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The conservation of polyol transporter proteins and their involvement in lichenized Ascomycota

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ABSTRACT

In lichen symbiosis, polyol transfer from green algae is important for acquiring the fungal carbon source. However, the existence of polyol transporter genes and their correlation with lichenization remain unclear. Here, we report candidate polyol transporter genes selected from the genome of the lichen-forming fungus (LFF) *Ramalina conduplicans*. A phylogenetic analysis using characterized polyol and monosaccharide transporter proteins and hypothetical polyol transporter proteins of *R. conduplicans* and various ascomycetous fungi suggested that the characterized yeast' polyol transporters form multiple clades with the polyol transporter-like proteins selected from the diverse ascomycetous taxa. Thus, polyol transporter genes are widely conserved among Ascomycota, regardless of lichen-forming status. In addition, the phylogenetic clusters suggested that LFFs belonging to Lecanoromycetes have duplicated proteins in each cluster. Consequently, the number of sequences similar to characterized yeast' polyol transporters were evaluated using the genomes of 472 species or strains of Ascomycota. Among these, LFFs belonging to Lecanoromycetes had greater numbers of deduced polyol transporter proteins. Thus, various polyol transporters are conserved in Ascomycota and polyol transporter genes appear to have expanded during the evolution of Lecanoromycetes.

1. Introduction

Lichen-forming fungi (LFFs) have evolved in various classes, including Arthoniomycetes, Coniocybomycetes, Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycetes and Lichinomycetes, in Ascomycota (James et al., 2006; Schoch et al., 2009a; Lumbsch and Rikkinen, 2017; Prieto et al., 2018). Lecanoromycetes is the largest class to which LFFs belong, with over 95 % of the species being lichenized (Miadlikowska et al., 2014). Although Dothideomycetes and Eurotiomycetes include mainly non-lichenized species, some classes, such as Trypetheliales (Dothideomycetes) and Verrucariales (Eurotiomycetes), include LFFs (Spatafora et al., 2006; Gueidan et al., 2007; Schoch et al., 2009b). Only one order in Coniocybomycetes, Coniocybales (Prieto et al., 2013), is entirely lichenized and associates with green algae (Rikkinen et al., 2016). On the basis of recent classifications, Lücking et al. (2016) suggested that 14–23 independent lichenization events occurred during the evolution of Ascomycota. Approximately 85 % of LFFs have green algae as their primary photosynthetic partners, and 3%–4 % are associated with both green algae and Cyanobacteria (Honegger, 2008). Although LFFs belong to many genera in seven classes of Ascomycota, their green algal partners belong to a more limited number of genera, including *Trebouxia*, *Asterochloris* and *Diplosphaera* in the class Trebouxiophyceae and *Trentepohlia* in the class Ulvophyceae (Friedl and Büdel, 2008). Thus, the fungal partners may diversify if they receive particular advantages from specific green algae, such as the acquisition of carbon sources.

A stable supply of carbon from a photosynthetic partner is a benefit of LFFs and probably the main reason why lichens expanded worldwide and retain their symbiotic relationships for long periods of time. Heterotrophs, such as fungi, cannot survive or grow in

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nature without a carbon source, and, in fact, the green algal partners release acyclic polyols, such as ribitol, sorbitol, and erythritol (Richardson et al., 1968), when they associate with a fungal partner (Smith, 1968; Green and Smith, 1974). For example, ribitol, which is absent in almost all plants (Negm and Marlow, 1985), is released from *Trebouxia* spp., the most common photosynthetic partners in lichens (Honegger, 2008). Observations of *Xanthoria aureola* (Richardson and Smith, 1968a, 1968b) and *Xanthoria calcicola* (Lines et al., 1989), which associate with green algae (*Trebouxia*), indicated that ribitol is converted to mannitol and arabitol through the pentose phosphate pathway of fungal metabolism. The other algal partners also release polyols, and polyols are present in many lichens (Lindberg et al., 1953; Lewis and Smith, 1967). Therefore, the polyol transport pathway is most likely essential to LFFs associated with green algae. However, the mechanisms of polyol transfer from green algae to fungi have not been elucidated, and, so far, a polyol transporter has not been isolated from LFFs, even though its function is required for symbiotic associations.

Polyols are widely present in diverse organisms (Lewis and Smith, 1967; Pfyffer and Rast, 1980; Honegger et al., 1993; Gustavs et al., 2011), and they function to remove reactive oxygen species (Parida and Das, 2005), to adjust the osmotic pressure of compatible solutes (Wegmann, 1986; Reed et al., 1987), and to store the reducing power provided by NADH or NADPH (Jennings, 1985; Williamson et al., 2002). Polyol transporters have been reported in bacteria, such as D-arabinitol and ribitol transporters from *Klebsiella pneumoniae* (Heuel et al., 1997), in red alga, such as sugar and mannitol transporters from *Galdieria sulphuraria* (Schilling and Oesterhelt, 2007), in plants, such as the first reported mannitol transporter from celery (Noiraud et al., 2001), and in humans, such as glycerol, mannitol, sorbitol and the other neutral solute channels (Tsukaguchi et al., 1999). The orthologs or paralogs of these proteins have also been characterized as polyol transporters. Recently, some transporters (or H^+ symporters), having the capabilities to transport acyclic polyols, were reported in ascomycetous yeast, such as *Debaryomyces hansenii* (Syl1 and 2; Pereira et al., 2014), *Ambrosiozyma monospora* (Lat1 and 2; Londesborough et al., 2014) and *Saccharomyces cerevisiae* (Hxt11, 13, 15, 16 and 17; Jordan et al., 2016). Some of these yeast proteins have no ability to transport sugars, even though their encoding genes were paralogous to monosaccharide transporters. Palma et al. (2009) revealed the lineages of hexose transporters in nine ascomycetous yeast, but the phylogenetic relationship among the characterized monosaccharide and polyol transporters were unclear in the Ascomycetes.

LFFs associating with green algae utilize polyols; therefore, we hypothesize that at least one polyol transport system is conserved in LFFs and it is required for the lichenization events that occur widely and independently in Ascomycota. Thus, the present study aimed to first evaluate phylogenetic relationships between polyol and monosaccharide transporters and then confirm the conservation of polyol transporters in ascomycetous fungi, including LFFs. On the basis of the analyses, we also speculated that there was a correlation between polyol transporters and lichenization in ascomycetous fungi, especially those associated with green algae. To determine the predicted polyol transporters, the eight genome sets of ascomycetous lichenized fungi were analyzed. Moreover, we also sequenced the whole genome of LFF *Ramalina conduplicans* and selected candidate polyol transporter genes. We also isolated the candidates that were expressing when *R. conduplicans* was cultured on a medium containing ribitol as the sole carbon source. Ramalinaceae, including *R. conduplicans*, is the fourth largest of the 115 families representing lichenized fungi (Lücking et al., 2016). The genus *Ramalina* grows on culture media relatively quickly (Komiya and Shibata, 1971) and has been used for chemical and biological activity studies of extracts and isolated compounds (reviewed in Moreira et al., 2015). Additionally, it is distributed worldwide (Kirk et al., 2008). Some metabolites of *R. conduplicans* have possible industrial, pharmaceutical and agricultural uses (Oh et al., 2006; Wei et al. 2008; Vinayaka et al., 2009; Luo et al., 2010), and *R. conduplicans* is associated with *Trebouxia* spp., the most common photosynthetic partner in lichens (Honegger, 2008). Therefore, *R. conduplicans* could be a lichen model for studying secondary metabolites and polyol utilization.

2. Materials and methods

2.1. Collection, isolation and culture conditions

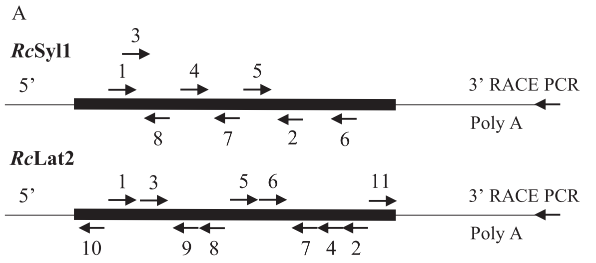
On 15 July 2014, *R. conduplicans* was collected from the shore of Lake Towada (Aomori, Japan, 40°26′N 140°55′E). Fungal cultures (strain ID: KY1401) were isolated from thallus fragments using the methodology of Yamamoto et al. (1985). The specimens were deposited into the herbarium of the Natural History Museum and Institute, Chiba, Japan (CBM) under the accession number CBM-FL-204754. The culture strain KY1401 was preserved in the Biological Resource Center, NITE (NBRC) under the accession number 113405. The culture was incubated in Malt/Yeast extract agar medium (Yoshimura et al., 2002) for DNA extraction and modified Czapek-Dox liquid medium [3 % ribitol as a sole carbon source, 0.2 % $NaNO_3$, 0.1 % K_2HPO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.05 % KCl, 0.001 % $FeSO_4 \cdot 7H_2O$] for RNA extraction with shaking at 200 rpm at 19 °C for 3 m.

2.2. DNA extraction and primers for molecular identifications

Genomic DNA was extracted from the culture strain KY1401 and the thalli of specimens. The culture was ground with a mortar and pestle in Buffer T1 from the NucleoSpin Tissue XS kit (TaKaRa Bio, Shiga, Japan), and we followed the kit manufacturer's instructions after grinding. For the thalli, the standard cetyltrimethylammonium bromide method was used. Extracted genomic DNA was used as the PCR template for the amplification of internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA) and 5.8S nrDNA with the primer pair, ITS5 and ITS4 (White et al., 1990).

2.3. RNA extraction and degenerate PCR primers

The fungal cultures were disrupted by mortar and pestle with 5 μL of 2-mercaptoethanol and 450 μL of RLC included in the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan), and the kit manufacturer's instructions were followed after grinding. First-strand cDNA was synthesized by reverse transcription PCR from 20-μL reactions that included the following: 4 μL 2.5-mM dNTP Mixture (TaKaRa Bio), 1 μL Oligo (dT)15 Primer (Promega, Madison, WI, USA), total RNA (1 μg), 4 μL 5× first-strand buffer, 1 μL 100-mM dithiothreitol, 1 μL SuperScript™ III (Invitrogen, Carlsbad, CA, USA) and RNase inhibitor (Toyobo, Osaka, Japan). The alignments of amino acid sequences were performed using GENETYX ver. 12 (Genetyx Co., Tokyo, Japan) between *D. hansenii*' (*Dh*) Syl1 and Syl2, and among *A. monospora*' (*Am*) Lat2 and the similar sequences selected from the LFF genomes. Degenerate primers were designed from the six to eight conserved amino acids that were visually identified from the alignments. The criteria for designing degenerate primers were high annealing temperatures and small variations in primers (under 1296 variants). All designed primer sequences and their relative positions are found in Fig. 1A and B. The primer pairs *Dh*Syl-dF/*Dh*Syl-dR and *Am*Lat2-dF1/*Am*Lat2-dR1 were used for degenerate PCR. The other pair of degenerate primers (*Am*Lat2-dF2/*Am*Lat2-dR2) was used nested PCR using the first PCR product (*Am*Lat2-dF1/*Am*Lat2-dR1) as the template.



B

No.	Primer name	Sequences (5'-3')	Usage
***Rc* Syl1**			
1	*Dh* Syl-dF	GCN GCN GTN CAR GGN ATG GA	Degenerate PCR
2	*Dh* Syl-dR	CCR CAR AAY TGY TGC ATR AAC AT	Degenerate PCR
3	*Rc* Syl1-GSP1	GCC GTA CAG GGA ATG GAT GA	3’ RACE
4	*Rc* Syl1-GSP2	AAT TTG GCA TTG ACG GCG AG	3’ and 5’ RACE
5	*Rc* Syl1-GSP3	AAA TCG TCG GGC AAT GCT CG	3’ RACE
6	*Rc* Syl1-GSP4	CAA GCG AAG GCA CCT GGG	3’ RACE
7	*Rc* Syl1-GSP5-P	P-GCC AAG GAC AGA ACA GCA GA	Reverse Transcription
8	*Rc* Syl1-GSP6	CGC CAT TCA CGA CGG TTT C	5’ RACE
***Rc* Lat2**			
1	*Am* Lat2-dF1	TTY TTY GGN TAY GAY CAR GG	Degenerate PCR
2	*Am* Lat2-dR1	ATY TCY TCN ARN GTN C	Degenerate PCR
3	*Am* Lat2-dF2	GAR TTY ACB YTB AAY ATH TTH GG	Degenerate PCR
4	*Am* Lat2-dR2	GGR AAN ATY TCV GMH GG	Degenerate PCR
5	*Rc* Lat2-GSP1	TAC CTC GCC ATG TTC TTC GG	3’ and 5’ RACE
6	*Rc* Lat2-GSP2	AAT CCG GCA AGC TGC ATA CT	3’ and 5’ RACE
7	*Rc* Lat2-GSP3-P	P-CAG TTG ATC CTT AGC GGC CA	Reverse Transcription
8	*Rc* Lat2-GSP4	GAC GTC CTG AAA CTC GGC TT	5’ RACE
9	*Rc* Lat2-GSP5	GCC CAA GAT GAA ACG ACC CT	5’ RACE
10	*Rc* Lat2-GSP6	CCA CAG CAT TGA CGG CAA TC	5’ RACE
11	*Rc* Lat2-GSP7	CAA GGA GCA GAA CCC CGA AT	3’ RACE

Fig. 1. List of primers designed in this study for the degenerate PCR and RACE of *Ramalina conduplicans*' Syl1 and Lat2. (A) Primer positions in each cDNA sequence. (B) List of primer names and sequences. Abbreviations: Syl = Symporter polyols (Pereira et al., 2014); Lat = L-Arabinose transporters (Verho et al., 2011); GSP = Gene-specific primer; RACE = Rapid amplification of cDNA ends; P = Phosphorylated.

2.4. PCR, cloning and sequencing

PCR reactions were performed using TaKaRa Ex Taq HS (TaKaRa Bio) and, in some cases, also Blend Taq-Plus (Toyobo) by following the manufacturer's protocol. PCR products were visualized on a Tris-Acetate-EDTA 1 %–2 % agarose gel stained with 5 μg mL^{-1} ethidium bromide. PCR products were purified using a FastGene Gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan) and cloned using a pGEM-T Easy Vector System (Promega) according to the technical manual. The alkaline lysis method was used for the isolation of the plasmids. The target sequences inside these plasmids were sequenced by using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) with M13 primers (forward: GTA AAA CGA CGG CCA GT and reverse: CAG GAA ACA GCT ATG AC) and a 3130xl Genetic Analyzer (Applied Biosystems). Sequences were edited using BioEdit ver. 7.2.5 (Hall, 1999). The pairwise sequence alignment of the ITS region and the 5.8S nrDNA between the cultured strain and the thalli were conducted using Global Alignment in EMBOSS Needle (https://www.ebi.ac.uk/services).

2.5. Rapid amplification of cDNA ends (RACE)

Using degenerate PCR and sequencing, we obtained partial sequences that were candidate polyol transporters (*R. conduplicans*' (*Rc*) Syl1 and *Rc*Lat2). Gene-specific primers (GSPs, Fig. 1) were designed from non-conserved region in these partial sequences and used in the following RACE. For 3′ RACE, first-strand cDNA anchored with an adaptor primer named “3′ RACE PCR” (GGC CAC GCG TCG ACT AGT AC; Integrated DNA Technologies, Coralville, IA, USA) behind a poly-A tail (Fig. 1A) was synthesized from total RNA using the “cDNA cloning primer” GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT TV (Integrated DNA Technologies). For the 3′ RACE, primer pairs of “3′ RACE PCR” and GSPs were used. For the 5′ RACE, first-strand cDNA was synthesized using 5′ phosphoryl-terminated GSP, and the ligation of single-stranded DNA was performed by T_4 RNA ligase (New England Biolabs, Tokyo, Japan) following the manufacturer's protocol. This ligation mixture was used for PCR with the GSPs, and the resulting PCR product was used for nested PCR. The PCR and sequencing protocols were as described in Section 2.4. If the PCR product was visualized as a single band, then it was used directly for sequencing. The complete cDNA sequences were translated into deduced amino acids using GENETYX ver. 12.

2.6. *Whole-genome* de novo *sequencing and the selection of polyol transporter genes*

Abundant genomic DNA was extracted from the KY1401 using NucleoSpin Plant II (TaKaRa Bio) following the kit manufacturer's instructions. The whole-genome sequencing was carried out on a PacBio Sequel System with the SMRTbell Express Template preparation kit and Sequel SMRT Cell 1M v2 Sequel Binding Kit 2.1 (Pacific Biosciences of California, Inc.). *De novo* assembly and gene prediction were performed by HGAP (hierarchical genome assembly process) ver. 4.0 (http://www.pacb.com/products-and-services/pacbio-systems/sequel) and Augustus ver. 3.3.2 (http://augustus.gobics.de/), respectively. The candidate polyol transporters similar to *Dh*Syl1 and *Am*Lat2 were selected from the genome by using local BLASTP ver. 2.7.1+.

2.7. *Amino acid sequences*

We obtained amino acid sequences of characterized polyol and monosaccharide transporters in ascomycetes from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Table 1) for the phylogenetic analysis. These proteins transport specific polyol or monosaccharides through cell membranes. Monosaccharide transporters are derived from all subphylum of Ascomycota, i.e., Saccharomycotina, Taphrinomycotina and Pezizomycotina. Acyclic polyols transporters were derived from three species of Saccharomycotina, *A. monospora*, *D. hansenii* and *Saccharomyces cerevisiae*. Transporters of inositols, which are cyclic polyols, were derived from both Saccharomycotina and Taphrinomycotina, and some transport both monosaccharides and polyols (Table 1). The similarities between these amino acid sequences and acyclic polyol transporters, *S. cerevisiae*' (*Sc*) Hxt15, *Dh*Syl1 and *Am*Lat2, were calculated using Global Alignment in EMBOSS Needle. If at least one similarity was ≥30 %, then the protein was retained for phylogenetic analysis. Moreover, we selected the amino acid sequences most similar to those of polyol transporters, namely *Sc*Hxt15, *Dh*Syl1, *Dh*Syl2 and *Am*Lat2 (Table 2) for the following phylogenetic analysis. These candidate polyol transporters were selected from the genomes of Taphrinomycotina and six classes of Pezizomycotina (Dothideomycetes, Eurotiomycetes, Leotiomycetes, Orbiliomycetes, Pezizomycetes and Sordariomycetes) using the Position-Specific Iterated-BLAST algorithm provided by NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and from the genomes of Xylonomycetes and each lichen-forming species, *Sclerophora sanguinea* (Coniocybomycetes), *Trypethelium eluteriae* (Dothideomycetes) and *Endocarpon pusillum* (Eurotiomycetes) using a BLASTP algorithm-based analysis performed on the MycoCosm portal, a fungal

Table 1
Characterized polyols and monosaccharide transporters used for the phylogenetic analysis.

Species	Protein name	Transportable substances[a]	GenBank	Reference
Saccharomycotina				
Ambrosiozyma monospora	Lat1	L-arabinose, **ribitol**, **L-arabitol**	AAX98667.1	Verho et al. (2011) Londesborough et al. (2014)
	Lat2	L-arabinose, **ribitol**, **L-arabitol**	AAX98668.1	Verho et al. (2011), Londesborough et al. (2014)
Candida albicans	Hgt1	glucose	CAA76406.1	Varma et al. (2000)
	STL1	**glycerol**	CAY08065.1	Kayingo et al. (2009)
Candida intermedia	GXF1	glucose, xylose	CAI77652.1	Leandro et al. (2006)
	GXS1	glucose, xylose	CAI44932.1	Leandro et al. (2006)
Debaryomyces hansenii	Sgl1	**galactitol**	CAG88649.2	Pereira et al. (2014)
	Stl1	**glycerol**	CAG87598.2	Pereira et al. (2014)
	Syi1	D-(+)-*chiro*-inositol	CAG90290.2	Pereira et al. (2014)
	Syl1	**sorbitol**, **mannitol**, **ribitol**, **arabitol**, **galactitol**	CAR65543.1	Pereira et al. (2014)
	Syl2	**sorbitol**, **mannitol**, **ribitol**, **arabitol**	CAG86001.1	Pereira et al. (2014)
Kluyveromyces lactis	Frt1	fructose	CAC79614.1	Diezemann and Boles (2003)
	Hgt1	glucose	AAC49461.1	Billard et al. (1996)
	Kht1	glucose	P18631.1	Milkowski et al. (2001)
	Kht2	glucose	CAA87389.1	Milkowski et al. (2001)
	Rag1	glucose	CAA37781.1	Wésolowski-Louvel et al. (1992)
Saccharomyces cerevisiae	Gal2	galactose, glucose	NP_013182.1	Nishizawa et al. (1995)
	Hxt1	glucose, fructose	NP_011962.1	Reifenberger et al. (1997)
	Hxt2	glucose, fructose	NP_013724.1	Kruckeberg and Bisson (1990), Reifenberger et al. (1997)
	Hxt3	glucose, fructose	NP_010632.1	Reifenberger et al. (1997)
	Hxt4	glucose, fructose	NP_011960.2	Reifenberger et al. (1997)
	Hxt5	glucose, fructose, mannose	NP_011964.1	Diderich et al. (2001)
	Hxt6	glucose, fructose	NP_010630.1	Reifenberger et al. (1997)
	Hxt7	glucose, fructose	NP_010629.3	Reifenberger et al. (1997)
	Hxt11	glucose, fructose, mannose, galactose, xylose, **xylitol**	NP_014486.1	Jordan et al. (2016)
	Hxt13	**sorbitol**, **mannitol**	NP_010845.1	Jordan et al. (2016)
	Hxt15	**sorbitol**, **mannitol**, **xylitol**	NP_010036.1	Jordan et al. (2016)
	Hxt16	**sorbitol**, **mannitol**	NP_012692.3	Jordan et al. (2016)
	Hxt17	**sorbitol**, **mannitol**	NP_014470.1	Jordan et al. (2016)
	ITR1	*myo*-inositol	BAA14366.1	Nikawa et al. (1991)
	ITR2	*myo*-inositol	BAA14367.1	Nikawa et al. (1991)
	STL1	**glycerol**	AAA57229.1	Ferreira et al. (2005)
Saccharomyces pastorianus	Fsy1	fructose	CAC08232.1	Gonçalves et al. (2000)
Scheffersomyces stipitis	Sut1	glucose, fructose, xylose	AAD00266.1	Weierstall et al. (1999)
	Sut2	glucose, xylose	AAD02831.2	Weierstall et al. (1999)
	Sut3	glucose, fructose, xylose, galactose	AAD00269.2	Weierstall et al. (1999)
Torulaspora delbrueckii	Lgt1	glucose, fructose	AAT95983.1	Alves-Araújo et al. (2005)
Zygosaccharomyces rouxii	Fsy1	fructose, **xylitol**, sorbose	XP_002495678.1	Leandro et al. (2013)
Taphrinomycotina				
Schizosaccharomyces pombe	Ght1	glucose, fructose	NP_587747.1	Lichtenberg-Fraté et al. (1997), Wood et al. (2002)
	Ght2	glucose, fructose	NP_596425.1	Heiland et al. (2000), Wood et al. (2002)
	Ght3	gluconate	NP_592790.1	Heiland et al. (2000), Wood et al. (2002)
	Ght5	glucose, fructose	NP_587740.1	Heiland et al. (2000), Wood et al. (2002)
	Ght6	glucose, fructose	NP_587739.1	Heiland et al. (2000), Wood et al. (2002)
	Itr1	*myo*-inositol	CAA67211.1	Niederberger et al. (1998)
	Itr2	*myo*-inositol	NP_593320.1	Niederberger et al. (1998)
Pezizomycotina				
Aspergillus nidulans	MstA	glucose	CAC80843.1	Forment et al. (2014)
	MstC	glucose	CAI54231.1	Forment et al. (2014)
	XtrD	xylose	XP_657854.1	Colabardini et al. (2014)
Aspergillus niger	MstA	glucose, mannose, xylose	AAL89822.1	Vankuyk et al. (2004)
Botrytis cinerea	Frt1	fructose	AAU87358.1	Doehlemann et al. (2005)
Colletotrichum graminicola	Hxt1	glucose	CBA11542.1	Lingner et al. (2011)
	Hxt2	glucose	CBA11543.1	Lingner et al. (2011)
	Hxt3	glucose	CBA11544.1	Lingner et al. (2011)
	Hxt5	glucose	CBA11546.2	Lingner et al. (2011)
Neurospora crassa	LAT1	L-arabinose	EAA30346.3	Li et al. (2015)
Tuber borchii	Hxt1	glucose, fructose, mannose	AAY26391.1	Polidori et al. (2007)
Trichoderma harzianum	Gtt1	glucose	CAC81782.1	Delgado-Jarana et al. (2003)
Tuber melanosporum	2281	glucose, fructose	KP718607.1	Ceccaroli et al. (2015)
	Hxt1	glucose, fructose	KF481921.1	Ceccaroli et al. (2015)
Thermothelomyces thermophila	LAT1	L-arabinose	AEO58453.1	Li et al. (2015)

[a] Acyclic polyols are shown in bold.

Table 2
List of candidate polyol transporter proteins used for the phylogenetic analysis.

Species	Query genes[a]	Reference sequence[b] (Gene ID[c])	Length	Transmembrane helices	
			(amino acids)	MEMSAT3	MEMSAT-SVM
Pezizomycotina					
Coniocybomycetes					
Sclerophora sanguinea CBS100924 v1.0	*Am*Lat2	241678	562	11	12
	*Dh*Syl1 & *Dh*Syl2	136594	658	10	11
	*Sc*Hxt15	184654	538	10	12
Dothideomycetes					
Diplodia corticola CBS 112549	*Sc*Hxt15	XP_020129849.1	571	12	11
Diplodia seriata DS831	*Dh*Syl1	KKY19172.1	653	11	11
Glonium stellatum CBS 207.34	*Dh*Syl2	OCL04378.1	637	10	11
Pseudocercospora fijiensis CIRAD86	*Am*Lat2	XP_007930618.1	556	12	12
Trypethelium eluteriae v1.0	*Am*Lat2	440123	543	12	12
	*Dh*Syl1 & *Dh*Syl2	500005	623	10	11
	*Sc*Hxt15	128538	592	5	10
Eurotiomycetes					
Aspergillus nomius NRRL 13137	*Am*Lat2	XP_015407194.1	754	12	12
Coccidioides immitis H538.4	*Dh*Syl1	KMU91130.1	844	12	12
Endocarpon pusillum Z07020	*Am*Lat2	4708	579	11	12
	*Dh*Syl1 & *Dh*Syl2	1090	623	11	11
	*Sc*Hxt15	5810	524	12	12
Penicillium nordicum DAOMC 185683	*Sc*Hxt15	KOS37549.1	563	12	11
Uncinocarpus reesii 1704	*Dh*Syl2	XP_002583310.1	649	10	11
Lecanoromycetes					
Cladonia grayi Cgr/DA2myc/ss v2.0	*Am*Lat2	3543	578	11	11
		3537	584	11	12
		8535	575	11	12
	*Dh*Syl1 & *Dh*Syl2	2664	614	11	11
		4326	665	11	11
		1127	643	10	11
		7345	619	4	10
	*Sc*Hxt15	1746	599	12	11
Lasallia pustulata v1	*Am*Lat2	contig 338, gene position 26576–28409 (g10505)	573	11	12
		contig 3496, gene position 17264–19336 (g11659)	620	9	12
	*Dh*Syl1 & *Dh*Syl2	contig 837, gene position 52680–62564 (g3990)	2207	NC	NC
		contig 1791, gene position 32590–34641 (g11434)	655	10	11
		contig 3577, gene position 7605–11916 (g6892)	1125	11	10
	*Sc*Hxt15	SLM39237.1	548	11	11
Lobaria pulmonaria Scotland reference genome	*Am*Lat2	1075510	562	12	12
		717725	573	11	12
	*Dh*Syl1 & *Dh*Syl2	1261078	645	10	11
		407424	648	11	11
		1270634	619	11	10
		638017	638	11	11
	*Sc*Hxt15	1267150	539	12	12
Ramalina conduplicans KY1401 (in this study)	*Am*Lat2	LC456214 (g3682)	574	11	12
		LC456215 (g4707) = LC375945 (Lat2)	570	11	12
		LC456216 (g6856)	621	6	12
	*Dh*Syl1	LC456217 (g806) = LC375944 (Syl1)	645	11	11
		LC456218 (g3796)	669	11	10
		LC456219 (g8137)	635	11	11
		LC456220 (g6460)	909	11	12
		LC456221 (g1008)	1583	NC	NC
Usnea florida ATCC18376 v1.0	*Am*Lat2	817483	576	12	12
		877507	573	11	12
	*Dh*Syl1 & *Dh*Syl2	816522	643	11	11
		880758	613	11	11
		873497	642	10	11
		717331	649	11	11
		891961	621	6	10
	*Sc*Hxt15	815118	547	12	11
Xanthoria parietina 46-1-SA22 v1.1	*Am*Lat2	1618183	614	12	12
		1578916	559	11	12
		1234738	566	11	12
	*Dh*Syl1 & *Dh*Syl2	1660360	674	10	10
		1645041	658	11	10
		1606405	654	11	10
		81021	627	11	11

Table 2 (*continued*)

Species	Query genes[a]	Reference sequence[b] (Gene ID[c])	Length	Transmembrane helices	
			(amino acids)	MEMSAT3	MEMSAT-SVM
		1572476	619	4	10
		1613179	608	11	11
	*Sc*Hxt15	1602355	531	12	12
Leotiomycetes					
Glarea lozoyensis ATCC 20868	*Am*Lat2	XP_008087870.1	552	11	12
Oidiodendron maius Zn	*Sc*Hxt15	KIN01525.1	567	11	11
Phialocephala scopiformis CBS 120377	*Dh*Syl1 & *Dh*Syl2	XP_018072650.1	614	10	11
Orbiliomycetes					
Arthrobotrys oligospora ATCC 24927	*Sc*Hxt15	XP_011123148.1	551	12	11
Dactylellina haptotyla CBS 200.50	*Am*Lat2	XP_011106430.1	555	12	12
	*Dh*Syl1 & *Dh*Syl2	XP_011106845.1	612	11	12
Pezizomycetes					
Pyronema omphalodes CBS 100304	*Am*Lat2	CCX06871.1	573	12	12
	*Dh*Syl1 & *Dh*Syl2	CCX32903.1	621	11	11
	*Sc*Hxt15	CCX04720.1	508	12	12
Sordariomycetes					
Daldinia sp. EC12	*Sc*Hxt15	OTB11998.1	539	12	11
Phaeoacremonium minimum UCRPA7	*Am*Lat2	XP_007914611.1	563	12	12
Thermothelomyces thermophila ATCC 42464	*Dh*Syl1 & *Dh*Syl2	XP_003667183.1	655	10	11
Xylonomycetes					
Symbiotaphrina kochii v1.0	*Am*Lat2	778756	540	12	12
	*Sc*Hxt15	658577	567	11	11
Trinosporium guianense CBS132537 v1.0	*Dh*Syl1	589593	613	10	11
Xylona heveae TC161 v1.0	*Dh*Syl2	33643	656	10	11
Taphrinomycotina					
Neolecta irregularis DAH-3	*Dh*Syl1 & *Dh*Syl2	OLL27129.1	623	9	11
Saitoella complicata NRRL Y-17804	*Am*Lat2	XP_019021487.1	520	12	12
Schizosaccharomyces japonicus yFS275	*Sc*Hxt15	XP_002175189.1	539	12	11

NC, not calculated.

[a] Abbreviations: *Am*Lat2 = *Ambrosiozyma monospora*' Lat2, *Dh*Syl1 & *Dh*Syl2 = *Debaryomyces hansenii*' Syl1 and Syl2, *Sc*Hxt15 = *Saccharomyces cerevisiae*' Hxt15.

[b] GenBank accession number or MycoCosm Protein ID.

[c] Constructed by Augustus.

genomics resource (https://genome.jgi.doe.gov/programs/fungi/index.jsf; Grigoriev et al., 2011, 2014). The genome of LFF, *Lasallia pustulata* (Lecanoromycetes, GenBank accession no. GCA_900169345.1) was used for anew gene prediction by Augustus ver. 3.3.2 and for the selection of plural candidate polyol transporters with local BLASTP ver. 2.7.1+. The plural candidate polyol transporters were also selected from the genomes of other LFFs (*Cladonia grayi*, *Lobria pulmonaria*, *Usnea florida* and *Xanthoria parietina*) belonged to the class Lecanoromycetes using a BLASTP algorithm-based analysis performed on the MycoCosm portal. The genomic DNAs of LFFs were extracted from cultures grown from isolated single or polyspore progeny (confirmed on the MycoCosm portal and each culture collection). Their primary photosynthetic partners were investigated and appeared to be green algae that released acyclic polyols. In this analysis, we did not use *Am*Lat1 because it is a low-affinity or nonsaturable transporter of pentitol when tagged with C-terminal fusions (Londesborough et al., 2014). We also did not use the *Sc*Hxt11, 13, 16 and 17 polyol transporters because their amino acid sequences are highly similar to that of *Sc*Hxt15. For the candidate polyol transporters, the amino acid sequence lengths and the protein family domains were examined using InterProScan (https://www.ebi.ac.uk/interpro/) and the number of transmembrane helices were examined using MEMSAT3 and MEMSAT-SVM (http://bioinf.cs.ucl.ac.uk/psipred/?memsatsvm=1, Jones, 2007; Nugent and Jones, 2009).

2.8. Alignment and phylogenetic analysis

A multiple alignment of 143 amino acid sequences obtained as described in Section 2.6 and 2.7 was executed using the MUSCLE in MEGA ver. 7 (Kumar et al., 2016). Ambiguous regions of the alignment were removed by Gblocks 0.91b (Castresana, 2000) under the least stringent setting in the platform phylogeny.fr (http://phylogeny.lirmm.fr/phylo_cgi/index.cgi; Dereeper et al., 2008). Final alignments are available at the following URL: http://purl.org/phylo/treebase/phylows/study/TB2:S23762. The best-fit protein model for the maximum-likelihood (ML) method was selected using MEGA. The parameters for the model selections were as follows: the tree used was automatic and a gaps/missing data treatment was used for all sites. The selected best model was LG (Le and Gascuel, 2008) with empirical frequencies (+F), a discrete Gamma distribution (+G) (Yang, 1994) and a proportion of invariant sites (+I). The phylogenetic analyses were conducted using ML method based on this selected model in raxmlGUI ver. 1.31 (Silvestro and Michalak, 2012), and maximum-parsimony method based on tree-bisection-regrafting algorithm (Nei and Kumar 2000) in MEGA. The node supports were evaluated by bootstrap tests using 1000 replications in each analysis.

2.9. The numbers of polyol transporter proteins

The numbers of proteins similar to *Am*Lat2 and *Dh*Syl1 were calculated using a BLASTP algorithm based on the BLOSUM-62 matrix (Henikoff and Henikoff, 1992) in 470 genome sets of Ascomycota fungal species registered in MycoCosm and in 2 LFFs (*R. conduplicans* and *L. pustulata*). Proteins similar to *Am*Lat2 and *Dh*Syl1 were defined as having values over 1000 as calculated by the BLOSUM-62 matrix. *Dh*Syl2 was not used in these analyses because it has a 90 % protein homology to *Dh*Syl1 (Pereira et al., 2014), and the results were almost the same when using *Dh*Syl1 in the pre-calculations.

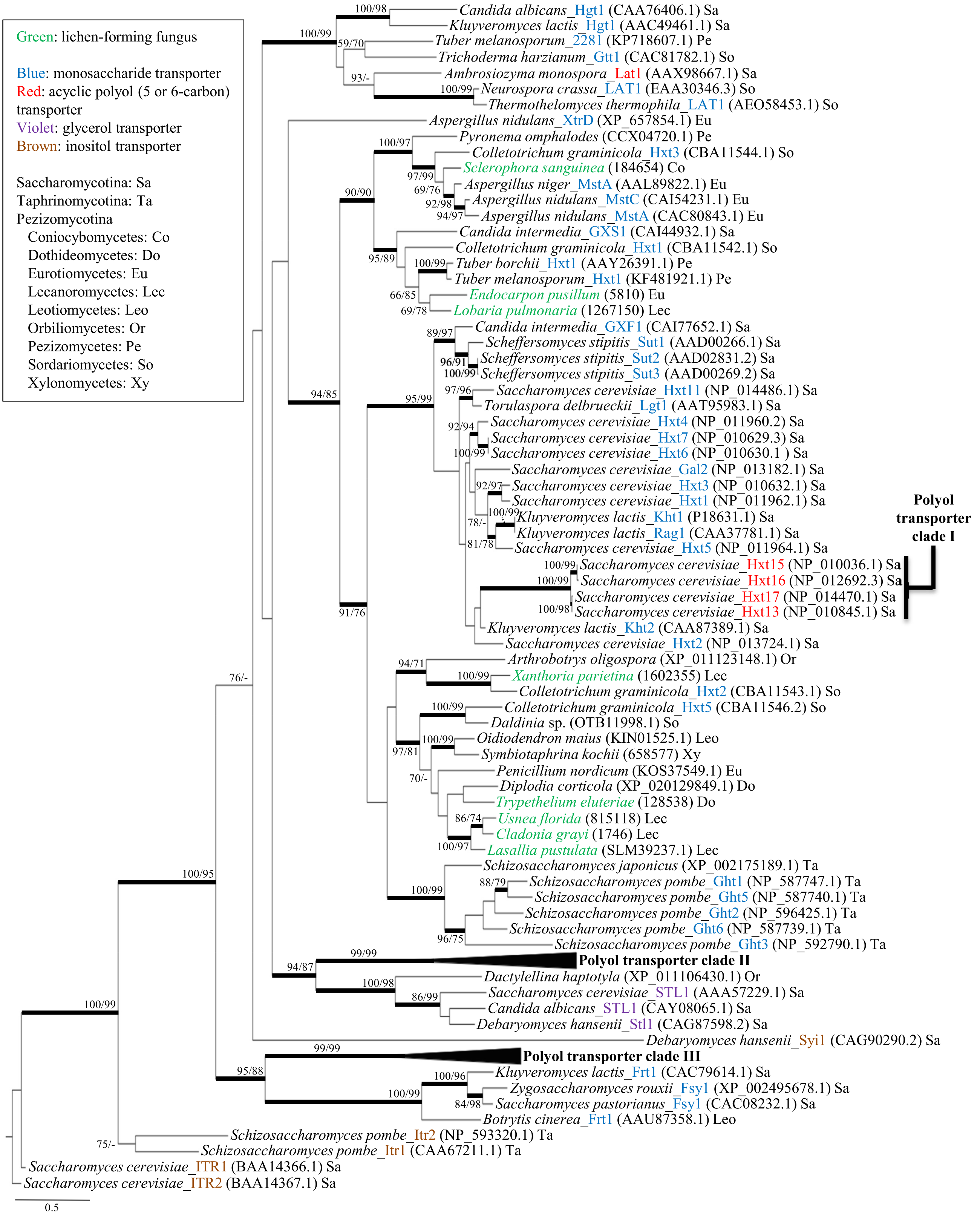


Fig. 2. A phylogenetic tree resulting from a maximum-likelihood (ML) analysis based on amino acid sequences of polyol and monosaccharide transporters in Ascomycota. Bootstrap values calculated from 1000 replications are shown near the nodes (≥50 %, ML method/maximum-parsimony (MP) method) when the values resulting from ML and/or MP methods are over 70. In addition, the nodes that supported by both ML and MP methods (>70 %) are depicted as thick lines. In each branch, characterized proteins that demonstrated the capability to transport monosaccharides and/or polyols listed in Table 1 are colored as follows: blue for monosaccharide, red for acyclic polyol (5 or 6-carbon), violet for glycerol, and brown for inositol transporters. The transporters that transport both monosaccharides and acyclic polyols, are distributed in accordance with the substance they transport more efficiently. Lichen-forming fungi are shown in green characters. Characterized transporters and selected candidates of polyol transporters are denoted with species' name, the protein's name for characterized transporters, GenBank accession number or MycoCosm protein ID, and fungal class or subphylum. The following abbreviations were used: Coniocybomycetes, Co; Dothideomycetes, Do; Eurotiomycetes, Eu; Lecanoromycetes, Lec; Leotiomycetes, Leo; Orbiliomycetes, Or; Pezizomycetes, Pe; Sordariomycetes, So; Xylonomycetes, Xy; Taphrinomycotina, Ta; and Saccharomycotina, Sa.

3. Results and discussion

3.1. Candidate polyol transporter genes used in the phylogenetic analysis

ITS regions and 5.8S nrDNA sequences from strain KY1401 and the specimen CBM-FL-204754, which was identified as *R. conduplicans* by morphological observations, were deposited in DDBJ (accession numbers: LC382022 and LC382023, respectively) and had 98.9 % nucleotide identity levels. Two partial sequences of candidate polyol transporters from KY1401 were obtained using each pair of degenerate primers (*Dh*Syl-dF/*Dh*Syl-dR and *Am*Lat2-dF2/*Am*Lat2-dR2), respectively. RACE using gene-specific primers designed from these partial sequences enabled us to obtain the complete cDNAs of the candidate polyol transporters from KY1401 (Table 2). These sequences were named as *Rc*Syl1 (accession number: LC375944) and *Rc*Lat2 (accession number: LC375945) and then translated to deduced amino acid sequences. The local BLASTP using predicted genes from the KY1401 genome revealed four and two additional sequences similar to that of *D. hansenii*' (*Dh*) Syl1 and *A. monospora*' (*Am*) Lat2, respectively (Table 2). An additional 75 candidate polyol transporter proteins similar to *Saccharomyces cerevisiae*' (*Sc*) Hxt15, *Dh*Syl1, *Dh*Syl2 and *Am*Lat2 were selected from Taphrinomycotina and the nine classes of Pezizomycotina (Table 2). The candidates were all members of the sugar/inositol transporter (IPR003663) protein family, contained 10 to 12 predicted transmembrane helices (Table 2), and had been annotated as putative monosaccharide transporters or hypothetical proteins, except for *Neolecta irregularis* (OLL27129.1, annotated as "putative polyol transporter 1"). In general, monosaccharide or polyol transporters are predicted as having 10 to 12 transmembrane domains. Therefore, the numbers of membrane-spanning regions were typical. However, the amino acid lengths varied from 508 to 2207 (Table 2), which implied that the lengths of the central loops and C or N-terminal tails are different in each protein.

3.2. Phylogenetic analysis

Fig. 2 shows a ML-based phylogeny of the amino acid sequences of 43 monosaccharide transporters, 12 acyclic polyols transporters, 5 inositol transporters (Table 1) and 83 candidate polyol transporters (Table 2). This tree formed three acyclic polyol transporter clades supported by high bootstrap values resulting from both ML and maximum-parsimony methods. Although polyol transporter clades do not include monosaccharide transporters, polyol transporter clade III was a sister clade of monosaccharide transporters (Fig. 2). In addition, some transporters, such as *Am*Lat1 and *Sc*Hxt11, which transport both polyols and monosaccharides, did not belong to the polyol transporter clade. Thus, the differences in transportable substance may result from partial amino acid changes. Clade II was a sister clade of the glycerol transporters (*Sc*STL1, *Dh*Stl1, and *Candida albicans*' STL1); therefore, transportable polyols were also affected by partial changes in amino acids. The polyol transporter clade I only included *Sc*Hxt polyol transporters that were closely related to *Sc*Hxt monosaccharide transporters but distinct from the proteins of various ascomycetous fungi selected by the BLASTP-based analysis in which *Sc*Hxt15 was used as the query (Table 2 and Fig. 2). This suggested that *Hxt* polyol transporter genes evolved mainly in *S. cerevisiae*.

Polyol transporter clades II and III (Fig. 2) included the proteins detected from genomes of every subphylum of Ascomycota, including eight or nine classes of Pezizomycotina (Fig. 3A and B), suggesting that multiple polyol transporters existed in the basal ancestor of Ascomycota and that these clades were probably formed by out-paralogs derived from this basal ancestor. In addition, the preservation of polyol transporter genes may be helpful in the lichenization process or a requirement for lichenization in Ascomycota. Mannitol is ubiquitous throughout the fungal kingdom (Solomon et al., 2007), and glycerol and erythritol are the major osmoregulatory solutes in the filamentous fungal species *Aspergillus nidulans* (Beever and Laracy, 1986). Thus, selected proteins may have the potential to transport these acyclic polyols. However, because *Dh*Syl1, *Dh*Syl2 and *Am*Lat2 transport ribitol (Pereira et al., 2014; Londesborough et al., 2014), their orthologs were also expected to transport ribitol. In this study, all species in Lecanoromycetes, including *R. conduplicans*, were associated with green algae that release ribitol, while the other LFFs were associated with sorbitol- or erythritol-releasing green algae. Therefore, some differences in transportable substances may be detected for each candidate protein. In our other study (Yoshino et al., In Press), *R. conduplicans* KY1401 was shown to grow well on Lilly and Barnett's medium that contained ribitol as a sole carbon source, as well as on a malt and yeast extract-based medium. In addition, this strain has maintained the same mycelial phenotype for over 4 y. Here, we obtained putative polyol transporters from *R. conduplicans* KY1401, which allows it to act as a model LFF for elucidating ribitol utilization. In addition, degenerate primers designed in this study can be used to amplify new candidate polyol transporters from various fungi. However, in the literature, there are some technical differences in characterizing polyol transporters. For example, the activity levels of *Dh*Syl1 and *Dh*Syl2 were only detected after 3 h of carbon starvation in yeast (Pereira et al., 2014), and *Am*Lat2's activity level showed a greater increase when assessed using C-terminal tags, such as red fluorescent protein, than when unmodified (Londesborough et al., 2014). Therefore, it is important to consider gene expression levels and gene or protein modifications that occur under survival conditions in each species.

3.3. The numbers of proteins similar to polyol transporters at the species' level

As shown in Fig. 3A and B, multiple candidates of polyol transporter selected from Lecanoromycetes, including *R. conduplicans*, belong to both polyol transporter clades II and III. Additionally, the transporters are expected to belong to these two clades when they have BLOSUM-62 matrix scores of roughly over 1000. In accordance with the results, the numbers of proteins similar to *Dh*Syl1 and *Am*Lat2 defined with scores over 1000 were examined using protein databases containing 46 species or strains of Saccharomycotina, 9 species of Taphrinomycotina, and 417 species (including a few variants and forms) of Pezizomycotina, including *R. conduplicans* KY1401 and *L. pustulata* v1 (Table S1). The numbers of proteins similar to *Dh*Syl1 ranged from 0 to 11, with most species having up to 6 proteins (Fig. 4). In the LFFs, there were from two to six proteins similar to *Dh*Syl1. However, there were only up to three proteins similar to *Am*Lat2, and 87.5 % of the species or strains had only one or no proteins that met this criterion. The species containing one protein similar to *Am*Lat2 included *E. pusillum* (Eurotiomycetes), *S. sanguinea* (Coniocybomycetes), *T. eluteriae* (Dothideomycetes) and *L. pustulata* (Lecanoromycetes), which are LFFs. By contrast, the remaining LFFs belonging to Lecanoromycetes had two or three proteins similar to *Am*Lat2 in their genomes. Wang et al. (2014) suggested that the numbers of most sugar transporter genes, such as glucose and fructose, are reduced in the *E. pusillum* genome. However, most lichen-forming species in Lecanoromycetes tend to contain duplicate polyol transporters similar to

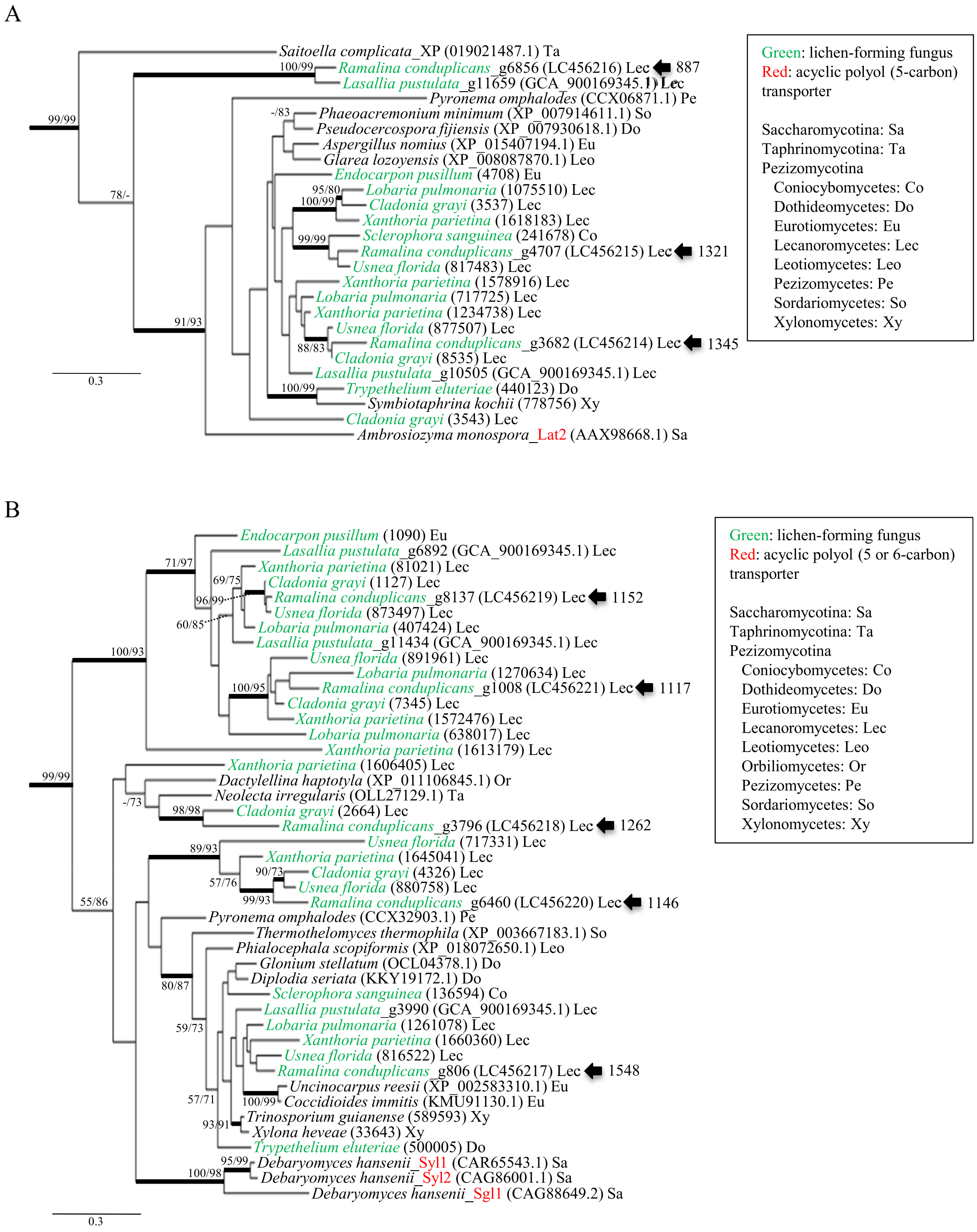


Fig. 3. Partial details of the phylogenetic tree found in Fig. 2, (A) Polyol transporter clade II constructed with *Ambrosiozyma monospora*' Lat2 and similar proteins. (B) Polyol transporter clade III constructed with *Debaryomyces hansenii*' Syl1, *D. hansenii*' Syl2, and similar proteins. Bootstrap values are shown near the nodes (≥50 %, maximum-likelihood (ML) method/maximum-parsimony (MP) method) when the values resulting from ML and/or MP methods are over 70. In addition, the nodes that supported by both ML and MP methods (>70 %) are shown thick internal branches. Characterized acyclic polyol (5 or 6-carbon) transporters are shown in red characters. Lichen-forming fungi are shown in green characters. Each branch contains the species' name, the protein's name for characterized transporters, gene ID constructed by Augustus for *Ramalina conduplicans* and *Lasallia pustulata*, GenBank accession number or MycoCosm protein ID, and fungal class or subphylum. The proteins selected from the genome of *Ramalina conduplicans* are indicated by arrows and the BLOSUM-62 matrix scores are included. The following abbreviations were used: Coniocybomycetes, Co; Dothideomycetes, Do; Eurotiomycetes, Eu; Lecanoromycetes, Lec; Leotiomycetes, Leo; Orbiliomycetes, Or; Pezizomycetes, Pe; Sordariomycetes, So; Xylonomycetes, Xy; Taphrinomycotina, Ta; and Saccharomycotina, Sa.

*Am*Lat2, while most of the other genomes did not contain multiple proteins. This expansion may be correlated with lichenization events in Lecanoromycetes. Because most fungi retain polyol transporter genes that may allow polyols to be used in the removal of reactive oxygen species (Parida and Das, 2005), as a compatible solute to adjust the osmotic pressure (Wegmann, 1986; Reed et al., 1987), and to store reducing power (Jennings, 1985; Williamson et al., 2002). However, LFFs also use polyols as their major carbon source (Lines et al., 1989); therefore, they depend on polyol transporter genes in their associations with green algae. In addition, only Lecanoromycetes have undergone lichenization associated with green algae for a long time. Gene duplication may have occurred randomly, but perhaps it was required by Lecanoromycetes to enhance the ability to transport polyols or associate with green

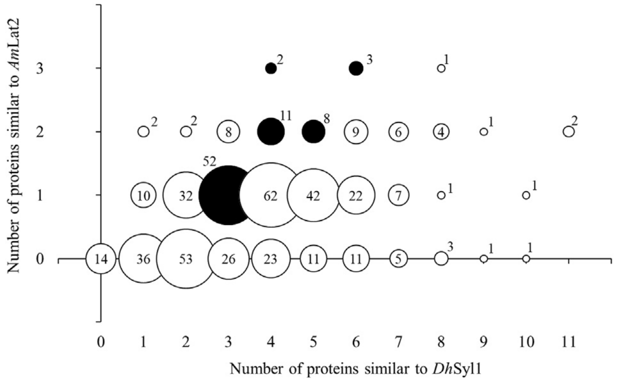


Fig. 4. Numbers of proteins similar to polyol transporters *Ambrosiozyma monospora*' Lat2 and *Debaryomyces hansenii*' Syl1 among 472 genome sets. Black circles include lichen-forming species belonging to Lecanoromycetes. White circles include other class of lichen-forming and non-lichenized fungi.

algae during their long symbiotic history. In general, multiple proteins derived from gene duplication events are expected to have some functional capabilities, such as more efficient transcription. Therefore, the expansion of *Am*Lat2-like proteins might enable the efficient utilization of polyols.

4. Conclusion

Polyol transporter genes are conserved in nearly all major ascomycetous lineages, regardless of whether they are lichen-forming (Fig. 3A and B). The maintenance of polyol transporter genes may explain why lichenization events have occurred many times in Ascomycota. However, LFFs belonging to Lecanoromycetes tend to retain multiple proteins similar to *A. monospora*' Lat2. Thus, Lecanoromycetes may have duplicated polyol transporter genes for stronger associations with green algae after a lichenization events. Degenerate primers designed based on conserved domains allowed the isolation of polyol transporter-like genes from the cDNA of *R. conduplicans* that corresponded with the genome sequences. This suggested that further candidate genes could be collected from LFFs, such as basidiolichens and cyanolichens, photosynthetic partners, lichenicolous fungi and endolichenic microorganisms, even if their genomic data are not available. In addition, broad experiments, including those elucidating the gene expression patterns, post-translational modifications and horizontal gene transfer events, are also needed to investigate the polyol transfer systems in LFFs and their symbiotic association processes with green algae. Finally, we have begun to uncover possible connections between the evolution of diverse polyol transporters in the phylum Ascomycota and the success of lichenized fungi.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.01.006.

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