C, D = 500 µm, E = 200 µm, F = 10 µm, G, H = 30 µm, I–L = 10 µm.

Material examined – Thailand, Hat Yai, on unidentified tree, 12 May 2018, V. Thiyagaraja, TV106 (MFLU 20-0542)

Notes – *Pseudopyrenula* was introduced by Müller (1883), with *P. diluta* as the type. Taxa in this genus are characterized by an ecorticate thallus and hyaline, transversely septate, astrothelioid ascospores with diamond-shaped lumina (Aptroot & Lücking 2016). The yellow oil droplets in the ascospore lumina and/or the hamathecium characterizes several species. This genus comprises 12 species (Species Fungorum 2021) and mainly occurs in tropical areas (Aptroot & Lücking 2016). In this study, *Pseudopyrenula* was recovered as a monophyletic clade in the phylogenetic analyses, which concurs with previous studies (Nelsen et al. 2014, Lücking et al. 2016b), with an additional new sequenced terminal corresponding to *Pseudopyrenula endoxanthoides.* The latter is distributed in the Eastern Palaeotropics including Thailand.

Discussion

Lichenization is an important phenomenon in the evolution of fungi (Lipnicki 2015, Lücking et al. 2017), particularly within the Ascomycota, where it has been repeatedly gained and lost (da Silva Cáceres et al. 2020, Nelsen et al. 2020, Thiyagaraja et al. 2020, 2021). Lichenized lineages are found in several classes within Ascomycota, including at least five major lineages within Dothideomycetes (Nelsen et al. 2009, 2011, Schoch et al. 2009). Within the latter, Trypetheliaceae is the most speciose and one of the nutritionally most diverse lineage that predominantly comprise lichenized taxa. In this family, the early diverging lineages chiefly represent non-lichenized or weakly lichenized taxa, including species of *Alloarthopyrenia, Arthopyrenia*, *Bogoriella*, *Constrictolumina*, *Polypyrenula*, and *Pseudobogoriella,* whereas the main clade is exclusively lichenized, with an anatomically more complex thallus organization (Nelsen et al. 2014, Hyde et al. 2016). While the diversity of nutritional strategies in Trypetheliaceae is comparable to that of Arthoniaceae and Stictidaceae, the underlying evolutionary histories are quite different. Thus, Stictidaceae is deeply nested within the predominantly lichenized Lecanoromycetes, clearly indicating its non-lichenized lineages as secondarily non-lichenized (Thiyagaraja et al. 2021). The origin of lichenization in Arthoniales is less clear, but also here the non-lichenized lineages within *Arthonia sensu lato* appear to be secondarily delichenized (Thiyagaraja et al. 2020). In contrast, Trypetheliaceae as a whole can be reconstructed as a de-novo lichenization event within Ascomycota, showing a clear progression from saprotrophic or weakly lichenized, early diverging lineages that apparently experimented with lichenization to a larger derived clade, including most species of the family, with stable lichenization and a complex thallus anatomy associated with this lifestyle (Lücking et al. 2016b).

The family status of Arthopyreniaceae and its phylogenetic relationships have been debated for a long time, owing to lack of molecular data. Based on the morphological characteristics of the type species, it was hitherto placed in Pleosporales, whereas non-type species with available molecular data clustered elsewhere (Nelsen et al. 2011, 2014, Hyde et al. 2013, Liu et al. 2014). Arthopyreniaceae presumably differs from Trypetheliaceae in several important characteristics, such as cellular pseudoparaphyses, broadly clavate asci and a non-refractive ocular chamber, whereas Trypetheliaceae possess trabeculate pseudoparaphyses, obclavate to cylindrical asci with a refractive ring with a wide ocular chamber (Hyde et al. 2013, Nelsen et al. 2014). However, these differences become diffuse when considering the anatomical variation of early diverging lineages now included in Trypetheliaceae, and so the now confirmed placement of *Arthopyrenia sensu stricto* within that family is not entirely surprising. The close relationship between *A. cerasi* and *Julella fallaciosa* sheds new light on the potential status of species currently included in *Julella sensu lato*. Following Barr (1985), Harris (1995) included *Julella* within Arthopyreniaceae, whereas before it was classified within various families, such as Amphisphaeriaceae (Lindau 1897), Pleosporaceae (von Arx & Müller 1975), and Thelenellaceae (Cannon & Kirk 2007). Presently only *Julella fallaciosa* has been sequenced and our results show that the muriform ascospores in this taxon have no taxonomic value at the genus level, as already suggested elsewhere (Harris 1995,

Aptroot et al. 2008) and also shown for other genera in Trypetheliaceae (Nelsen et al. 2011, Hongsanan et al. 2020b).

Arthopyrenia salicis, described by Massalongo (1852), has been recorded as lichenized, non-lichenized and sometimes both within the same population. A detailed morphological description was provided by Coppins & Orange (2009), who included several important diagnostic features such as ascomata often with a depressed ostiole lined with periphysoids, an involucrellum reacting brown in K, and the absence of interascal hyphae. *Arthopyrenia salicis* thus differs from *Arthopyrenia sensu stricto* in various important characters (Nelsen et al. 2009, 2011, Hyde et al. 2016), which is in accordance with its different phylogenetic placement (Nelsen et al. 2009, 2011, Liu et al. 2014). Molecular sequence data support its close relationship to *Roussoella* and *Roussoellopsis* (Liu et al. 2014, Nelsen et al. 2014). However, *Arthopyrenia salicis* differs from both genera in the absence of paraphysoids and in the oblong to fusiform, hyaline spores lacking striations (Hyde et al. 2016). Hence its exact generic position remains unclear. Unfortunately, the available sequence data for this species are also partly inconsistent, requiring studies of additional specimens before a conclusion on its genus-level taxonomy can be drawn.

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