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Revision of *Xylonaceae* (*Xylonales*, *Xylonomycetes*) to include *Sarea* and *Tromera*

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ABSTRACT

The resinicolous fungi *Sarea difformis* and *S. resinae* (*Sareomycetes*) were taxonomically revised on the basis of morphological observations and phylogenetic analyses of the nucleotide sequences of the nSSU-LSU-*rpb1*-*rpb2*-mtSSU genes. The results of phylogenetic analyses show that *S. difformis* and *S. resinae* are grouped with members of *Xylonomycetes*. According to the results of phylogenetic analyses and their sexual and asexual morphs resemblance, *Sareomycetes* is synonymized with *Xylonomycetes*. Although *Tromera* has been considered a synonym of *Sarea* based on the superficial resemblance of the sexual morph, we show that they are distinct genera and *Tromera* should be resurrected to accommodate *T. resinae* (= *S. resinae*). *Xylonomycetes* was morphologically re-circumscribed to comprise a single family (*Xylonaceae*) with four genera (*Sarea*, *Trinosporium*, *Tromera*, and *Xylona*) sharing an endophytic or plant saprobic stage in their lifecycle, ascostroma-type ascomata with paraphysoid, *Lecanora*-type bitunicate asci, and pycnidial asexual morphs. Phylogenetic analyses based on ITS sequences and environmental DNA (eDNA) implied a worldwide distribution of the species. Although *Symbiotaphrinales* has been treated as a member of *Xylonomycetes* in previous studies, it was shown to be phylogenetically, morphologically, and ecologically distinct. We, therefore, treated *Symbiotaphrinales* as *Pezizomycotina incertae sedis*.

Keywords: ecological diversity; evolution; ontogeny; systematics

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1. Introduction

The resinicolous genus *Sarea* Fr. was established by Fries (1825). This genus is characterized by having orange or black, rounded ascomata, clavate, polysporic asci with a *Lecanora*-type ascus apex and round aseptate ascospores, and a pycnidial asexual morph (Hawksworth & Sherwood, 1981). Two species, *S. difformis* (Fr.) Fr. (generic type) and *S. resinae* (Fr.) Kuntze, have been accepted in the modern taxonomic treatment, with both species occurring on pine resin (Ellis & Ellis, 1997; Hawksworth & Sherwood, 1981; Suto, 1985). The latter species originally described as the type species of *Tromera* A. Massal. ex Körb. [as *T. resinae* (Fr.) Körb.] by Körber (1865). Although Hawksworth and Sherwood (1981) reported that the asexual morph of *S. resinae* was different from *S. difformis* in having papillate ostiole and multilocular conidiomata (ostiole lacking and unilocular conidiomata in *S. difformis*), they considered these differences were not an important character for generic circumscription and merged the two genera with *Sarea*. The broad generic concept of *Sarea* sensu Hawksworth and Sherwood (1981) has been used by several authors (Beimforde et al., 2020; Ellis & Ellis, 1997; Suto, 1985).

Sarea difformis and *S. resinae* have been reported worldwide and are known to form ascomata or conidiomata on the pine resin of gymnosperms (Ellis & Ellis, 1997; Hawksworth & Sherwood, 1981; Suto, 1985). In previous studies, these species have been reported as endophytes in the stems of gymnosperms (Arhipova et al., 2011; Arhipova et al., 2015; Jusino et al., 2015; Konrad et al., 2007; Lygis et al., 2014; Lygis et al., 2004; Vasiliauskas et al., 2005) and in pine needles (Bowman & Arnold, 2018; Larkin et al., 2012; Sanz-Ros et al., 2015). However, studies on the environmental DNA (eDNA) and endophytic diversity have indicated that these species are also found on monocotyledons (Sánchez Márquez et al., 2008), seaweeds (unpublished; see Supplementary Table S1), and the thallus of lichen-forming fungi (Arhipova et al., 2011; Arhipova et al., 2015; Burņeviča et al., 2016; Koukol et al., 2011; Lygis et al., 2014; Lygis et al., 2004; Masumoto & Degawa, 2019; Peršoh & Rambold, 2011; Sánchez Márquez et al., 2008; Vasiliauskas et al., 2005; see Supplementary Table S1). Although these eDNA and endophyte studies have suggested that *Sarea* utilize a wider array of habitats, its geographic distribution and substrate preferences at the population level had not been compared.

The familial position of *Sarea* has been a long-standing topic of controversy. In early studies, *Sarea* was placed within *Acarosporaceae* (*Lecanorales*, *Lecanoromycetes*), based on the polysporic asci

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with a thickened ascus apex (Poelt, 1974). Because the genus closely resembles *Agyrium* Fr. in its polysporic asci, peridium structure, and plant saprobic habitats, Hawksworth and Sherwood (1981) proposed that *Sarea* be placed within *Agyriaceae* (*Lecanorales*). Ultrastructural observation of the ascus apex by Bellemére (1994) placed the genus in an uncertain position within *Lecanorales*, and Eriksson et al. (2004) classified it within *Agyriales* with no explanation. In their phylogenetic studies using small subunit nuclear ribosomal DNA (18S; nSSU), large subunit nuclear ribosomal DNA (28S; LSU) and DNA-directed RNA polymerase II second largest subunit (*rpb2*) genes, Reeb et al. (2004) showed that *Sarea* did not group with *Lecanoromycetes* and treated the genus as *Pezizomycotina incertae sedis*. Subsequently, Hodkinson & Lendemer (2011) provisionally placed *Sarea* in *Trapeliaceae* based on its morphology, as they believed that the sequences of *Sarea* generated by Reeb et al. (2004) could potentially have been contaminated. Miadlikowska et al. (2014) confirmed the placement of *Sarea* outside *Lecanoromycetes*. Thus, the class, order, and familial position of the genus remain unresolved due to a lack of informative sequence data suitable for a higher rank taxonomic analysis. A basic local alignment search tool (BLAST) search of the internal transcribed spacer (ITS) sequences of *Trinosporium guianense* Crous & Decock suggested that the species was related to *S. difformis* and *S. resinae* (Crous et al., 2012).

Most recently, Beimforde et al. (2020) established a new class (*Sareomycetes*) to accommodate *Sarea* emphasizing the results of their phylogenetic analyses. Although the monophyly of *Sareomycetes* was confirmed in these analyses, the classes used in the taxon sampling were limited and biased in member selection. Additionally, the statistical supports for most of the classes were lacking in their analyses because few gene regions were used in their analyses. In other previous studies, the nucleotide sequences of ribosomal RNA-coding genes (nSSU and LSU), single-copy protein coding genes [DNA-directed RNA polymerase I largest subunit (*rpb1*) and *rpb2*], as well as mitochondrial small subunit ribosomal DNA (mtSSU) were used for phylum-level phylogenetic analyses to resolve relationships of respective classes among *Ascomycota* (Prieto et al., 2013; Schoch et al., 2009; Spatafora et al., 2017; Voglmayr et al., 2018). In addition, Beimforde et al. (2020) did not mention that the phylogenetic relationship of *Sareomycetes* and *Xylonomycetes*, although *Sarea* was suggested phylogenetically related to members of *Xylonomycetes* in a BLAST search of ITS (Crous et al., 2012) and was morphologically similar to *Trinosporium* Crous & Decock and *Xylona* Gazis & P. Chaverri in having pycnidial conidiomata and unique conidiogenous cells.

The resinicolous habitats and polysporic asci are important features of *Sareomycetes* to distinguish it from other known classes by Beimforde et al. (2020). Those features, however, occur scattered throughout many classes within the *Ascomycota*. The resinicolous fungi are known in *Dothideomycetes* (Boehm et al., 2009), *Eurotiomycetes* (Rikkinen & Poinar, 2000; Seifert & Hughes, 2000), *Leotiomycetes* (Hawksworth & Sherwood, 1981), and *Sordariomycetes* (Lombard et al., 2009). The morphological convergence of polysporic asci has been reported in *Candelariomycetes* (Bellemére, 1994; Voglmayr et al., 2018), *Dothideomycetes* (Barr, 1972), *Leotiomycetes* (Quijada et al., 2019), *Sordariomycetes* (Réblová & Mostert, 2007). Thus, the validity and circumscription of *Sareomycetes* sensu Beimforde et al. (2020) seem to be questionable. The ontogenetic approaches are useful for ascomycetes systematics (Luttrell, 1981), and asexual morph features can help circumscribe of higher rank taxonomy in *Ascomycota* (Hashimoto et al., 2017a, b, 2018). As alternative approaches to resolve these problems, comparing the ontogeny of ascomata and the asexual morph morphology may re-evaluate or support the uniqueness of *Sareomycetes*.

Here, we re-evaluated (1) the validity of *Sareomycetes* based upon morphological observations such as ascomatal development, and molecular phylogenetic analyses based on nSSU, ITS, LSU, *rpb1*, *rpb2*, and mtSSU; (2) its ecological niches combined with previous eDNA and endophytic studies using ITS sequences.

2. Materials and methods

2.1. Field sampling

Bark exuding pine resin was collected in the winter to early summer months from subalpine or high altitude zones in Japan. At times samples were collected randomly and observed using a stereomicroscope in the laboratory. When ascomata were found under good conditions, these samples were preserved as specimens and used for isolation.

2.2. Isolation

A single apothecium without the substrate was removed using a needle. The ascoma was glued using a piece of agar to the inner surface of the lid of a petri dish plated with water agar (FUJIFILM WAKO, Osaka, Japan) or potato dextrose agar (PDA; Nissui, Tokyo, Japan). The discharged ascospores were confirmed using a × 40 objective lens. Handmade needles were used to obtain single- or multi-spore isolates. The single or multiple ascospore isolates were then transferred to PDA plate and incubated at 20 °C in the dark.

A total of five specimens, of two single-spore isolate (culture AH 1107 and AH 1278) and three multi-spore isolates (culture AH 1149, AH 1164, and AH 1309), were used for phylogenetic analyses (Table 1). Specimens were deposited in the Mycological Herbarium of the National Museum of Nature and Science, Japan (TNS). Cultures were deposited in the Japan Collection of Microorganisms RIKEN BioResource Research Center (JCM).

2.3. Morphological observation

Five of our newly obtained specimens of *Sarea* spp. (Table 1) were used for morphological observation in this study. Fungal structures except ascomata were observed in preparations mounted in distilled water. Ascomata were sectioned using a freezing microtome FX-80 (Yamato, Saitama, Japan) and mounted in lactophenol

Table 1. Specimens, strains and new sequences obtained in this study

Species	Original Specimen no.	Specimen no.	Strain no.	GenBank no.					
				nSSU	ITS	LSU	mtSSU	*rpb1*	*rpb2*
Sarea difformis	AH 1278	TNS-F-89129	JCM 39114	**LC513856**	**LC513861**	**LC513866**	**LC513871**	**LC513876**	**LC513881**
	AH 1309	TNS-F-89130	JCM 39115	**LC513857**	**LC513862**	**LC513867**	**LC513872**	**LC513877**	**LC513882**
Tromera resinae	AH 1107	TNS-F-89131	JCM 39116	**LC513858**	**LC513863**	**LC513868**	**LC513873**	**LC513878**	**LC513883**
	AH 1149	TNS-F-89132	JCM 39117	**LC513859**	**LC513864**	**LC513869**	**LC513874**	**LC513879**	**LC513884**
	AH 1164	TNS-F-89133	JCM 39118	**LC513860**	**LC513865**	**LC513870**	**LC513875**	**LC513880**	**LC513885**

cotton blue. To observe their ontogeny, 10 to 20 ascomata at various stages were sectioned for *S. difformis* and *S. resinae*. Field and macroscopic images were obtained using a mirrorless interchangeable-lens camera (X-M1; FUJIFILM, Tokyo, Japan) with QZ-35M (TAMRON, Saitama, Japan), a compact digital camera (COOLPIX 4500; Nikon, Tokyo, Japan) with a macro conversion lens (MSN-202; Raynox, Saitama, Japan), and a Nikon SMZ-10A stereomicroscope with DP12 (Olympus, Tokyo, Japan). The morphological characteristics of the samples were observed by differential interference microscopy OPTIPHOT 2 (Nikon). Images were captured using a digital camera (DS-L2; Nikon). Free-hand drawings were made using scaled paper (LA-R50N; Sakae technical paper, Tokyo, Japan) with PIGMA Micron Pen (Sakura Color Products, Osaka, Japan).

2.4. DNA extraction, PCR, and Sequencing

DNA extraction from pure culture was carried out using an ISOPLANT II kit (Nippon Gene, Toyama, Japan) based on the manufacturer's protocol. Amplicons of small subunit nrDNA (18S; nSSU), ITS, large subunit nrDNA (28S; LSU), DNA-directed RNA polymerase I largest subunit (*rpb1*), and DNA-directed RNA polymerase II second largest subunit (*rpb2*), mitochondrial small subunit ribosomal DNA (mtSSU) were obtained by PCR with the primer pairs NS1/NS4 (White et al., 1990), ITS5/ITS4 (White et al., 1990), LR0R/LR7 (Rehner & Samuels, 1994; Vilgalys & Hester, 1990), RPB1-Af/RPB1-Cr (Matheny et al., 2002; Stiller & Hall, 1997), fRPB2-5F/fRPB2-7cR (Liu et al., 1999), and mrSSU1/mrSSU3R (Zoller et al., 1999), respectively. Amplifications were performed in 25 μL consisting of 2 μL of 2 ng/μL DNA extract, 12.5 μL of 2× Buffer for KOD FX Neo, 5 μL of 2 mM dNTPs, 1 μL of each 20 pM primer, 3 μL MilliQ water, and 0.5 μL KOD FX Neo (TOYOBO, Tokyo, Japan). PCRs were carried out on a GeneAmp PCR System 9700 (Applied Biosystems, California, US) as follows: 94 °C for 2 min; followed by 35 cycles of 10 s at 98 °C, 30 s at the designated annealing temperature (42 °C for nSSU, 61.5 °C for ITS, 46 °C for LSU, 56 °C for mtSSU and *rpb1*, and 58 °C for *rpb2*), and 1 min at 68 °C; and a final extension of 7 min at 68 °C. PCR products were purified using an FastGene Gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan) based on the manufacturer's protocol. Purified DNA was cycle-sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher, Warrington, UK) with the same primers as in PCR or for nSSU with NS2 and NS3(White et al., 1990), ITS3 for ITS (White et al., 1990), LR3R, LR4, LR5, LR6 for LSU (Vilgalys & Hester, 1990). Sequencing was performed on SeqStudio by default setting (Thermofisher, Tokyo, Japan). Newly generated nucleotides were deposited in DDBJ (Table 1).

2.5. Taxon sampling

Two alignments were generated. The first analyses were conducted to resolve the phylogenetic relationship of *Sareomycetes* in *Ascomycota* and their relationship with *Xylonomycetes*. Since taxon sampling and gene selection of Beimforde et al. (2020) seem to be problematic, we selected the examined taxa by the following criteria. (1) All known classes of *Pezizomycotina* [*Arthoniomycetes*, *Candelariomycetes*, *Coniocybomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Geoglossomycetes*, *Laboulbeniomycetes*, *Lecanoromycetes*, *Leotiomycetes*, *Lichinomycetes*, *Orbiliomycetes*, *Pezizomycetes*, *Sareomycetes* sensu Beimforde et al. (2020), *Sordariomycetes*, *Xylobotryomycetes*, and *Xylonomycetes*] were used for ingroup taxa. (2) Referring to the taxon sampling by Schoch et al. (2009), whose dataset is well-balanced and showed a strong phylogenetic support for each class, we paid the balance for genetic distances and the number of members incorporated for each class. (3) Avoid integrating sequence of the same taxon from different origins. (4) Specimens with multi-locus (nSSU, ITS, LSU, *rpb1*, *rpb2* and mtSSU) with at least LSU region.

Referring to Schoch et al. (2009) and Spatafora et al. (2017), two taxa of *Saccharomycotina*, two taxa of *Taphrinomycotina*, eight taxa of *Basidiomycota*, and *Entorrhiza parvula* Vánky, and *Mortierella verticillata* Linnem. were used were as an outgroup of *Pezizomycotina*, *Dikarya*, and the outgroup of tree, respectively (Table 1; Supplementary Table S2).

The second analysis was conducted to clarify the tendency among geographic distribution, substrate preferences and endophytic or plant saprobic lifestyle of *S. difformis* and *S. resinae*. Because the ITS region had been used for eDNA and endophytic analyses in previous studies of *Sarea* spp. (Supplementary Table S1), ITS data was used for this analysis and consisted of five sequences from our newly obtained data and 41 sequences downloaded from GenBank. The GenBank accessions of MH856727 (*Hormococcus conorum* (Sacc. & Roum.) Robak, CBS 504.50) and MH854935 (*Zythia pinastri* P. Karst., CBS 217.27) were hit in the BLAST search of *Sarea* spp. and seemed to be misidentified strains due to the morphological similarity of the asexual morph. Although these strains were misidentified, these sequences were incorporated into our dataset, because of providing ecological information of *Sarea* spp.

2.6. Sequence alignment

Sequences for each data set were aligned using MAFFT version 7.429 as the default setting (Katoh et al., 2017). Ambiguously aligned portions of the alignments were manually removed using MEGA7 (Kumar et al., 2016). For the first analyses, genes were combined using Kakusan4 software (Tanabe, 2011).

2.7. Phylogenetic analyses

To evaluate the validity of *Sareomycetes* sensu Beimforde et al. (2020) and its relationship with *Xylonomycetes*, we tested the following four alternative hypotheses using maximum-likelihood (ML) analyses: (1) monophyly of *Sareomycetes*; (2) monophyly of *Xylona* + *Trinosporium* [= *Xylonomycetes* sensu Gazis et al. (2016)]; (3) monophyly of *Xylona* + *Trinosporium* + *Symbiotaphrina* Kühlw. & Jurzitza ex W. Gams & Arx [= *Xylonomycetes* sensu Baral et al. (2018)]; and (4) monophyly of *Xylona* + *Trinosporium* + *Sarea* (*Xylonomycetes* sensu this study). Hypothesis tests were performed using the same alignment with the first dataset. Additionally, alternative phylogenetic analyses were conducted for comparison with previous studies as follows: LSU [same locus as Baral et al. (2018)], LSU-ITS [same locus as Gazis et al. (2016) and Beimforde et al. (2020)], nSSU-LSU, nSSU-ITS-LSU, nSSU-LSU-*rpb2*, nSSU-ITS-LSU-*rpb2* [same locus as Beimforde et al. (2020)], nSSU-LSU-*rpb1*-*rpb2*-mtSSU, and nSSU-ITS-LSU-*rpb1*-*rpb2*-mtSSU.

Phylogenetic analyses using nSSU-LSU-*rpb1*-*rpb2*-mtSSU were conducted using the ML, maximum parsimony (MP), and Bayesian methods for the first dataset. The optimum substitution models for each data set were estimated using Kakusan4 (Tanabe, 2011) based on the corrected Akaike information criterion (AICc; Sugiura, 1978) for the ML analysis, and the Bayesian information criterion (BIC; Schwarz, 1978) for the Bayesian analysis. All molecular characteristics were given equal weight, and gaps were treated as missing data for the MP analysis.

The ML analysis was performed using the RAxML-HPC2 v.

8.2.10 on Cipres Science Gateway (Miller et al., 2015; Stamatakis, 2014) based on the models selected with the AICc4 parameter (a separate codon nonpartitioned model). The first data set used GTR+G for nSSU, LSU, *rpb1*, *rpb2*, and mtSSU. Bootstrap probability (BPs) were obtained using 1000 bootstrap replications.

The MP analysis was performed using the PAUP* 4.0a166 on Cipres Science Gateway (Miller et al., 2015; Swofford, 1991). For the MP analysis, the heuristic searches were conducted with 1000 random-addition-sequences (RAS), tree-bisection-reconnection (TBR) branch swapping and MulTrees option in effect, rearrangement limit 8, and collapsing branches with maximum branch length are zero. BPs were obtained using 1000 bootstrap replications.

The Bayesian analysis was performed with MrBayes v. 3.2.7a on Cipres Science Gateway (Miller et al., 2015; Ronquist et al., 2012) using substitution models containing the BIC4 parameter (i.e. proportional codon proportional model). GTR+G was used for LSU, mtSSU, and the all codon position of *rpb1* and *rpb2*. SYM+G was used for nSSU. Two simultaneous and independent Metropolis-coupled Markov chain Monte Carlo (MCMC) runs were performed for 4 million generations with the tree sampled for every 1000 generations of the analyses. The convergence of the MCMC procedure was assessed from the average standard deviation of split frequencies (< 0.01) and the effective sample size scores (all > 100) using MrBayes and Tracer v. 1.6 (Rambaut et al., 2014), respectively. The first 25% of the trees were discarded as burnin, and the remainders were used to calculate the 50% majority-rule trees and to determine the posterior probabilities (PPs) for individual branches.

For the second dataset, phylogenetic analyses were conducted using ML and Bayesian methods. The ITS sequences were divided into ITS1-5.8S-ITS2 regions for substitution model estimations and analyses. The optimum substitution models for each data set were estimated using Kakusan4 based on the AICc for the ML analysis and on the BIC for the Bayesian analysis.

The ML analysis was performed using the RAxML-HPC2 v. 8.2.10 on Cipres Science Gateway based on the models selected with the AICc4 parameter (equal rate model, GTR+G for ITS1-5.8S-ITS2). BPs were obtained using 1000 bootstrap replications.

Bayesian analysis was performed with MrBayes v. 3.2.7a on Cipres Science Gateway, using substitution models containing the BIC4 parameter (proportional model, SYM+G for ITS1 and ITS2, and K80+G for 5.8S). Bayesian analysis was conducted according to the methods as described above. These two alignments were submitted to TreeBASE under study number S25330.

3. Results

3.1. *Phylogenetic evaluation of* Sareomycetes

The ML, MP, and Bayesian phylogenetic analyses were conducted using an aligned sequence dataset composed of 933 nucleotides from nSSU, 1170 from LSU, 549 from *rpb1*, 1089 from *rpb2*, and 590 from mtSSU for the first analyses. The alignment contained a total of 112 taxa which consisted of 97 taxa (86.6%) in nSSU, 112 (100%) in LSU, 77 (68.8%) in *rpb1*, 83 (74.1%) in *rpb2*, and 65 (58.0%) in mtSSU (Table 1; Supplementary Table S1). Of the 4331 characters included in the alignment, 2920 were variable, 1354 were conserved, and 2428 were parsimony-informative. The ML tree with the highest log likelihood (–128577.3717) is shown in Fig. 1. The topology recovered by the Bayesian analysis did not contain any topological conflicts with significant support. The MP analysis had the lowest supports, especially at deeper internodes (Fig. 1).

LSU, LSU-ITS, nSSU-LSU, and nSSU-ITS-LSU phylogenies produced low resolution results or failed to reconstruct most of the nodes at the class and order level (Table 2; Supplementary Fig. S1A–D). Phylogenies built from nSSU-LSU-rpb2 and nSSU-ITS-LSU-rpb2 showed moderate support for clades for major classes, while the monophyly of *Candelariomycetes*, *Eurotiomycetes*, *Lecanoromycetes*, and *Lichinomycetes* were either weakly supported (< 80% in ML BP) or failed (Table 2; Supplementary Fig. S1C, D). The loci nSSU-LSU-*rpb1*-*rpb2*-mtSSU and nSSU-ITS-LSU-*rpb1*-*rpb2*-mtSSU were able to distinguish all classes (Table 2; Supplementary Fig. S1E, F). We removed the ITS region from our analyses considering the rapid substitution causing false homologies.

The results of our hypothesis tests significantly rejected the monophyly of *Xylona* + *Trinosporium* + *Symbiotaphrina* [= *Xylonomycetes* sensu Baral et al. (2018)]. The monophyly of *Sareomycetes* was moderately supported and *Xylonomycetes* sensu Gazis et al. (2016) was strongly supported. The monophyly of *Xylona* + *Trinosporium* + *Sarea* (include *S. difformis* and *S. resinae*) was moderately to robustly supported in the multi-locus analysis (Table 3; Supplementary Fig. S1A–H). The results of our phylogenetic analysis based on the nSSU-LSU-*rpb1*-*rpb2*-mtSSU sequences (Fig. 1; Supplementary Fig. 1G) was largely in accordance with the findings of previous studies (Schoch et al., 2009; Voglmayr et al., 2018), with the exception for the position of *Symbiotaphrinales*. *Symbiotaphrinales* was placed outside of *Xylonomycetes* by all of our analyses. Our phylogenetic study indicated that *S. difformis* and *S. resinae* formed a strongly supported clade with *T. guianense* and *X. heveae* Gazis & P. Chaverriand belonged to *Xylonaceae* (*Xylonales*, *Xylonomycetes*). Monophyly of these four species were strongly supported (100% in ML BP, 88% in MP BP and 1.00 in Bayesian PP), although the monophyly of *S. difformis* and *S. resinae* was moderately supported (84% in ML BP, below 60% in MP BP and 0.99 in Bayesian PP). Therefore, *Sareomycetes* seems to be a synonym of *Xylonomycetes* and retained *Tromera* to accommodate *S. resinae* as *Tromera resinae*.

3.2. *Evolutionary ecological aspects of* Sarea difformis *and* Tromera resinae

ML and Bayesian phylogenetic analyses were performed using the second dataset consisting of 46 sequences with 480 nucleotide positions composed of 174 nucleotides from ITS1, 158 from 5.8S, and 148 from ITS2. Of these positions, 99 were variable and 380 were conserved. The topology recovered by the ML and Bayesian analyses was identical. According to sequence comparison, the strain of CBS 504.50 (MH856727) and CBS 217.27 (MH854935) should be renamed as *S. difformis* and *T. resinae*, respectively.

Sarea difformis and *T. resinae* formed strongly supported clades (100% in ML BP and 1.00 in Baysian PP in Fig. 2), and both species consisted of several distinct groups (three for *S. difformis*, two for *T. resinae*, each termed “Group” in Fig. 2).

Group 1 of *S. difformis* was moderately supported (63% in ML BP and 0.97 in Baysian PP), and consisted from two saprobic samples on *Larix kaempferi* (Lamb.) Carrière collected in Japan, one endophytic sample on *Pinus* sp. in the US, and four endolichenic samples on *Lecanoromycetes* spp. in Europe. Group 2 was robustly supported (97% in ML BP and 1.00 in Baysian PP), and consisted of three samples on *Pinus* spp. in Europe and the US and two endolichenic samples on *Lecanoromycetes* spp. in Europe. Group 3 was strongly supported (93% in ML BP and 1.00 in Baysian PP) and consisted of mainly endophyte samples of *Pinus* spp. in Europe and US, except for two samples that derived from monocots and seaweed.

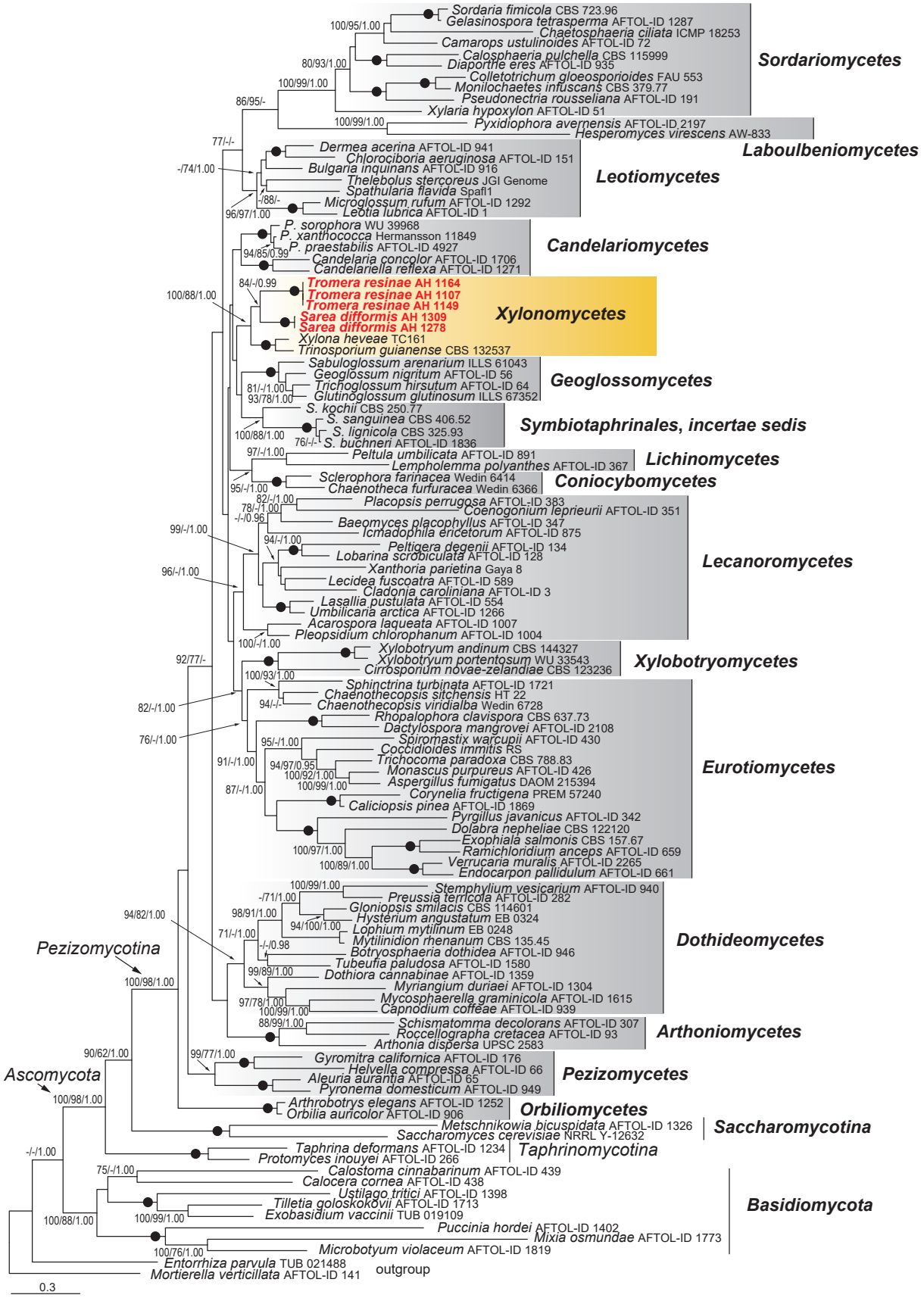

Fig. 1. Maximum-likelihood (ML) tree of *Ascomycota* based on the nSSU-LSU-*rpb1*-*rpb2*-mtSSU sequences. A ML bootstrap probability (BP) greater than 60%, Maximum parsimony (MP) bootstrap probability above 60% and Bayesian posterior probabilities (PP) above 0.95 are presented at the nodes as ML BP/MP BP/Bayesian PP. The circle (●) indicates nodes with 100% ML BP/100% MP BP/1.00 Bayesian PP. A hyphen ("-") indicates values lower than 60% ML BP and MP BP or 0.95 PP. The newly obtained sequences are shown in bold. The scale bar represents nucleotide substitutions per site.

Table 2. Comparisons in Maximum-likelihood (ML) bootstrap probability (BP) when single copy regions were added. Detailed tree topologies are shown in Supplementary Fig. 1. ML BP values were shown as LSU, LSU+ITS, nSSU+LSU, nSSU+ITS+LSU, nSSU+LSU+*rpb2*, nSSU+ITS+LSU+*rpb2*, nSSU+LSU+*rpb1*+*rpb2*+mtSSU, nSSU+ITS+LSU+*rpb1*+*rpb2*+mtSSU, respectively. NA indicates that the node could not reconstructed in the dataset. Class, phylum and subphylum are represented in the order of Fig 1.

	LSU[a]	LSU +ITS[b]	nSSU +LSU	nSSU +ITS +LSU	nSSU +LSU +*rpb2*	nSSU +ITS +LSU +*rpb2*[c]	nSSU +LSU +*rpb1* +*rpb2* +mtSSU	nSSU +ITS +LSU +*rpb1* +*rpb2* +mtSSU
Sordariomycetes	100	100	100	100	100	100	100	100
Laboulbeniomycetes	45	43	100	100	100	100	100	100
Leotiomycetes	NA	NA	NA	NA	81	81	96	98
Candelariomycetes	NA	NA	NA	NA	NA	NA	54	61
Xylonomycetes	NA	NA	48	53	88	89	100	100
Geoglossomycetes	80	81	96	95	100	99	100	99
Symbiotaphrinales	98	99	100	100	83	90	100	100
Lichinomycetes	100	100	100	100	27	24	97	96
Coniocybomycetes	97	99	99	99	100	100	100	100
Lecanoromycetes	NA	15	NA	NA	72	74	96	93
Xylobotryomycetes	99	98	99	98	100	89	100	100
Eurotiomycetes	78	74	91	87	NA	NA	76	70
Dothideomycetes	NA	NA	NA	NA	88	88	94	95
Arthoniomycetes	100	100	99	98	99	99	100	100
Pezizomycetes	79	94	85	95	99	100	100	99
Orbiliomycetes	100	100	100	100	100	100	100	100
Saccharomycotina	94	99	100	100	100	100	100	100
Taphrinomycotina	100	100	100	100	100	100	100	100
Basidiomycota	79	91	100	100	99	98	100	100

[a] same locus as Baral et al. (2018)
[b] same locus as Gazis et al. (2016) and Beimforde et al. (2020)
[c] same locus as Beimforde et al. (2020)

Table 3. Relationship of *Sareomycetes* and *Xylonomycetes* based on comparisons in Maximum-likelihood (ML) bootstrap probability (BP) when single copy regions were added. Detailed tree topologies are shown in Supplementary Fig. 1. ML BP values were shown as LSU, LSU+ITS, nSSU+LSU, nSSU+ITS+LSU, nSSU+LSU+*rpb2*, nSSU+ITS+LSU+*rpb2*, nSSU+LSU+*rpb1*+*rpb2*+mtSSU, nSSU+ITS+LSU+*rpb1*+*rpb2*+mtSSU, respectively. NA indicates that the node could not reconstructed in the dataset.

	LSU[a]	LSU +ITS[b]	nSSU +LSU	nSSU +ITS +LSU	nSSU +LSU +*rpb2*	nSSU +ITS +LSU +*rpb2*[c]	nSSU +LSU +*rpb1* +*rpb2* +mtSSU	nSSU +ITS +LSU +*rpb1* +*rpb2* +mtSSU
Monophyletic "*Sareomycetes*"	80	64	85	NA	62	71	84	87
Monophyletic *Xylona*+ *Trinosporium* = *Xylonomycetes* sensu Gazis et al. (2016)	99	100	100	100	100	100	100	100
Monophyletic *Xylona* + *Trinosporium* + *Symbiotaphrina* = *Xylonomycetes* sensu Baral et al. (2018)	45	NA	NA	NA	NA	NA	NA	NA
Monophyletic *Xylona* + *Trinosporium* + *Sarea* + *Tromera* = *Xylonomycetes* sensu this study	NA	NA	48	53	88	89	100	100

[a] same locus as Baral et al. (2018)
[b] same locus as Gazis et al. (2016) and Beimforde et al. (2020)
[c] same locus as Beimforde et al. (2020)

Group 1 of *T. resinae* was robustly supported (97% in ML BP and 1.00 in Baysian PP), and consisted of three saprobic samples on *L. kaempferi* in Japan, three endophytic samples on *Picea abies* (L.) H.Karst in Europe and *Pinus* sp. in the US, and three endolichenic samples on *Lecanoromycetes* spp. in Asia. Group 2 was weakly supported (below 60% in ML BP and below 0.95 in Baysian PP), and mainly occurred as an endophyte of *Pinus* spp. in the US and Europe and *Pseudotsuga menziesii* (Mirb.) Franco in the UK.

3.3. Developmental morphology

The matured ascomatal detailed structures, ascomatal ontogeny, and ascus development have been studied in *S. difformis* (Figs. 3, 5, 7A–D, 8A–C) and *T. resinae* (Figs. 4, 6, 7E–I, 8D–F).

The young ascocarp of *S. difformis* developed on the host tissue (Fig. 5A–D). The generative fungal tissue composed of radially arranged hyaline hyphae was embedded in the gel, which was already pigmented (Fig. 5C, 7A), and enclosed the ascogonium (Fig. 5D, 7B). The primordium then formed paraphysoids in the prospective subhymenium region (Fig. 5E, F), which was derived from the generative fungal tissue and a centrum was filled with gel. Young asci grew into the hymenium (Figs. 5G–I, 7C). Young ascoma with an opening cortical layer and the tips of paraphysoids were secondarily developed, and the epihymenium and excipulum were pigmented (Figs. 5J, 7D).

The first stage of *T. resinae* development was found in the host tissue (Fig. 6A–E). The generative fungal tissue enclosed the ascogonium (Figs. 6C–E, 7F). An ascogonium formed a hook cell in the center (Fig. 6C). Paraphysoids were produced in the prospective subhymenium region and were rapidly filled the primordium

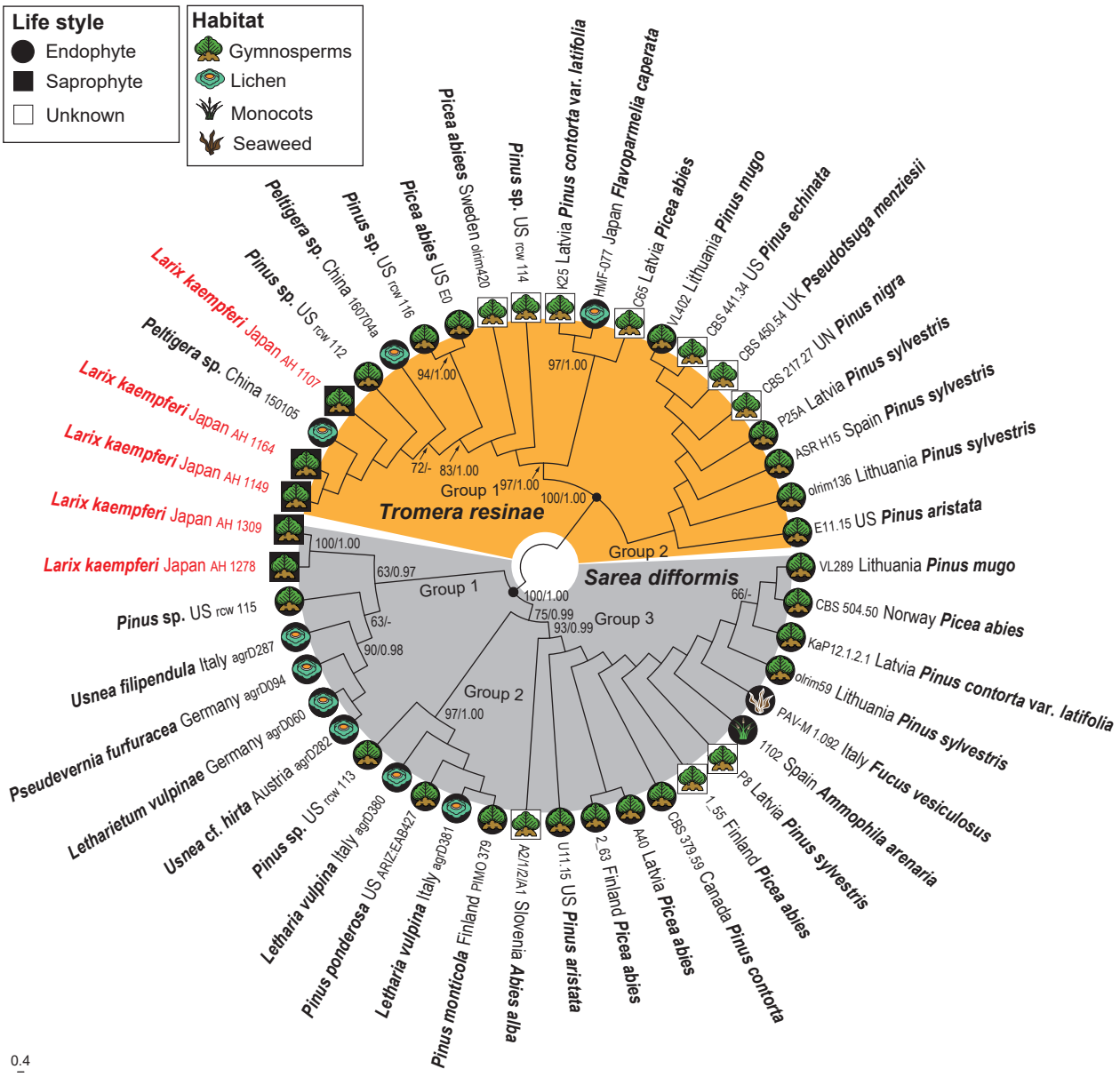

Fig. 2. Maximum-likelihood (ML) tree of *Sarea* and *Tromera* spp. based on ITS sequences. A ML bootstrap probability (BP) greater than 70%, and Bayesian posterior probabilities (PP) above 0.95 are presented at the nodes as ML BP/Bayesian PP. *Sarea difformis* was shown grey clade. *Tromera resinae* was shown yellow clade. Each taxon was shown in order "host species name", "collected country", "strain/sample name", "lifestyle and habitats". Lifestyle and habitats were shown in the combination of outer box and illustration as shown in the upper right box. The circle (●), black square (■), white square (□) indicates the difference of lifestyle as endophytic, saprophytic, and unknown, respectively. Four types of illustration icons indicate the difference of habitat as gymnosperms, the thallus of lichen-forming fungi unknown lifestyle. Habitat showed gymnosperms, lichen (= the thallus of lichen-forming fungi), monocots, and seaweed, respectively. The newly obtained sequences are shown in red. The scale bars represent nucleotide substitutions.

(Figs. 6F–H, 7G). The hymenium extended and the peridium developed, but no asci were observed (Figs. 6I, J, 7H). Young asci grew into the hymenium when hamathecium was well-developed (Fig. 6K–M). Young ascoma with an opening cortical layer and tips of paraphysoides were secondarily developed. The epihymenium, hyphothecium, and excipulum were pigmented (Figs. 6M, 7I).

4. Discussion

4.1. Recircumscription of Xylonomycetes

Xylonomycetes was originally proposed to accommodate a single species *X. heveae*, a sapwood endophyte in *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. (Angiosperms, *Malpighiales*) with non-ostiolate pycnidial conidiomata, and hyaline aseptate conidia (Gazis et al., 2012). Subsequently, two additional genera, *Symbiotaphrina* and *Trinosporium* were reported to be phylogenetically related to this class (Gazis et al., 2016). A yeast-like fungus *Symbiotaphrina* is well-known as an intracellular symbiont in anobiid beetles (Noda & Kodama, 1996). More recently, a sexual morph of *Symbiotaphrina* was found as *Tromeropsis* Sherwood, which is characterized as black cup-like ascomata with polysporic asci and a thin ascus apex, and *Tromeropsis* is synonymized under *Symbiotaphrina* (Baral et al., 2018). The genus *Trinosporium* is characterized by having ostiolate pycnidial conidiomata and trigonous brown conidia (Crous et al., 2012). *Xylonomycetes* has been recog-

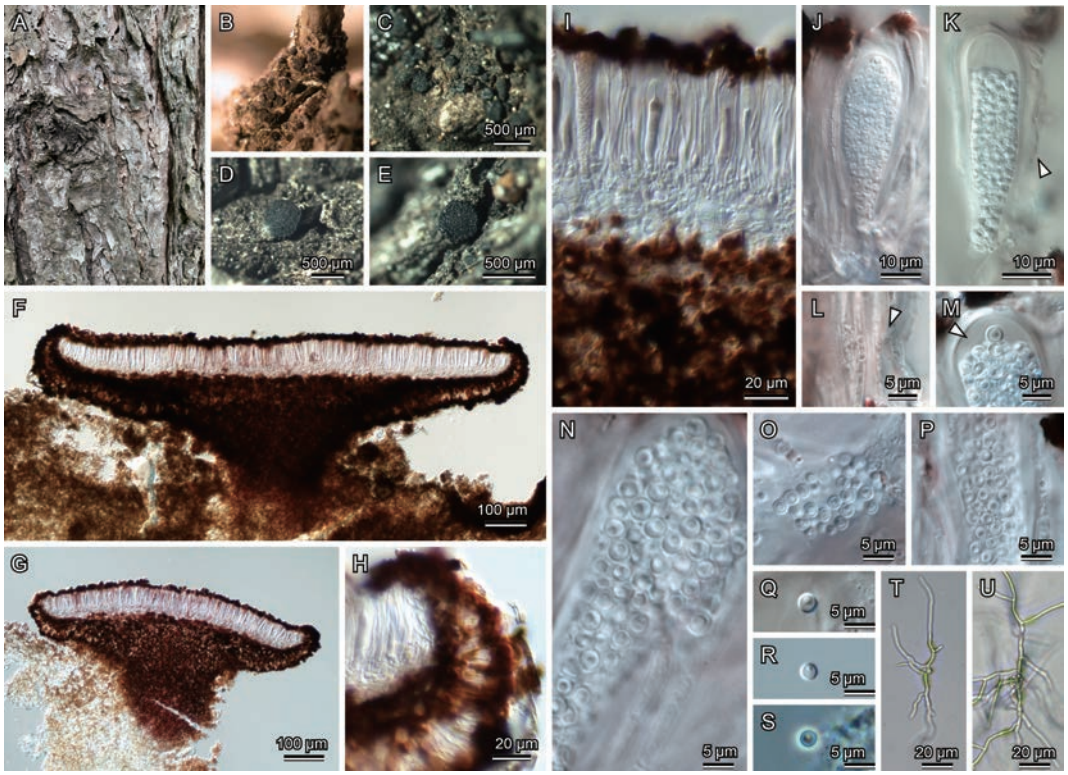

Fig. 3. *Sarea difformis*. A: Habitat of *Sarea difformis*; B–D: Ascomata on substrate; F, G: Ascomata in longitudinal section; H: Lateral peridium of ascomata; I: Hymenium of ascoma; J, K: Ascus (arrowhead indicates peridial gel in K); L: paraphysoides (arrowhead indicates anastomose of paraphysoides); M: Ascus apex (arrowhead indicates border line of C and D layer in M); N–S: Ascospores; T, U: Germinating ascospores. A, B, D, F–I, K, M, N–Q, T from AH 1278; C, E, J, L, R, S, U from AH 1309.

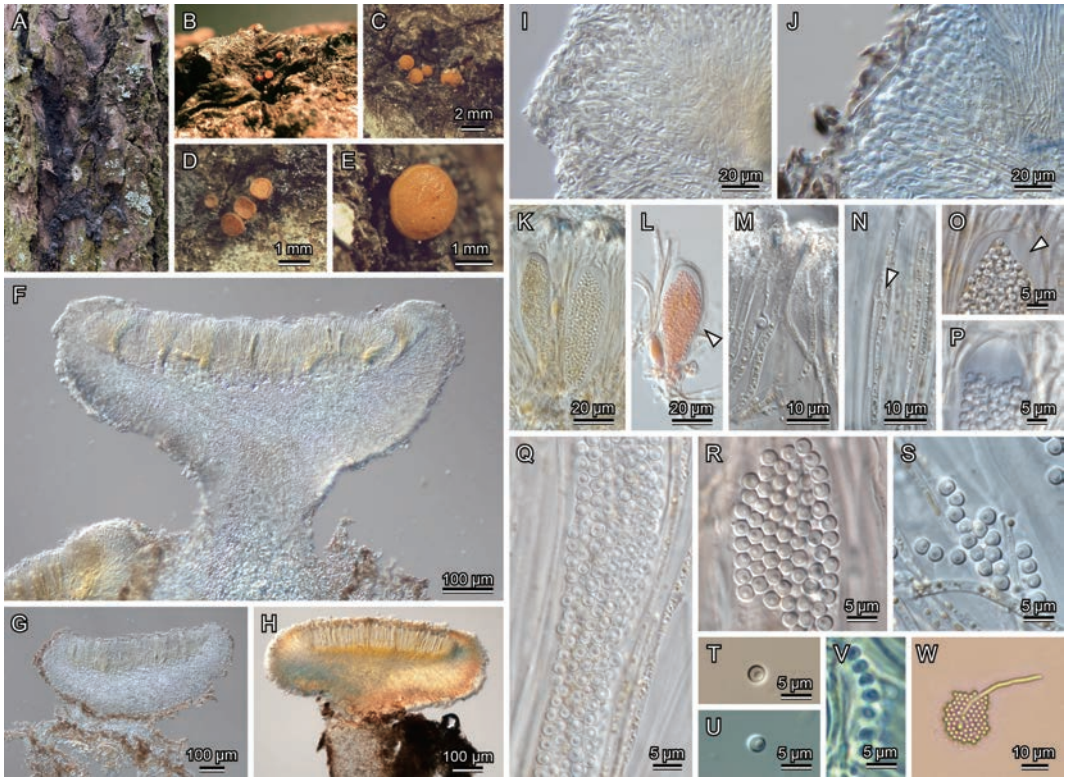

Fig. 4. *Tromera resinae*. A: Habitats of *Tromera resinae*; B–E: Ascomata on substrate; F–H: Ascomata in longitudinal section; I, J: Lateral peridium of ascomata; K, L: Ascus (arrowhead indicates peridial gel in L, stained with congo red in L); M, N: paraphysoides (arrowhead indicates anastomose of paraphysoides); O, P: Ascus apex (arrowhead indicates border line of C and D layer in N, Meltzer's solution positive in P); Q–V: Ascospores; W: Germinating ascospore. A, C, F, I, U–W from AH 1164; B, E, H, K–S, from AH 1107; D, G, J, T from AH 1149.

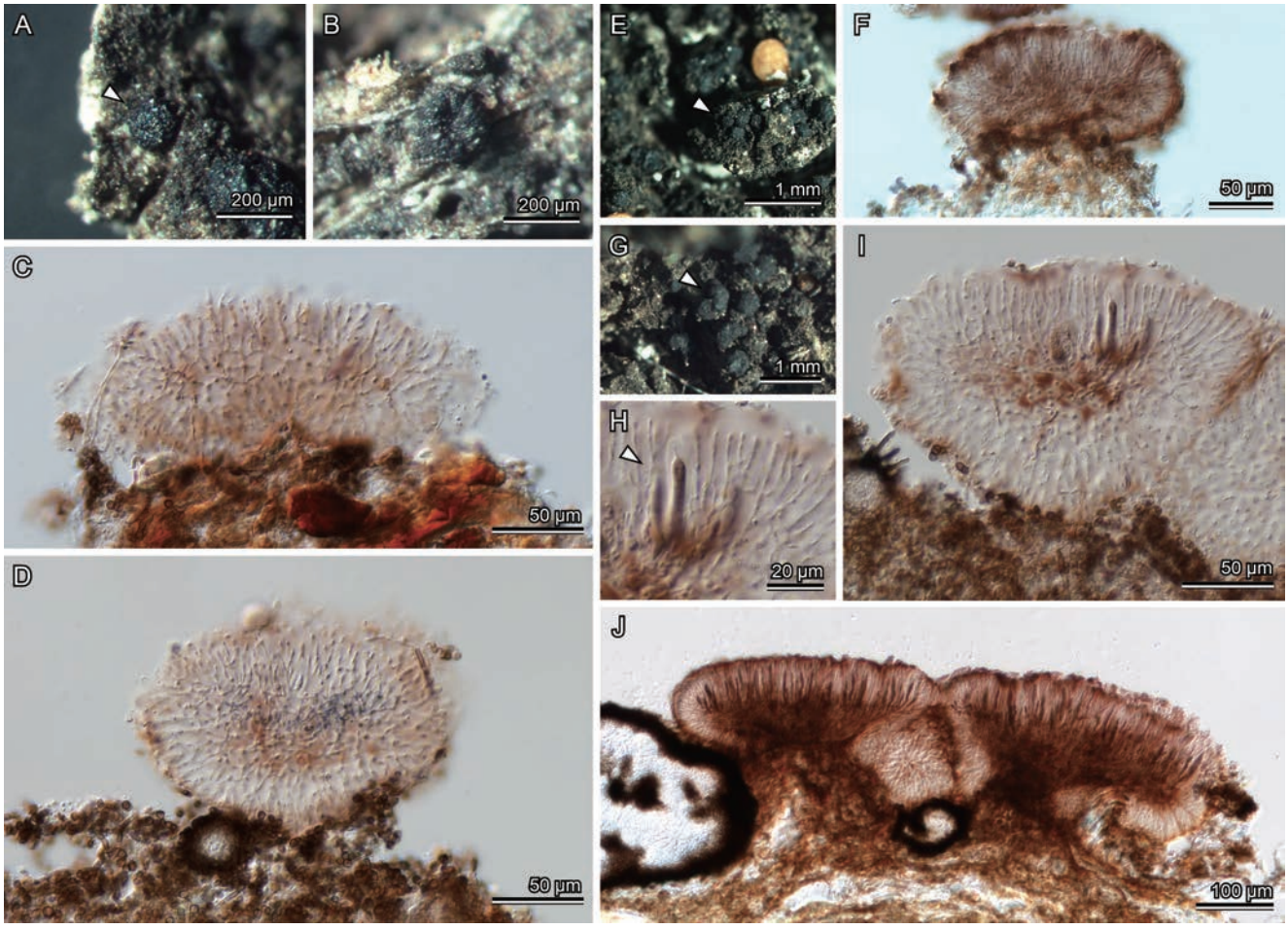

Fig. 5. Ascomatal development of *Sarea difformis*. A–D: Initial stage of ascomatal development. A, B: Young ascomata on the host tissue. C, D: Generative tissue. Note the primordium grows center of generative tissue in D. E–I: Development of paraphysoids. Note young asci grow into the hymenium in F. Paraphysoids derived from the generative hyphae and a centrum filled with gel (arrowhead indicates earlier developed paraphysoids in H). J: Young ascoma in longitudinal section. A–J from AH 1278.

nized as an ecologically and morphologically diverse group (Adl et al., 2019; Baral et al., 2018; Naranjo-Ortiz & Gabaldon, 2019).

Sareomycetes sensu Beimforde et al. (2020) was not supported or reconstructed in our dataset. We confirmed that analyses based on ITS-LSU or nSSU-ITS-LSU-*rpb2* were insufficient for subphylum-level analyses (Fig. 1; Table 3; Supplementary Fig. S1). Phylogenetic relatedness of *Sareomycetes* to *Xylonomycetes* was robustly demonstrated, supporting *Sarea* as a member of *Xylonaceae* (*Xylonales*, *Xylonomycetes*, 100% in ML BP, 100% in MP BP and 1.00 in Bayesian PP; Fig. 1). We therefore treat *Sareomycetes* as a synonym of *Xylonomycetes* following the strong results of our phylogenetic analyses and the ecological and morphological similarity of the two classes. Species in this class bear several common features, including an endophytic or plant saprobic stage in their lifecycle, sexual morphs with ascostroma-type ascomata with paraphysoid, bitunicate, polysporic asci with a *Lecanora*-type ascus apex, and asexual morphs with pycnidial conidiomata and enteroblastic conidiogenous cells (Table 4). The monophyly of the genera *Sarea*, *Trinosporium*, *Tromera*, and *Xylona* was strongly supported by the results of our phylogenetic analyses (Fig. 1), with all four taxa accepted here in *Xylonaceae*, *Xylonomycetes*. Therefore, *Sareales* and *Sareaceae* are also synonymized under *Xylonales* and *Xylonaceae*, respectively (see Taxonomy).

Previously, the lack of a sexual morph caused the *Xylonomycetes* as an enigmatic class that could not morphologically compared with other ascomycetous classes (Gazis et al., 2012). In the present study, we presented the ascomatal development in *Xylonomycetes* for the first time (Figs. 5–7). The ontogeny of the ascomata in *Xylonomycetes* resembles that of the locule in *Dothideomycetes* prior to the formation of the asci (Luttrell, 1953, 1981). *Sarea* and *Tromera* were found to possess locular paraphysoids, differing from dothideomycetous development (Figs. 3L, 4M, N) and instead resembling the developmental pattern of the *Lecanoromycetes* (Henssen et al., 1981), although *Xylonomycetes* does not possess a thallus. The anatomical structures of the ascus of *Xylonomycetes* were first observed by Bellemére (1994), who found that the asci of *S. difformis* and *T. resinae* (as *S. difformis*) are bitunicate with rostrate type dehiscence from the ascus apex, using TEM. Collectively, these features of *Xylonomycetes* do not align with those of any other class of *Pezizomycotina*, *Ascomycota*.

On the basis of morphological resemblance, *Sybiotaphrina microtheca* (P. Karst.) Baral, E. Weber & G. Marson was provisionally treated as a member of *Sarea* and *Tromera* due to its polysporic asci with a *Lecanora*-type ascus apex (Karsten, 1888; Kuntze, 1898). Hawksworth and Sherwood (1981) claimed that structureless peridium and an iodine staining-positive ascus were important for generic circumscription, and established *Tromeropsis* to accommodate a single species, *Tromeropsis microtheca* (P. Karst.) Sherwood. Because they were unable to confidently assign the genus to an appropriate order or family, *Tromeropsis* has long been treated as

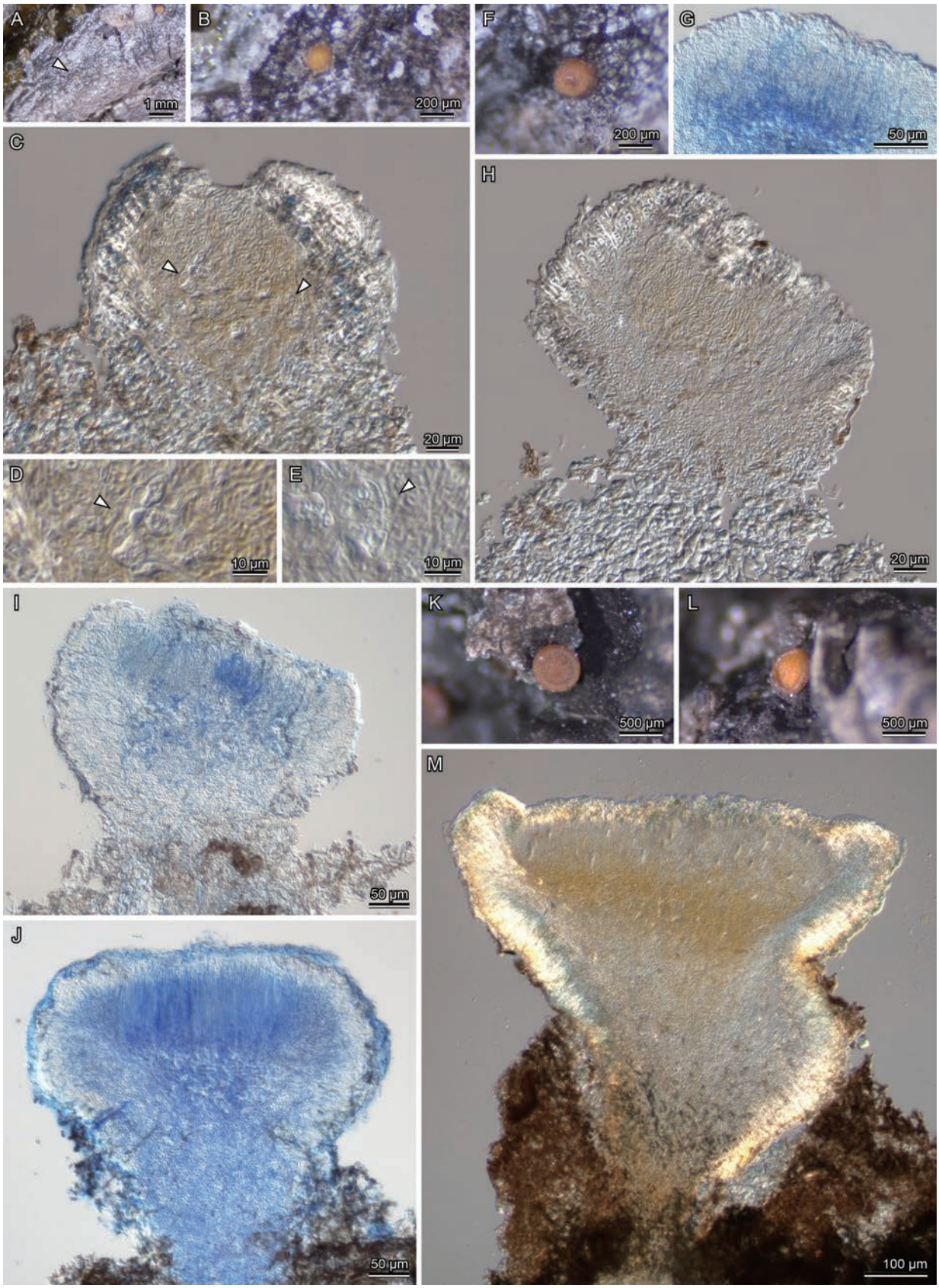

Fig. 6. Ascomatal development of *Tromera resinae*. A–E: Initial stage of ascomatal development. A, B: Young ascomata on the host tissue. C: Generative tissue with ascogonia (arrowhead indicates ascogonia). D, E: Ascogonium. F–H: Development of paraphysoid. F: Young ascomata on the host tissue. G: Earlier developed paraphysoids. H: Primordium with paraphysoids. I, J: Development of hymenium. Note hymenium is filled with paraphysoids (in J). K–M: Asci developmental stage. K, L: Young ascomata with opened cortical layer; M: Young ascoma in longitudinal section. Note young ascus developed in hymenium. A–M from AH 1164.

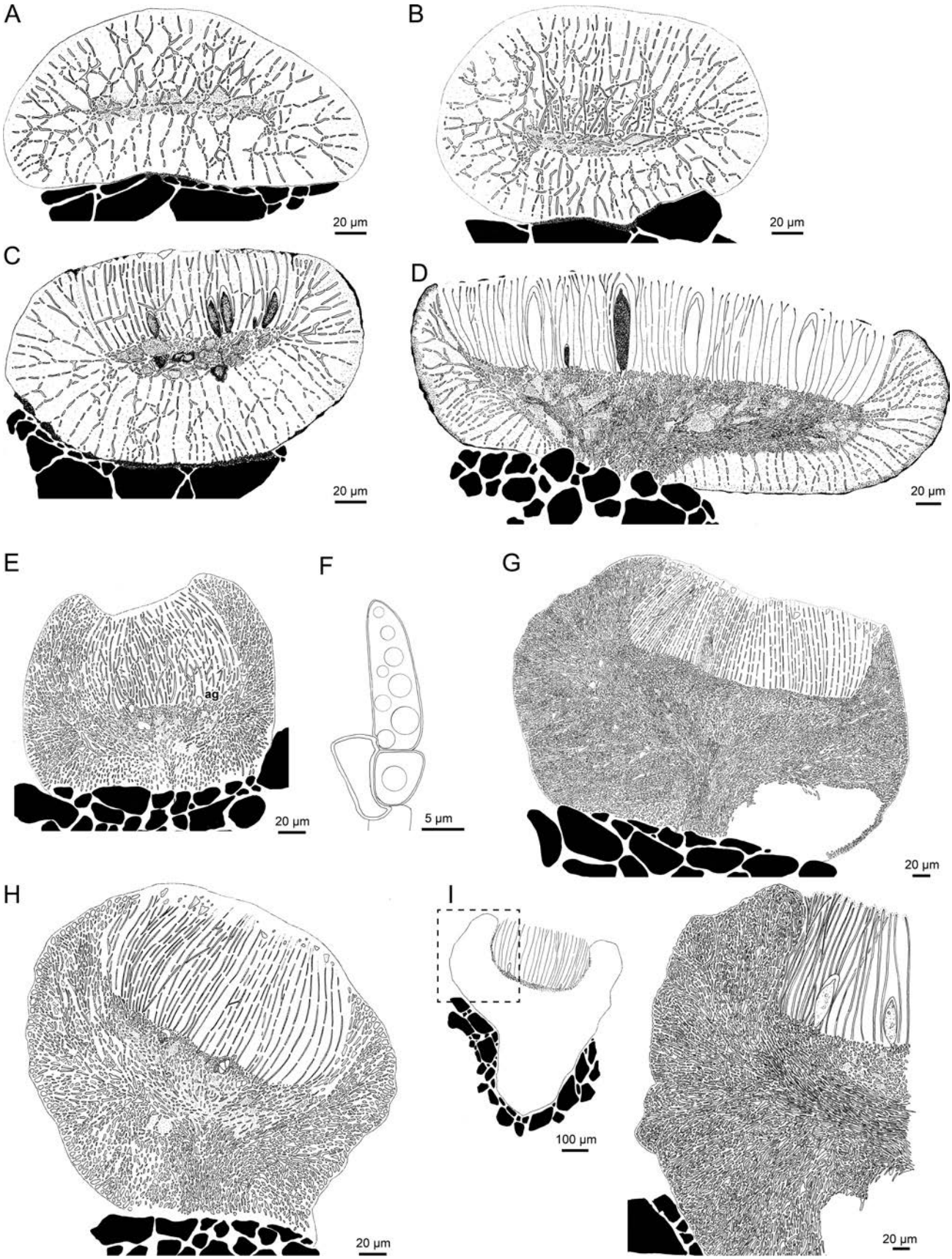

Fig. 7. Ascomatal development of *Sarea difformis* (A–D) and *Tromera resinae* (E–I). A: Initial stage of development of ascoma. B: Early stage of development of paraphysoids. C: Development of hymenium with immature asci. D: Young Ascoma. Note hymenium is opened. E: Initial stage of development of ascoma. Ascogonia indicated "ag". F: Ascogonia. G: Early stage of development of paraphysoids. H: Development of hymenium. I: Young Ascoma with immature asci. Note hymenium is opened. A–D from AH 1278. E–I from AH 1164.

Table 4. Morphological comparison of genera of *Xylonomycetes* accepted in this study.

	Sarea	*Trinosporium*	*Tromera*	*Xylona*
Habitats	Saprobic on pine resin. eDNA occurrence also see Fig. 2 and Supplementary Table S2.	Obtained as contaminant	Saprobic on pine resin. eDNA occurrence also see Fig. 2 and Supplementary Table S2.	Endophyte in sapwood
Sexual morph				
Ascomata	Ascostroma-type ascomata (Figs. 5, 7A–D), up to 500 μm diam.	NA	Ascostroma-type ascomata (Figs. 6, 7E–I), up to 750 μm diam.	NA
Peridium	Composed of pigmented hyphae with gel (Figs. 5C, D, 7A, B)	NA	Composed of hyaline hyphae without gel (Figs. 6C, H, 7E, G)	NA
Hamathecium	Asci and hamathecium are formed simultaneously (Figs. 5I, J, 7C, D)	NA	Developed after hamathecium is matured (Figs. 6I, J, M, 7H, I)	NA
Ascus	Hyaline in all developmental stage (Fig. 8A–C), bitunicate, polysporus asci with a *Lecanora*-type ascus apex and up to 3.3 μm thick of peridial gel (Fig. 3J–M)	NA	Well-pigmented from its initial stage (Fig. 8D, E), bitunicate, polysporus asci with a *Lecanora*-type ascus apex and up to 1.3 μm thick of peridial gel (Fig. 4K–P)	NA
Asexual morph				
Conidiomata	Pycnidia lacking an ostiole	Ostiolate pycnidia	Ostiolate pycnidia	Pycnidia lacking an ostiole
Conidiophore	Hyaline, hyaline	Hyaline, branched	Hyaline, branched	Absent
Conidiogenous cells	Phialidic and/or annelidic	Phialidic	Phialidic or rarely annelidic	Phialidic
Conidia	Subglobose, pale brown conidia with catenate	Heart-shaped, brown	Subglobose, hyaline conidia	Brown and heart-shaped conidia
References	This study Hawksworth and Sherwood (1981)	Crous et al. (2012)	This study Hawksworth and Sherwood (1981)	Gazis et al. (2012)

NA indicates not observed in previous study.

an *Ascomycota* genus *incertae sedis* (Hawksworth & Sherwood, 1981; Kirk et al., 2009; Lumbsch & Huhndorf, 2010; Wijayawardene et al., 2017). Recently, Baral et al. (2018) found *Tromeropsis microtheca* is phylogenetically close to *Symbiotaphrina* based on nSSU, LSU, and 5.8S sequences. The asexual morphology of *Tromeropsis microtheca* matched that of *Symbiotaphrina*, and *Tromeropsis* was therefore synonymized under *Symbiotaphrina*. *Symbiotaphrinales* can be distinguished from *Xylonomycetes* by its paraphyses, which are not thickened at the apex, and its poorly differentiated bitunicate asci and thin-walled apex (up to 4.5 μm) in sexual morph found on the surfaces of the dead xeric wood of gymnosperms and angiosperms; the yeast stage in the asexual morph is symbiotic in the gut of arthropods (Baral et al., 2018; Hawksworth & Sherwood, 1981; Noda & Kodama, 1996). We therefore treated *Symbiotaphrinales* as *Pezizomycotina incertae sedis* in this study. To clarify the precise class position of *Symbiotaphrinales*, additional sequences, as well as the further discovery of hidden lineages, will be required.

4.2. *Relationships between* Sarea *and* Tromera

Hawksworth and Sherwood (1981) emphasized the resinicolous habitat and bitunicate, polysporic asci with *Lecanora*-type ascus apex and treated *Tromera* as a synonym of *Sarea*. This broad taxonomic concept was subsequently supported by later studies (Beimforde et al., 2020; Bellemére, 1994; Ellis & Ellis, 1997; Kirk et al., 2009; Suto, 1985; Wijayawardene et al., 2018). Our phylogenetic analysis of *Sarea* and *Tromera* showed that their monophyletic status was moderately supported in several analyses (e.g. 84% in ML BP, below 60% in MP BP and 0.99 in Bayesian PP; Fig. 1), but the monophyly of each genus was robustly supported (100% in ML BP, below 60% in MP BP and 1.00 Bayesian PP; Fig. 1). Several morphological differences were noted for these genera, shown below and in Table 4. *Sarea difformis* has smaller ascomata (up to 500 μm diam; Fig. 3H) with peridium composed of gelatinous hyphae, while *S. resinae* has ascomata reaching 750 μm diam (Fig. 4I, J) and a peridium composed of radially arranged cellular hyphae. The ontogeny of the ascomata differs between the two genera. The peridium of *T. resinae* is composed of hyaline hyphae without gel at all stages of development (Figs. 6C, I, 7E, F), while in *S. difformis*, the peridium is composed of pigmented hyphae with gel present from the initial stage (Figs. 5C, D, 7A, B). In *T. resinae*, the asci are formed after the development of the hamathecium (Figs. 6I, J, M, 7H), whereas in *S. difformis*, the asci and hamathecium are formed simultaneously (Figs. 5F, I, J, 7C, D). Beimforde et al. (2020) mentioned the thickness of peridium of ascomata and these cells varied in *S. difformis* and *T. resinae* (as *S. resinae*). Because of the wide range of growth capacity (up to 500 μm diam in *S. difformis* and 0.5–2 mm diam in *T. resinae*), the variability of thickness of the peridium can be agreed and may be unstable characteristics depending on different conditions or environment. The contexture of the peridium and thickness of these cells, however, are always stable even on different conditions at matured ascomata (Fig. 3F, G in *S. difformis*, and Fig. 4F–H in *T. resinae*). The thickness of peridial cells of ascomata would change during their developmental stage (Figs. 5–7), and should be measured using only matured ascomata.

In both genera, the asci are initially polysporic and lacking an intervening primary ascospore (Fig. 8). The asci of *T. resinae* were found to be well-pigmented from the initial stage of development (Fig. 8D, E), but those of *S. difformis* were not pigmented (Fig. 8A–C). Bellemére (1994) compared the anatomical structures of the ascus of both species. Although Bellemére (1994) considered the ascus structures of both groups to be superficially similar (by the presence of a thickened ascus apex), differences in the anatomical structure were overlooked in the TEM. Our light microscope observations show a well-developed thickened peridial gel (up to 3.3 μm thick) in *S. difformis* (Fig. 3K), with a similar but thinner (up to 1.3 μm thick) structure in *T. resinae* (Fig. 4L). These differences in structure may be related to a positive reaction following iodine staining. Additionally, the morphological characters of their asexual morphs are also different; *S. difformis* has ostiole lacking and unilocular conidiomata, while *T. resinae* has papillate ostiole and multilocular conidiomata (Hawkswoth & Sherwood, 1981; Table 4). Thus, we treat *Tromera* and *Sarea* as separate genera based on the morphological differences in the sexual/asexual morphs and their developmental stage.

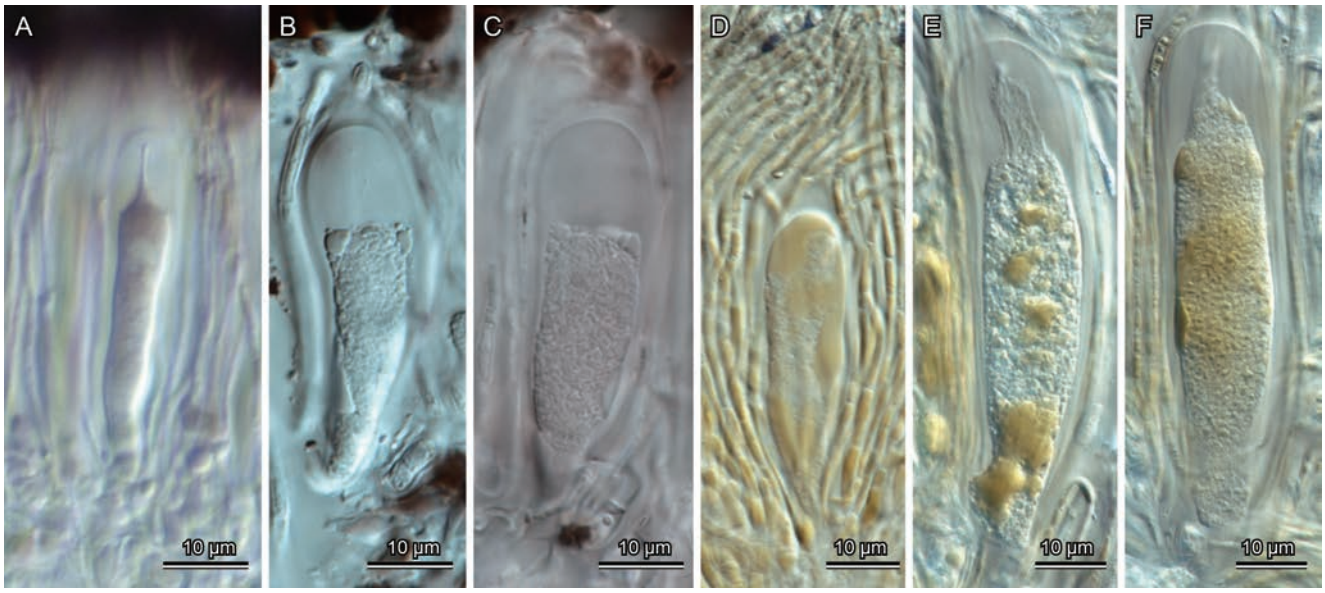

Fig. 8. Ascus development of *Sarea difformis* (A–C) and *Tromera resinae* (D–F). A: Initial stage of ascus development. B, C: Immature asci. D, E: Initial stage of ascus development. Note inside of the ascus is hyaline and pigmented in *S. difformis* and *T. resinae*, respectively. F: Immature ascus. Note ascospores developed in ascus. A, B from AH 1278; C from AH 1309; D–F from AH 1164.

4.3. Re-examination of the ecological niche of Sarea and Tromera

Phylogenetic analyses clarified the worldwide distribution of *S. difformis* and *T. resinae* (Fig. 2). The population occurring in the gymnosperms and the thallus of lichen-forming fungi in both species cannot be phylogenetically segregated by ITS sequences. The high frequency of isolation/detection of *Sarea* and *Tromera* from the thallus of lichen-forming fungi may implicate their hidden habitats as mentioned by Masumoto and Degawa (2019). Few samples were detected from unexpected habitats, i.e. monocots and seaweeds, in *T. resinae*. Their occurrence in these "unexpected habitats" may be attributed to the presence of DNA (most likely in the form of spores) present in these environments, which does not imply that they are natural of the habitat for their lifecycle. To clarify the diversity of their natural habitat, reproducible of isolation/detection or clarifying their mode of existence in each environment, will be required.

Taxonomy

Based on the present study, following taxonomic changes are required.

Xylonomycetes Gazis & P. Chaverri, Mol. Phylogen. Evol. 65: 301, 2012.
= *Sareomycetes* Beimforde, A.R. Schmidt, Rikkinen & J.K. Mitch. Fung. Syst. Evo. 6: 29, 2020
Type order: *Xylonales* Gazis & P. Chaverri

Xylonales Gazis & P. Chaverri, Mol. Phylogen. Evol. 65: 301, 2012.
= *Sareales* Beimforde, A.R. Schmidt, Rikkinen & J.K. Mitch. Fung. Syst. Evo. 6: 29, 2020
Type family: *Xylonaceae* Gazis & P. Chaverri

Xylonaceae Gazis & P. Chaverri, in Gazis, Miądlikowska, Lutzoni, Arnold & Chaverri, Mol. Phylogen. Evol. 65: 301, 2012.
= *Sareaceae* Beimforde, A.R. Schmidt, Rikkinen & J.K. Mitch. Fung. Syst. Evo. 6: 29, 2020
Endolichenic on Lecanoromycetes, endophytic or saprobic on various plants.
Sexual morph: *Ascomata* superficial, discoid, orange or black. *Peridium* composed of radially arranged hyphae and pigmented amorphous granules. Subhymenium composed of pseudoparenchymatous, composed of hyaline to dark brown cells. *Paraphysoid* filiform, septate, mostly unbranched, rarely anastomosed, the apices cemented in a dark brown gel. *Asci* bitunicate, clavate, with a thick inner layer, with a short stipe, apically rounded with a broad apical dome, polyspored. *Ascospores* spherical, hyaline, smooth. **Asexual morph:** *Conidiomata* pycnidial, astromatic; Peridium composed of thin-walled cells, ostiole lacked or central. *Conidiophores* hyaline, branched or absent. *Conidiogenous cells* holoblastic or phialidic, annelidic, hyaline. *Conidia* ellipsoidal to apically rounded with two lateral obtuse projections appearing heart-shaped, narrower and truncated at base, hyaline or dark brown, aseptate.
Type genus: *Xylona* Gazis & P. Chaverri, in Gazis, Miądlikowska, Lutzoni, Arnold & Chaverri
Notes: Our phylogenetic analyses using the nSSU-LSU-*rpb1-rpb2*-mtSSU sequences suggest that *Xylonomycetes* encompasses *Sarea*, *Trinosporium*, *Tromera*, and *Xylona*, whereas *Symbiotaphrinales* forms a lineage distinct from all known classes in *Pezizomycotina* (Fig. 1).

Sarea Fr., Syst. orb. veg. (Lundae) 1: 86, 1825.
Type species: *Sarea difformis* (Fr.) Fr.
Notes — The genus *Sarea* was informally proposed as a provisional name because no species was mentioned in the original description (Fries, 1825). Two species were later assigned to this genus: *S. complanata* (Fr.) Fr. and *S. difformis* without a type designation (Fries, 1828). *Sarea complanata* was transferred to *Helotium* Pers., and *S. difformis* remained as the original element of the genus (Kuntze, 1898). *Tromera resinae* and 11 species were also transferred to *Sarea*. The earliest lectotypification of *Sarea* appears to have been made by Hawksworth and Sherwood (1981), who designated *S. difformis* as the type species based on Art. 10.2 (Shenzhen Code). Twenty-two taxa were listed in Index Fungorum (http://www.

indexfungorum.org/; accessed Dec 1, 2019). Most of the species were transferred to other leotiomycetous or lecanoromyceteous genera (Baral et al., 2018; Bayliss Elliott, 1914; Carmer, 1975; Dennis, 1956; Fuckel, 1870; Hafellner, 1994; Hawksworth, 1980; Hawksworth & Sherwood, 1981; Killermann, 1935; Korf & Abawi, 1971; Kuntze, 1898; Poelt, 1958; Sánchez, 1967; Schröter, 1893; Seifert, 1985; Seifert & Carpenter, 1987; Sydow, 1887; Wong & Brodo, 1990). As such, the genus presently comprises a single species: *S. difformis.*

Sarea difformis (Fr.) Fr., Elench. fung. 2: 14, 1828.
For synonyms, see Hawksworth and Sherwood (1981).
Endolichenic, endophytic or saprobic on cortex of *Pinaceae*. ***Sexual morph*:** *Ascomata* scattered, superficial, sessile or rarely stipitate, black, 400–500 μm diam, 300 μm high in section, circular in outline. *Peridium* 50–60 μm thick, composed of radially arranged 2.5 μm thick of hyaline hyphae embedded in thickened gel, surrounding by brown to blackish amorphous granules. *Subhymenium* 85–95 μm thick, gelatinous, pseudoparenchymatous, composed of dark brown cells. *Paraphysoid* numerous, to 78.5 μm high, 2–2.5 μm wide, filiform, septate, mostly unbranched, rarely anastomosed, the apices cemented in a dark brown gel to form an epithecial layer, turning deep blue in iodine. *Asci* bitunicate, clavate, with a thick inner layer, I-, outer layer with 1.6–3.3 μm of peridial gel, I+, 43–65 × 12–26.5 μm ($\overline{x}$ = 51.1 × 15.7 μm, n = 14), with a short stipe, apically rounded with a broad apical dome, up to 12 μm thick, polyspored from the first. *Ascospores* spherical, 2.3–2.7 μm diam ($\overline{x}$ = 2.44 μm diam, n = 110), hyaline, smooth, aseptate. ***Asexual morph*:** Not observed among the examined specimens.
Specimens examined: JAPAN, Iwate, Morioka, Yanagawa, near route 106, on cortex of *Larix kaempferi*, 28 May 2019, A. Hashimoto & H. Masumoto, AH 1278 (= TNS-F-89129; single ascospore isolate culture AH 1278, JCM 39114); Tono, Tsuchibuchi, Tochinai, route 340, near Ontoku river, on cortex of *Larix kaempferi*, 28 May 2019, A. Hashimoto & H. Masumoto, AH 1309 (= TNS-F-89130; multi ascospores isolate culture AH 1309, JCM 39115).
Notes: In the original description of *Peziza difformis* Fr., Fries (1822) mentioned the habitats as *Pinus* and rarely *Abies* but did not give any information on locality. Due to the lack of type specimen and authentic materials, Hawksworth and Sherwood (1981) neotypified Rehm's specimen, which lack host and locality information.

The Japanese specimens above were identified as *S. difformis*. The size of the ascospores in our materials was almost identical to that of *S. difformis* reported by Hawksworth and Sherwood (1981), for the neotype. This is the first report of *S. difformis* from Japan.

Tromera A. Massal. ex Körb., Parerga lichenol. (Breslau) 5: 453, 1865.
= *Tromera* Massal. in Arnold, Flora 41: 507, 1858. Nom. inval. (Article 32.1, Shenzhen Code)
= *Pycnidiella* Höhn., Sber. Akad. Wiss. Wien, Math.-Naturw. Kl. Abt. 1 124: 91, 1915.
Type species: *Tromera resinae* (Fr.) Körb.
Notes — *Tromera* was informally introduced by Arnold (1858) without type designation to include two species, *T. sarcogynoides* A. Massal. and *T. xanthostigma* A. Massal., whose names are invalid according to Art. 35.1 (Shenzhen Code). Later, Körber (1865) provided a formerly generic diagnosis for the genus and accepted a single species *T. resinae* by treating the previous two invalid names as its synonyms. In a monograph of a resinicolous species, Hawksworth and Sherwood (1981) treated *T. sarcogynoides* also as a synonym of *S. difformis*.

Twelve taxa are listed in Index Fungorum (http://www.indexfungorum.org/; accessed Dec 1, 2019). Most of the species have been transferred to *Biatorella* De Not. (as *B. fossarum* (Dufour) Th. Fr.; Lindau, 1923), *Claussenomyces* Kirschst. (as *C. olivaceus* (Fuckel) Sherwood; Hawksworth & Sherwood, 1981), *Stereocaulon* Hoffm. (as *S. cumulatum* (Sommerf.) Timdal (= *T. perfidiosa* (Nyl.) Räsänen); Timdal, 2002), *Symbiotaphrina* (as *Symb. microtheca*; Baral et al., 2018), and *Sarea* (as *Sarea difformis*; this study), or synonymized to *Claussenomyces atrovirens* (Pers.) Korf & Abawi (= *T. myriospora* (Hepp) Anzi, *T. ligniaria* P. Karst.; Hawksworth & Sherwood, 1981), *S. difformis* (= *T. sarcogynoides*; Hawksworth & Sherwood, 1981), and *Tromera resinae* (= *T. xanthostigma*; Hawksworth & Sherwood, 1981). One species, *Tromera aurellae* Werner, which has been reported as a lichenicolous fungi on *Candelariella aurella* (Hoffm.) Zahlbr. (Werner, 1934), was excluded from this genus by Hawksworth and Sherwood (1981). *Tromera sampaio* (Gonz. Frag.) Keissl., which was originally described as *Comesia sampaioi* Gonz. Frag. by González Fragoso (1924), should also be excluded from the genus because it is characterized by lichenicolous habitats on the thallus of *Physma chalazanellum* (Nyl.) Erichsen and produces numerous ascoconidia within the asci (González Fragoso, 1924; Keissler, 1928); whereas *Tromera* does not produce ascoconidia (Hawksworth & Sherwood, 1981; Fig. 8D–F in this study). Thus, the genus is presently a monotypic genus composed of the species *T. resinae*.

Hawksworth and Sherwood (1981) observed both the species of *Sarea* and *Tromera*, and thus treated *Tromera* as a synonym of *Sarea*. However, they did also recognize differences in the anatomical structures of the ascus and conidiogenous cell types (mostly anellidic in *Sarea* vs. mostly phialidic in *Tromera*). This treatment was supported by subsequent monographic studies (Bellemére, 1994; Ellis & Ellis, 1997; Kirk et al., 2009; Suto, 1985; Wijayawardene et al., 2018). Our phylogenetic, ontogenetic, and morphological studies clarify that the two genera should be separated. Thus, we retained *Tromera* in *Xylonaceae*.

Tromera resinae (Fr.) Körb., Parerga Lichenol. (Breslau) 5: 453, 1865.
≡ *Lecidea resinae* Fr., Observ. Mycol. (Havniae) 1: 180, 1815.
≡ *Sarea resinae* (Fr.) Kuntze, Revis. Gen. Pl. (Leipzig) 3(3): 515, 1898.
= *Cytospora resinae* Ehrenb., Sylv. Mycol. Berol. (Berlin): 28, 1818.
≡ *Pycnidiella resinae* (Ehrenb.) Höhn., Sber. Kaiserl. Akad. Wiss. Wien, Mat. Nat. Klasse, Abt. 1, 124: 91, 1915.
= *Peziza myriospora* Hepp, Flecht. Europ.: no. 332, 1857. Nom. illegit., Art. 52.1 (Shenzhen Code)
≡ *Tromera myriospora* (Hepp) Anzi, Cat. Lich. Sondr.: 117, 1860. Nom. illegit., Art. 52.1 (Shenzhen Code)
= *Sphaeria resinae* Fr., Observ. Mycol. (Havniae) 1: 180, 1815.
≡ *Zythia resinae* (Fr.) P. Karst., Meddn Soc. Fauna Flora fenn. 16: 104, 1890 (1889).
= *Tromera xanthostigma* A. Massal., Flora, Regensburg 41: 507, 1858. Nom. illegit., Art. 38.1 and 38.5 (Shenzhen Code)
For other synonyms, see Hawksworth and Sherwood (1981).
Endolichenic, endophytic or saprobic on cortex of *Pinaceae*. ***Sexual morph*:** *Ascomata* scattered, superficial, sessile or rarely stipitate, pale orange, 0.5–2 mm diam, 450 μm high in section, circular in outline. *Peridium* composed of radially arranged cellular hyphae composed of 1.5 μm thick of elongated, thin-walled, hyaline cells, surrounding by orange-red amorphous granules. *Subhymenium* 80–110 μm thick, gelatinous, almost pseudoparenchymatous, composed of elongated, thin-walled, hyaline cells. *Paraphysoid* numerous, up to 85 μm high, 1–1.5 μm wide, filiform, septate, mostly un-

branched, rarely anastomosed, the apices cemented into a gel, with orange granules forming an epithecial layer, turning weakly blue in iodine. *Asci* bitunicate, clavate, with a thick inner wall, I+, outer layer with 0.5–1.3 μm thick of peridial gel, I+, 69.5–106 × 12–22.5 μm ($\bar{x}$ = 81.1 × 19.7 μm, n = 10) with a short stipe, apically rounded with a broad apical dome, up to 14.5 μm thick, polyspored from the first. *Ascospores* spherical, 2–2.6 μm diam ($\bar{x}$ = 2.39 μm diam, n = 116), hyaline, smooth, aseptate. ***Asexual morph*:** Not observed among the examined specimens.

Specimens examined: JAPAN, Nagano, Ueda, Sugadaira Research Station, Mountain Science Center, University of Tsukuba, on cortex of *Larix kaempferi*, 9 Sep 2018, A. Hashimoto & H. Masumoto, AH 1107 (= TNS-F-89131; single ascospore isolate culture AH 1107, JCM 39116); ibid., on cortex of *Larix kaempferi*, 1 Feb 2019, H. Masumoto, AH 1149 (= TNS-F-89132; multi ascospores isolate culture AH 1149, JCM 39117); Tochigi, Utsunomiya, near Akagawa dam, 5 Jan 2019, A. Hashimoto, K. Yamamoto, K. Seto, & Y. Takashima, AH 1164 (= TNS-F-89133; multi ascospores isolate culture AH 1164, JCM 39118).

Notes: The nomenclatural history of *T. resinae* is complicated. The species was originally described as *Lecidea resinae*, from pine resin of *Pinus* and *Abies* (Fries, 1815). However, as no type specimen was specified, Hawksworth and Sherwood (1981) selected one of the original specimens as the lectotype material.

Our Japanese materials were identified as *T. resinae*, based on the description by Hawksworth and Sherwood (1981). Although *T. resinae* has already been reported from Japan by Suto (1985), it was only briefly described. Therefore, a detailed description and illustrations are presented in this study based on fresh specimens.

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Disclosures

All authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of the country in which they were performed.

Author Contributions

Conceived and designed the experiments: AH; HM.
Field Research: AH; HM.
Performed the experiments: AH.
Methodology: AH.
Review and editing: AH; HM; RE; YD; MO.
Supervision: MO.
Wrote the paper: AH.

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