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Phylogenomic analysis of 2556 single-copy protein-coding genes resolves most evolutionary relationships for the major clades in the most diverse group of lichen-forming fungi

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Abstract

Phylogenomic datasets continue to enhance our understanding of evolutionary relationships in many lineages of organisms. However, genome-scale data have not been widely implemented in reconstructing relationships in lichenized fungi. Here we generate a data set comprised of 2556 single-copy protein-coding genes to reconstruct previously unresolved relationships in the most diverse family of lichen-forming fungi, Parmeliaceae. Our sampling included 51 taxa, mainly from the subfamily Parmelioideae, and represented six of the seven previously identified major clades within the family. Our results provided strong support for the monophyly of each of these major clades and most backbone relationships in the topology were recovered with high nodal support based on concatenated dataset and species tree analyses. The alectorioid clade was strongly supported as sister-group to all remaining clades, which were divided into two major sister-groups. In the first major clade the anzioid and usneoid clades formed a strongly supported sister-group relationship with the cetrarioid + hypogymnioid group. The sister-group relationship of *Evernia* with the cetrarioid clade was also strongly supported, whereas that between the anzioid and usneoid clades needs further investigation. In the second major clade *Oropogon* and *Platismatia* were sister to the parmelioid group, while the position of *Omphalora* was not fully resolved. This study demonstrates the power of genome-scale data sets to resolve long-standing, ambiguous phylogenetic relationships of lichen-forming fungi. Furthermore, the topology inferred in this study will provide a valuable framework for better understanding diversification in the most diverse lineage of lichen-forming fungi, Parmeliaceae.

Keywords Fungi · Lecanorales · Lichenized fungi · Parmeliaceae · Parmelioideae · Phylogeny · Systematics

Introduction

Progress in sequencing technology has facilitated the efficient generation of genome-scale DNA sequence data and has revolutionized biological research in a wide range of

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fields. In phylogenetic systematics, genomic data has provided unprecedented insight into evolutionary relationships with the potential to generate thousands of independent phylogenetic markers distributed across genomes (Eisen and Fraser 2003; Murphy et al. 2004; Delsuc et al. 2005; Philippe et al. 2005; Meusemann et al. 2010; Torruella et al. 2012; Misof et al. 2014; Spatafora et al. 2017). Types of loci used in phylogenomic analyses range from restriction site-associated DNA sequencing [RADseq; (Andrews et al. 2016)] to target enrichment to capture known protein-coding regions distributed across the genome (Bragg et al. 2016) or ultraconserved elements (UCEs) (Faircloth et al. 2012).

Single-copy genes, or single-copy gene families, are valuable molecular markers for inferring relationships of previously unresolved lineages across Eukaryota (Shen et al. 2013; Salas-Leiva et al. 2014; Zeng et al. 2014; Ren et al. 2016). Generally single-copy genes are easily amplified and sequenced, because they occur only once in the genome and they are highly conserved across species. Whole genome sequencing can facilitate the identification of single-copy genes as potential phylogenetic markers. Two decades after the first fungal genome was sequenced, genomes from over 800 fungal species are now available (Spatafora et al. 2017). These data have been widely used to address phylogenetic relationships in fungi, especially Ascomycota (Robbertse et al. 2006; Liu et al. 2009; Wang et al. 2009; Medina et al. 2011; Ebersberger et al. 2012; Ekanayake et al. 2013; Zheng et al. 2013; Payen et al. 2014; Hettiarachchige et al. 2015; Luo et al. 2015; Lachance et al. 2016; Whiston and Taylor 2016; Choi and Kim 2017; Menardo et al. 2017; Spatafora et al. 2017; Teixeira et al. 2017). However, the number of phylogenomic studies addressing evolutionary relationships in lichen-forming fungi, which represent an important part of ascomycete diversity (Ott and Lumbsch 2001; Nash 2008; Jaklitsch et al. 2016), is still low and limited to resolving relationships among closely related species (Leavitt et al. 2016c; Grewe et al. 2017).

Here we generated a phylogenomic data set to address evolutionary relationships among major clades in the subfamily Parmelioideae of Parmeliaceae, which represents one of the most diverse clades of lichen-forming fungi (Kraichak et al. 2015; Divakar et al. 2017; Kraichak et al. 2017). Parmeliaceae includes almost 2800 currently accepted species (Jaklitsch et al. 2016), but the number of species is probably higher due to the frequent presence of cryptic species in this group (Kroken and Taylor 2001; Crespo and Perez-Ortega 2009; Divakar et al. 2010b; Lumbsch and Leavitt 2011; Molina et al. 2011a, b; Nuñez-Zapata et al. 2011; Amo de Paz et al. 2012; Leavitt et al. 2012, 2016a, b; Altermann et al. 2014; Singh et al. 2015; Alors et al. 2016; Del-Prado et al. 2016). Estimates suggest that the family originated during the Cretaceous with increase diversification rates after the Cretaceous-Paleogene (K-Pg) boundary and diversification within genera predominantly during the Miocene, resulting in the current hyper-diversity of the family (Amo de Paz et al. 2011; Kraichak et al. 2015; Huang et al. 2017).

Parmeliaceae occurs in all terrestrial ecosystems and all continents, including Antarctica, but has its highest species diversity in tropical montane areas and temperate to subtropical regions with Mediterranean-type climate (Crespo et al. 2010b; Thell et al. 2012). Members in the family grow on various substrates, including tree bark, wood, rocks, and soil. Species of Parmeliaceae have been widely used as bioindicators for air pollution and ecological health (Hawksworth and Rose 1970, 1976; Crespo et al. 1999). Additional human uses of Parmeliaceae include dyes, perfumes and medicinal remedies with pharmacological potential in folk medicines (Gomez-Serranillos et al. 2014).

The circumscription of the family Parmeliaceae, its sister-group relation, and the classification into genera within the family has been controversial and much discussed (as reviewed in (Elix 1993; Crespo et al. 2010b; Thell et al. 2012; Singh et al. 2013). However, phylogenetic studies (e.g., Mattsson and Wedin 1999; Wedin et al. 1999; Arup et al. 2007; Amo de Paz et al. 2010a, b, 2011; Crespo et al. 2010a, b; Divakar et al. 2010a; 2012, 2013, 2015, 2017; Thell et al. 2012; Singh et al. 2013; ; Kirika et al. 2015, 2016a, b) resulted in a general consensus on the circumscription of the family, and the sister-group relationship with Gypsoplacaceae (Singh et al. 2013). More recently, two subfamilies have been proposed—Protoparmelioideae and Parmelioideae (Divakar et al. 2017), including a revised circumscription of genera based on strongly supported monophyletic groups of comparable ages (Divakar et al. 2017).

Within the large subfamily Parmelioideae, which includes the bulk of species in Parmeliaceae, a number of strongly supported monophyletic clades have been identified in previous studies (Crespo et al. 2007, b; Divakar et al. 2015). These were given preliminary clade names derived from a characteristic genus, e.g., the cetrarioid clade including the genus *Cetraria* Ach. or the parmelioid clade including the genus *Parmelia* Ach. A recent multi-gene study based on six loci and 293 operational taxonomic units (OTU) recognized seven strongly supported major clades within Parmelioideae, most of which were also identified in a previous study (Crespo et al. 2007): (1) Alectorioid [incl. *Alectoria* Ach., *Bryocaulon* Kärnefelt, *Bryoria* Brodo & D.Hawksw., *Nodobryoria* R.S. Common & Brodo, *Pseudephebe* M. Choisy], (2) Anzioid [incl. *Anzia* Garovagl., *Pannoparmelia* (Müll. Arg.) Darbishire, *Protousnea* (Motyka) H.Krog, *Raesaenenia* D.Hawksw., Boluda & H. Lindgr.—recently (Divakar et al. 2017)

included in *Protousnea*], (3) Cetrarioid [incl. five genera following (Divakar et al. 2017), namely *Cetraria, Dactylina* Nyl., *Esslingeriana* Hale & M.J.Lai, *Melanelia* Esslinger, *Nephromopsis* Müll.Arg.], (4) Hypogymnioid [incl. *Arctoparmelia* Hale, *Brodoa* Goward, *Hypogymnia* (Nyl.) Nyl., *Pseudevernia* Zopf], (5) Parmelioid [incl. c. 25 genera following (Divakar et al. 2017)], (6) Psiloparmelioid [incl. *Everniopsis* Nyl., *Psiloparmelia* Hale], and (7) Usneoid [incl. *Cornicularia* (Schreb.) Hoffm., *Dolichousnea* (Ohmura) Articus, *Eumitria* Stirt., *Usnea* Adans.]. While these monophyletic clades with more than one genus received strong support in multi-gene studies, relationships among the major clades largely remained unsupported.

The aim of this study was to use phylogenomic data to resolve evolutionary relationships among major clades in the diverse subfamily Parmelioideae. Our specific objectives were twofold: (1) identify genome-wide single-copy protein-coding genes and infer relationships among the major clades within Parmeliaceae and (2) assess if phylogenomic datasets can effectively be obtained from metagenomic sequences (Huson et al. 2009; Mitra et al. 2009) to elucidate evolutionary relationships in symbiotic fungi. For this purpose, we used a low-coverage shotgun sequencing (i.e., metagenome skimming) approach to recover genome-wide phylogenetic markers for 46 lichen-forming fungi, representing six of the seven major clades of the subfamily Parmelioideae identified previously (Divakar et al. 2015, 2017).

Materials and methods

Taxon Sampling

A total of 51 lichen-forming fungal species were included in this study (Supplementary Table S1). We selected 46 samples representing six of the seven previously identified major clades in subfamily Parmelioideae of Parmeliaceae (Divakar et al. 2015, 2017). The psiloparmelioid clade was not included, because no fresh material was available. We included genome sequences from seven additional species belonging to other families in Lecanorales (*Cladonia grayi*, *C. macilenta*, *C. metacorallifera* and *Rhizoplaca melanopthalma*), and the orders Teloschistales (*Gyalolechia flavorubescens* and *Xanthoria parietina*) and Umbilicariales (*Lasallia hispanica*; Dal Grande et al. 2018), which also belong to the class Lecanoromycetes, as outgroups.

DNA extraction and sequencing

Total genomic DNA of 43 specimens, except *Evernia prunastri*, *Parmelina carporrhizans* and *Pseudevernia furfuracea* (data already available) was extracted from apothecia or thalli devoid of any visible damage, areas with black dot, contamination or dissimilar colors of the entire thallus using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA) following the manufacturers' instructions. DNA concentration was calculated using the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific, San Diego, CA), resulting in a total yield between 1 and 4 µg DNA. Paired-end libraries (250-bp) were built either using TrueSeq or Nextera XT DNA library preparation kits (Illumina, San Diego, CA) for most of the samples. However, in addition, two mate-pair libraries with average insert length of 3 and 8 kb were built for some samples (as *Alectoria, sarmentosa, Cetraria islandica, Evernia prunastri Lasallia hispanica* and *Pseudevernia furfuracea*). Sequencing of Nextera XT libraries was carried out by the University of Illinois at Chicago Research Resource Center (Chicago, IL) on Illumina's NextSeq platform and TruSeq libraries were sequenced on Illumina MiSeq platform at the Pritzker Laboratory for Molecular Systematics and Evolution at The Field Museum, Chicago, IL, USA. Raw sequences were downloaded from an Illumina BaseSpace application and quality-trimmed and filtered using Trimmomatic-0.36 (http://ww.usadellab.org/cm/?page=trimmomatic) (Bolger et al. 2014). Bases were trimmed when the average quality of 5-base sliding windows was below 20 and bases at the start and end of reads had a quality below 3 and 10, respectively. Subsequently, all trimmed reads shorter than 36 bp were filtered out.

Genome assembly and taxonomy assignment

Trimmed paired-end reads were assembled using MetaSPAdes (Nurk et al. 2017) using default parameters and checking the suitability of k-mer (K21, K33, K55 and K77). To filter the assembly from potential contaminants, and to extract contigs of fungal origin, assemblies were subjected to BLASTX searches using DIAMOND (Buchfink et al. 2015) against a custom database comprising the protein sets of Archaea, Bacteria, Eukaryota, and Viruses of the NCBI nr database (downloaded in August 2016), in addition, 150 complete fungal genomes and 20 algal genomes were added from JGI. Four additional unpublished Parmeliaceae genomes generated from axenic cultures from species within Parmeliaceae [*Cetraria islandica, Parmelina carporrhizans,* unpublished; *Evernia prunastri* and *Pseudevernia furfuracea* (Meiser et al. 2017)] were used as reference genomes for taxonomy assignment, taking only scaffolds belonging to Parmeliaceae. The results of the DIAMOND search were then used as input for MEGAN6 (Huson et al. 2016) for taxonomic assignment (parameters: min-support = 1, min-score = 50, top-hit = 10%, no low complexity filtering). Contigs that were

assigned as Parmeliaceae were extracted and genome statistics were generated using QUAST v. 4.3 (Gurevich et al. 2013), as shown in Supplementary Table S2a–b. We estimated the coverage of the lichen-forming fungus in each metagenome by mapping the filtered Illumina reads against the respective Parmeliaceae-binned contigs.

Genome completeness and ortholog identification

Evaluation of the completeness of the genome assemblies was carried out using the BUSCO v3 pipeline (Simao et al. 2015). This program is useful to detect genome duplication or two genomes in a same sample due to high number of duplicated genes. Every genome was assessed using 3156 single-copy genes present in at least 90% of the 50 reference genomes in OrthoDB v9 (Waterhouse et al. 2013). BUSCO uses reciprocal best hit (RBH), creating a Hidden Markov Model (HMM) profile using the protein sequences of 50 reference genomes for each single-copy gene. Every HMM profile generated was then used as query in tBLASTn searches against each genome to find the putative genomic region. An AUGUSTUS (Stanke et al. 2004) prediction was performed for each of the genomic regions. Predicted genes were then aligned to the HMM profiles of the BUSCO gene, and only the gene with the highest bitscore was kept. The complete BUSCO single-copy genes predicted in each genome were extracted; duplicated and fragmentary genes were removed using an in-house Perl script. A supermatrix was created concatenating every BUSCO single-copy gene using FASconCAT.pl. (Misof et al. 2013).

Every BUSCO single-copy gene recovered from each genome was aligned using MAFFT L-INS-i (–localpair –maxiterate *1000*). In order to reduce the effects of missing data, alignments with more than 20% of missing data were removed. A supermatrix was produced concatenating every BUSCO single-copy gene using FASconCAT.pl. Ambiguous regions within the alignment were removed using Gblocks v. 0.91b (Castresana 2000) implementing default parameters with the exception of using the option 'with-hal' gaps and a 'minimum length of a block' of 5.

Phylogenomic analyses

Evolutionary relationships were estimated both from a supermatrix comprising the concatenated single-locus alignments under maximum likelihood (ML) and using species tree interferences implementing the multispecies coalescent model (Degnan and Rosenberg 2006). ML analyses of the partitioned-marker supermatrix were run using IQTree v.1.5.5 with standard model selection (Nguyen et al. 2015), implementing the GTR + G + I model using ModelFinder (Chernomor et al. 2016). For each analysis, 1000 bootstrap replicates were calculated using the fast bootstrapping option implemented in IQTree. Trees were drawn with FigTree v. 1.4.2 (Rambaut 2009). Based on previous results (Miadlikowska et al. 2014), the resulting tree was rooted with *Lasallia hispanica.*

Since phylogenetic inferences from concatenated datasets may differ from species tree approaches (Knowles 2009), we inferred species-trees for Parmeliaceae using the summary coalescent approach ASTRAL-II (Mirarab and Warnow 2015). We used ASTRAL-II with multi-locus bootstrapping (MLBS) option. Nucleotide substitution models were inferred for each locus using the program PartitionFinder v1.1.1 (Lanfear et al. 2012) with Akaike Information Criterion model selection.

Results and discussion

Genomic data and genome assembly

The genome assembly metrics for the four specimens represented by axenic culture—*Cetraria islandica, Evernia prunastri, Parmelina carporhizans* and *Pseudevernia furfuracea*—and used as part of the reference genome databased used in the taxonomic assignment, as well as the assembly statistics for the 41 binned metagenomes included in this study are reported in Supplementary Tables S1a–b. The length of the assembled mycobiont genomes ranged from 24.1 (*Usnea antarctica*) to 47.6 (*Pannoparmelia angustata*) Mbp, with a mean value of 32.8 Mbp and a %GC-content between 47.3 (*Arctoparmelia centrifuga*) to 56.1% (*Oropogon seacalonicus*), with a mean value of 49.2%). Average coverage of the reconstructed mycobiont genomes varied from 9.8- (*Bulbothrix sensibilis*) to 95.3-fold (*Hypogymnia subphysodes*) (mean = 38.6-fold).

The percentage of complete BUSCO genes among 51 genomes varied from 70.51 to 98.4% of 3156 Pezizomycotina BUSCO genes, with an average of 84.14% (Fig. 1). From the total of 3156 BUSCO genes, between 2213 and 3015 could be recovered for each individual genome. Only two of the 51 lichen forming fungi had < 80% of 3156 Pezizomycotina BUSCO genes, with the assemblies of *Bulbothrix sensibilis* (2223 genes) and *Usnea antarctica* (2213 genes) representing the lowest values of completeness with more than ~ 900 BUSCO genes "missing", "fragmented" or "duplicated" (Fig. 1). Out of 3156 BUSCO genes, 2556 were present in 80% of taxa and were used for the construction of individual gene trees or included into a concatenated super matrix (Supplementary Table S3).

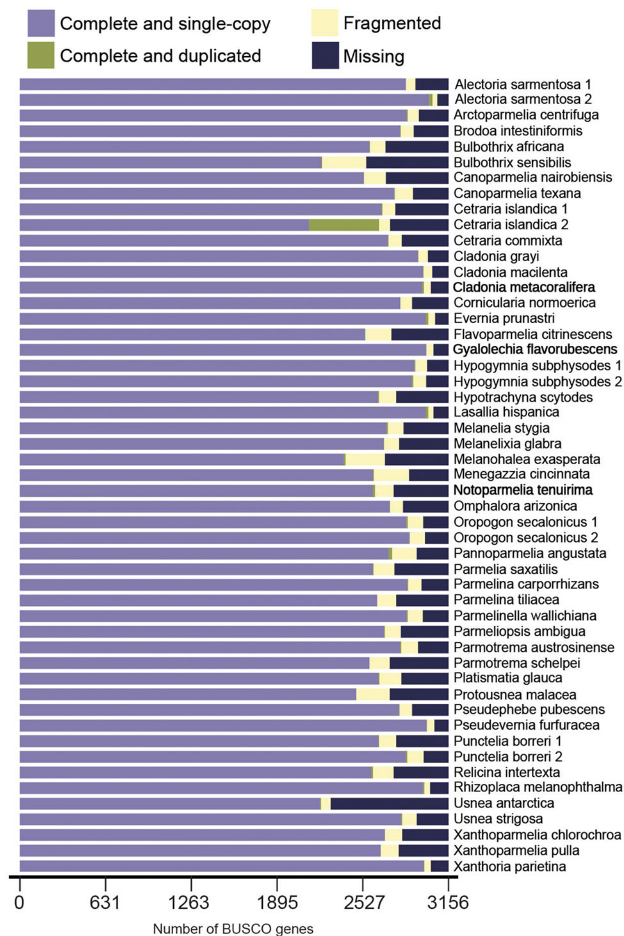

Fig. 1 Genomic quality assessment of the 51 genomes of lichen-forming fungi used in this study. The plot shows the number of BUSCO single-copy genes, which are "complete", "missing", "duplicated", and "fragmented" in each genome

Phylogenomic dataset

The complete and Gblocks-filtered BUSCO data sets were deposited in FigShare: (https://figshare.com/s/0f50e0d44f8bec621d10). The raw BUSCO matrix included 2556 single-copy genes, including introns and small portions of upstream and downstream regions, with a median protein length ranging from 77 to 4912 aa (mean = 629 ± 432.6 aa). The raw alignment had a total size of 10.04 Mbp. Informative and coding sites represented 34.8 and 48.7% of the total length, respectively. After filtering the alignment with Gblocks, the supermatrix consisted of 4,506,888 sites, including 59.4% informative and 94.1% coding sites. The number of unambiguous nucleotide positions in each data set, variable sites and the best fitting models of evolution selected in PartitionFinder, and the average bootstrap tree length, average branch length, variance in branch length, slope, R-Square, percentage of constant sites and number of taxa in each data set are summarized in Supplementary Table S4.

Phylogenomic inferences

Phylogenies inferred from the concatenated phylogenomic dataset and the species tree evaluations recovered highly similar topologies, with few exceptions (Fig. 2a, b). We only consider clades receiving bootstrap support of at least 90% and multilocus-bootstrap of at least 0.98 as strongly supported and focus on these well-supported relationships here. The monophyly of the six major clades of Parmeliaceae represented in this study was strongly supported, with each major clade recovered with 100% bootstrap support and 1.0 multilocus-bootstrap (MLBP). Relationships among these major clades generally received strong support in our phylogenomic analyses (Fig. 2). A comparison of phylogenetic relations and node support between a previously published multi-gene study and the current phylogenomic study is depicted in Supplementary Table S5. The sister-groups of the usneoid and alectorioid clades were unresolved in a previously published multi-gene study, and the genus *Menegazzia* had a well-supported sister-group relationship with *Coelopogon* (not included in this study). However, this clade had no strongly supported close relative. Our study was not entirely conclusive in this regard. While in the species tree analysis *Menegazzia* grouped within the Alectorioid clade (Fig. 2b), in the concatenated phylogenomic dataset analysis the genus *Menegazzia* represented the earliest diverging group in the family, followed by the alectorioid clade (Fig. 2a). In both phylogenomic analyses, the alectorioid clade was strongly supported as sister-group to all remaining clades. The remaining clades were divided into two major sister-groups, (i) *Evernia* + cetrarioid + hypogymnioid + usneoid + anzioid clades, and (ii) *Oropogon* + *Platismatia* + *Omphalora* + parmelioid clade.

In the first major group, the usneoid clade was strongly supported (93% bootstrap support) as sister-group to the anzioid clade. This relation was, however, not supported in the species tree inference (0.55 MLBP). The anzioid and usneoid clades formed a strongly supported (93% bootstrap support, 1.0 MLBP) sister-group relationship with the cetrarioid + hypogymnioid group. Within this latter group, the sister-group relationship of *Evernia* with the cetrarioid clade, which lacked support in a previous study (Divakar et al. 2015), received strong support with both methods here (Fig. 2). The clade including *Evernia* + the cetrarioid clade formed a strongly supported sister-group relationship with the hypogymnioid clade. Both the cetrarioid and the hypogymnioid clades have their centre of distribution in the northern Hemisphere (Goward 1986; Hale 1986; Thell

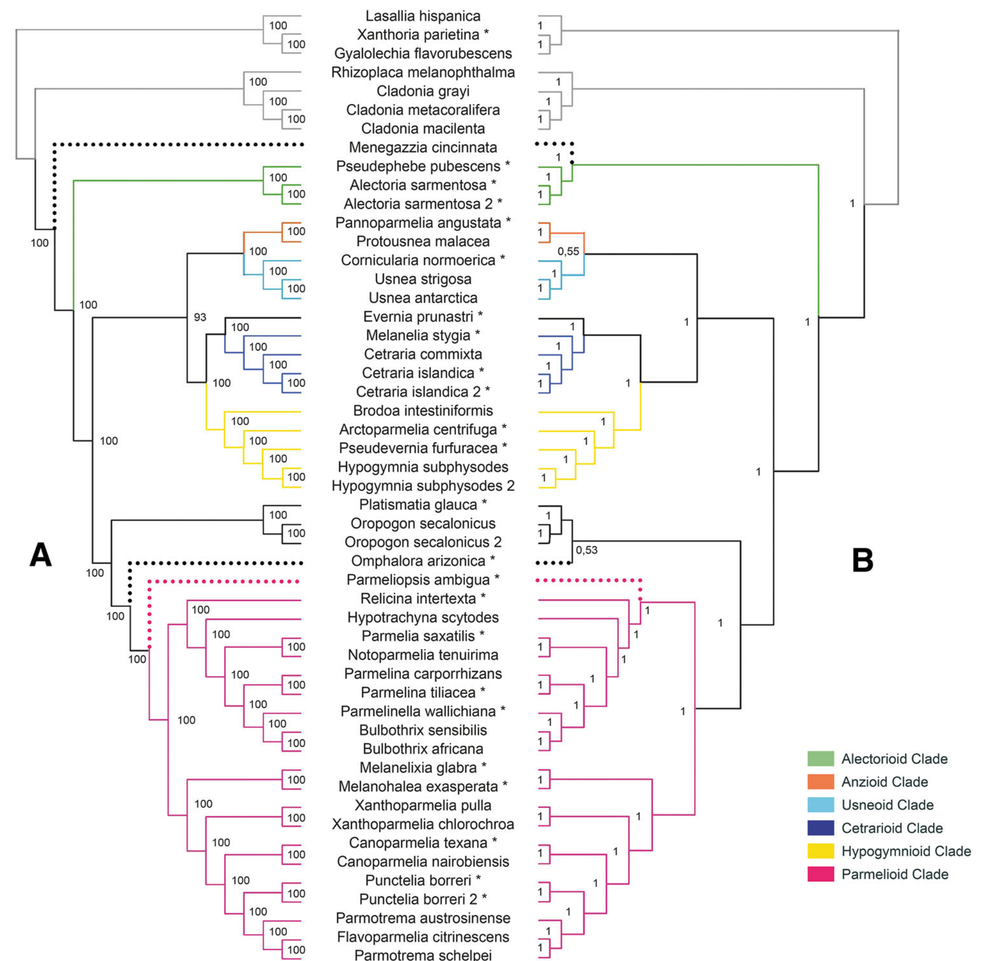

Fig. 2 Evolutionary relationships of six major clades (represented by differently coloured branches) in the subfamily Parmelioideae (Parmeliaceae) based on a concatenated Gblocks alignment of 2556 single-copy protein-coding genes. The tree on the left (**a**) is a ML tree with ML bootstrap values (1000 bootstrap pseudoreplicates) from a IQTree analysis added at nodes (Concatenation-based phylogeny). The tree on the right (**b**) is an ASTRAL species tree based on the ML inferred individual gene trees from the 51 taxa of the Parmelioideae (Coalescence-based phylogeny). Dot lines show conflicts between coalescence-based phylogeny and concatenation-based phylogeny. The type species of genera are marked with an asterisk

et al. 2009, 2012; Miadlikowska et al. 2011; Nelsen et al. 2011; Randlane et al. 2013; Egan 2016).

In the second major group, the position of *Omphalora arizonica* was not clearly established. In the concatenated phylogenomic approach *Omphalora arizonica* was strongly supported as sister to the entire parmelioid clade, which represents the most speciose clade in the family. *Oropogon* and *Platismatia* were sister to the parmelioid + *Omphalora* group (Fig. 2a). In the species tree, instead, *O. arizonica* grouped without support (0.53 MLBP) with the *Oropogon* + *Platismatia* group, which formed a strongly supported sister-group relationship with the entire parmelioid clade (1.00 MLBP; Fig. 2b). Within the parmelioid clade, the position of *Parmeliopsis ambigua* deserves further investigation.

Our taxon sampling is insufficient to discuss phylogenetic relationships within major clades since only two to four species were included, with the exception of the parmelioid clade. Interestingly, the relationships of previously recognized lineages within the parmelioid clade (Blanco et al. 2006; Crespo et al. 2010b) lacked support in our phylogenomic analyses. Whether this is due to a rapid radiation at the origin of the clade or can be addressed using a larger taxon sampling remains to be investigated.

Utility of metagenome skimming for phylogenomic analyses

Genome skimming consists of using a single sequencing library layout to sequence to shallow depth individual species' genomes (Elgar et al. 1999). This approach has been successfully used in phylogeographic and phylogenomic studies at different taxonomic levels, from intraspecific (i.e., ultra-barcoding) to family-wide analyses (Male et al. 2014; Dodsworth 2015; Weitemier et al. 2015; Denver et al. 2016; Gardner et al. 2016; Grandjean et al. 2017). Particularly when working with multi-species assemblages of non-model organisms, this method represents a cost-effective approach to survey the genomes of the individual species in the mixture (Greshake et al. 2016). The skimming approach is increasingly used in lichens, including multispecies fungal–algal symbioses harbouring complex fungal and bacterial microbiomes to tackle genome-wide phylogenetics (Leavitt et al. 2016c; Grewe et al.

2017) and population genetics (Dal Grande et al. 2017), as well as biotechnological aspects of the lichen symbiosis (Kampa et al. 2013).

Recently, it has been shown that metagenome skimming is a valid approach to retrieve almost the entire gene space of lichen-forming fungi from whole lichen thalli (Meiser et al. 2017). Thus the approach is suitable for extracting genome-wide phylogenetic markers. The genome completeness estimates of most of the reconstructed fungal genomes in our study are in line with reports from a previous study (Meiser et al. 2017), i.e. ~ 85–90% (Fig. 1). Furthermore, here we showed that the implemented metagenomic approach was able to recover a common set of single-copy protein coding genes in species with varying coverage depths. Per-genome missing loci represented on average only 6.2(± 5.6)% of the total gene set used for phylogenomic inference. Even in reconstructed genomes with low coverage (e.g., ~ 17-fold for *Pannoparmelia angustata, Flavoparmelia citrinescens, Notoparmelia tenuirima*) the percentage of missing loci did not exceed 7.2%, suggesting that the single-copy fraction of the fungal nuclear genome was readily recovered even at low sequencing depths.

Further, an evaluation of the minimum number of targeted markers required to obtain a robust phylogeny is recommended for phylogenomics study [e.g. Rokas et al. (2003)]. In a recent study, Ai and Kang (2015) developed a robust approach (including random sampling of genes starting from 25 with increments of 25, until the percentage of correct trees reached 100), based on 11 species with 830 single-copy nuclear genes to infer the need of minimum numbers of both random and selected genes to obtain a robust phylogeny. Given the large sizes of many of our subset data sets (i.e., computational limitations) and a desire to employ the same methodology when generating nodal support for all concatenated datasets analyses of the randomly sampled genes, we had little choice to use this methodology. Nonetheless, the single gene trees of the selected 2556 single-copy genes had an average bootstrap above 80% (Supplementary Table S4), indicating the outperformance of these markers to resolve phylogenetic relations in Lecanoromycetes and in Ascomycota in general. Gene selection strategies based on single-gene tree performance are strongly recommended in phylogenomic analyses (Ai and Kang 2015).

The comprehensive data set of 2556 homologous single-copy genes created in this study is a valuable genomic resource. It can, e.g., be used to identify new phylogenetic markers, extract optimal sets of phylogenetic markers, re-evaluate the resolution power of existing markers, or to identify the minimum number of loci or variable sites necessary to reconstruct well-resolved and highly supported phylogenies in the Lecanoromycetes (and potentially other ascomycete classes). Furthermore it may spur investigations into the evolution of functional genes and gene families in lichen-forming fungi. It is the first phylogenomic study addressing evolutionary relationships at the family level in lichen-forming fungi, focusing on the hyperdiverse family, Parmeliaceae (Kraichak et al. 2015). Extended taxon sampling of metagenomic data and additional phylogenomic approaches, such as RADseq and target enrichment of ultra-conserved elements provide promising avenues for resolving problematic phylogenetic relationships in this species-rich fungal subfamily.

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