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Standard Paper

Genetic variability in the *Physconia muscigena* group (*Physciaceae*, Ascomycota) in the Northern Hemisphere

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Abstract

The principal goal of our study was to test whether ecologically and chemically different populations of lichens in the *Physconia muscigena* group belong to a single, or multiple, species. We used sequence data from three markers (ITS rDNA, mtSSU rDNA and TEF1-α) for the reconstruction of phylogenetic trees based on a sampling of mostly European and Canadian populations of *P. muscigena* (Ach.) Poelt, *P. muscigena* var. *bayeri* (Nádv.) Poelt and *P. isidiomuscigena* Essl. In addition, we sought any possible geographical or ecological trends among chemotypes and haplotypes. Results show that: 1) sequence data of ITS rDNA and TEF1-α show large genetic variation in the *Physconia muscigena* group, which does not correlate with geographical distribution or thallus chemistry; 2) *Physconia muscigena* var. *bayeri* and *P. isidiomuscigena* appear undifferentiated with *P. muscigena* in our phylogenetic trees, and the three species cannot be distinguished on the basis of ITS rDNA, mtSSU rDNA and TEF1-α sequences. We therefore synonymized *Physconia muscigena* var. *bayeri* with *P. muscigena* and we recombine *P. isidiomuscigena* as a variety of *P. muscigena*.

Key words: cryptic species, ITS rDNA, lichen, phylogeny, *Physconia isidiomuscigena*, *Physconia muscigena* var. *bayeri*, TEF1-α

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Introduction

The interpopulational genetic structure of most lichen species is poorly known, which is due at least in part to their large distributional ranges and broad habitat diversities. Genetic structure of individual populations has been found to be related to the interaction of climatic and geographical factors, with locally adapted algal and fungal partners (Galloway & Aptroot 1995; Fernández-Mendoza *et al.* 2011; Sork & Werth 2014; Werth & Sork 2014; Núñez-Zapata *et al.* 2015).

Species in the genus *Physconia* (*Physciaceae*, *Lecanorales*) are foliose macrolichens with heteromeric thalli characterized by a greyish brown upper surface covered by white pruina. The genus consists of *c.* 30 species, all of them thought to associate with the unicellular green alga *Trebouxia* as a photobiont (Cubero *et al.* 2004).

Physconia species are distributed worldwide, except in the tropics (Otte *et al.* 2002). Species of the genus occur in a wide range of habitats, some being epiphytic or corticolous growing on various deciduous trees with nutrient-rich bark (e.g. *P. perisidiosa* (Erichsen) Moberg, *P. enteroxantha* (Nyl.) Poelt) and others preferring open sunny habitats on rocks, bare soil or bryophytes (e.g. *P. muscigena* (Ach.) Poelt, *P. rossica* Urbanav., *P. isidiomuscigena* Essl.). *Physconia* species are relatively poor in secondary metabolites. Many species do not contain substances that can be detected by thin-layer chromatography (TLC), a commonly used technique in lichenology (Brodo *et al.* 2001; Moberg 2002; Smith *et al.* 2009). However, *P. enteroxantha*, *P. isidiomuscigena* and *P. kurokawae* Kashiw. occasionally contain secalonic acid A, variolaric acid and gyrophoric acid (Esslinger 2000; Otte *et al.* 2002; Chen & Hu 2003).

Physconia muscigena grows on substrata having neutral to high pH among mosses or directly on mossy rocks (Fig. 1), in two different ecological habitats; in the xerothermic and temperate lowlands, and in open alpine or arctic environments (Moberg 2002; Türk & Obermayer 2006). The centre of its distribution is probably in the Northern Hemisphere, with other records reported from South America and South Africa (Thomson 1963; Moberg 1987; Otte *et al.* 2002; Chen & Hu 2003; Cubero *et al.* 2004; Flakus *et al.* 2012).

Physconia muscigena is distinguishable from similar species by the lack of vegetative propagules (isidia, soredia). Fragmentation of the thallus represents its only type of vegetative reproduction, which is rather unusual among foliose lichens. Apothecia occur rarely: Esslinger (2002) noted apothecia are 'common but not rarely missing'. Moberg (1987) did not observe apothecia in *P. muscigena* collections from Africa and suggested that apothecia are 'fairly rare' among specimens from Finland (Moberg 2002). Nádvorník (1947) did not find fertile specimens from the Czech Republic.

The taxonomy of the genus *Physconia* is rather outdated. Most comprehensive treatments were written decades ago (Nádvorník 1947; Poelt 1957, 1965; Moberg 1977). DNA-based studies have focused only on small sections of the genus. For instance, *Physconia muscigena* appears to be a polyphyletic taxon (Cubero *et al.* 2004) and two morphologically similar species pairs (*P. venusta*/*P. perisidiosa* and *P. detersa*/*P. distorta*) cannot

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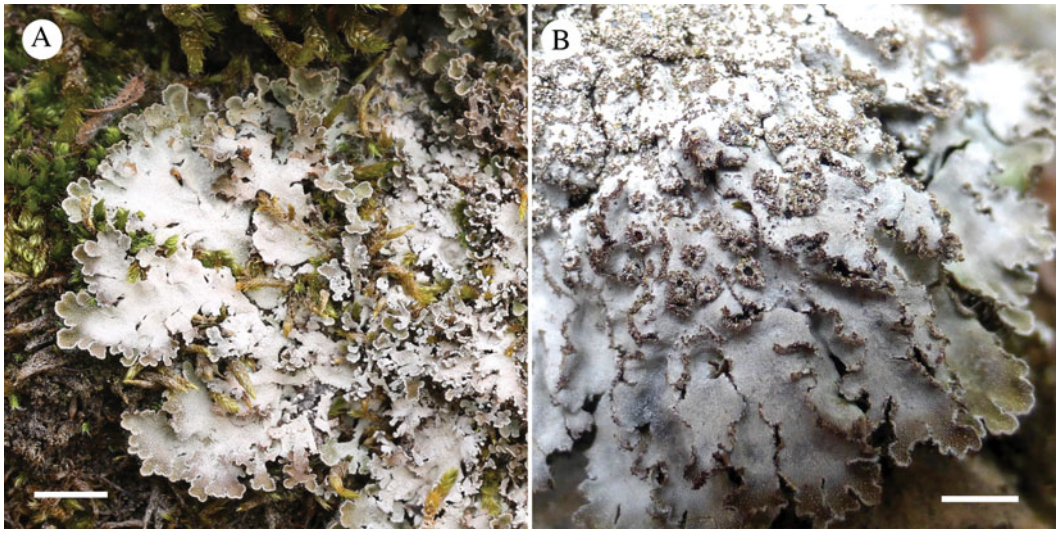

Fig. 1. A, *Physconia muscigena*. B, *P. isidiomuscigena* with sorediate-isidiate propagules on the upper surface. Scales = 0.5 mm.

be distinguished by the ITS rDNA marker (Cubero *et al.* 2004; Lohtander *et al.* 2007).

In the 1940s, *Physcia bayeri* Nádv. was newly described from the vicinity of Prague (Nádvorník 1947). This species, growing on calcareous bedrocks in sunny and warm temperate lowlands, was suggested to differ from *Physcia muscigena* in having a thinner thallus and a yellow reaction of the medulla in KOH (Nádvorník 1947). The reaction was later attributed to the presence of secalonic acid A (Otte *et al.* 2002). This species was recombined as *Physconia muscigena* var. *bayeri* (Poelt 1957, 1965) and the variety has been generally recognized, although some authors have rejected the taxon without explanation.

Two species were described recently both morphologically and ecologically very similar to *Physconia muscigena* and *P. muscigena* var. *bayeri*: *P. rossica* from Russia (Lohtander *et al.* 2007) and *P. isidiomuscigena* reported from the south-western United States (Arizona, California, Colorado, Idaho) and Canada (British Columbia) (Esslinger 2000; J. Hollinger & C. Björk, personal communication). The phylogenetic position of *P. rossica* was confirmed by ITS and mtSSU (Lohtander *et al.* 2007); *P. isidiomuscigena* does not appear in recent phylogenetic studies focused on *Physconia*. *Physconia isidiomuscigena* may be distinguished by sorediate-isidiate propagules on the upper surface ridges and laminae (Fig. 1). Ecologically it is similar to *P. muscigena*, growing on mosses in open sunny habitats (Esslinger 2000), though it appears to be limited to warmer/drier climates and may be more restricted to calcareous substrata.

Due to the lack of any recent taxonomic treatment of *P. muscigena* and related species, our study aimed to: 1) determine the phylogenetic relationships of *Physconia muscigena* var. *bayeri*, *P. muscigena* and *P. isidiomuscigena* and to assess whether *P. muscigena* var. *bayeri* forms a phylogenetically separate lineage; 2) elucidate the intraspecific variability of *P. muscigena*.

Material and Methods

Selected material and chemical analyses

We focused primarily on the European species, in addition to several from North America. We collected fresh material of *P. muscigena* from European localities in the Czech Republic, Kosovo, Italy, Serbia, Slovakia and Slovenia. Fresh material from North American populations of *P. muscigena* and *P. isidiomuscigena* was collected in British Columbia (see Table 1 and Supplementary Material Table S1, available online). Further material of *P. muscigena* and other species studied was kindly provided by curators from the following herbaria: B, BP, BRA, BRNM, BRNU, GZU, H, OLM, PRA, PRC, PRM, UBC, UCR, UPS and several personal herbaria (Table 1). In the case of *Physconia muscigena* var. *bayeri*, specimens collected by Nádvorník were used as comparative material (topotypes PRC2557 and PRM756193) and we examined additional specimens from the type locality (Praha, Butovice; Supplementary Material Table S1, available online).

Freshly collected specimens were cleaned to remove other lichen thalli, air-dried and examined under a stereomicroscope. Secondary metabolites of *Physconia muscigena*, *P. muscigena* var. *bayeri* and *P. isidiomuscigena* were analyzed using thin-layer chromatography (TLC) following the protocol of Orange *et al.* (2010). Extracted lichen compounds were transferred onto a set of two glass plates (Merck TLC Silicagel 60 F254) and placed into solvents A and B.

DNA isolation, PCR-amplification and sequencing

Total DNA was extracted from freshly collected as well as herbarium specimens using the Spin Plant Mini Kit (Invitek) according to the manufacturer's protocol. Altogether 113 samples were used for the analyses. We amplified one nuclear ribosomal region (ITS rDNA), one mitochondrial region (mtSSU rDNA), and the nuclear gene coding for translation elongation factor-1α (TEF1-α). Four PCR primer pairs were tested and used for amplification, one of which was newly designed (Table 2). Preliminary testing of TEF1-α primers has shown low success with older specimens; amplification of samples older than 5 years were mostly unsuccessful. This was partially solved by using newly designed TEF1-α primers (Table 2). DNA amplification followed the instructions described in the polymerase manufacturer's protocols (MyTaq Bioline). PCR products were cleaned with AMPure XP (Agencourt®), then sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), followed by analysis with an Applied Biosystems 3500 Genetic Analyzer.

Table 1. Specimens of *Physconia* used for this study. Voucher specimens, location information, herbarium codes and GenBank Accession numbers are also listed. The DNA numbers are unique to this study and function as labels in the phylogenetic trees.

Species	Herbarium	Collection	Collection date	Location	DNA no.	ITS rDNA	TEF1-α
Anaptychia ciliaris	GenBank			Spain		KC559095	
A. palmatula	GenBank	AFTOL-ID 648					DQ883776
Physconia americana	Hollinger	JPH 15390	2016	USA, California	147	LS483185	
P. americana	Hollinger	JPH 15389	2016	USA, California	150		LS483089
P. americana	Hollinger	JPH 15777a	2016	USA, California	151	LS483187	LS483090
P. americana	Hollinger	JPH 15777b	2016	USA, California	152		LS483091
P. americana	Hollinger	JPH 15445	2016	USA, California	153	LS483188	
P. detersa	PRA	*Palice* 19716	2015	Ukraine, Zakarpatska Oblast	100	LS483166	LS483073
P. detersa	Malíček	*Malíček* 10476	2017	Spain, Castilla La Mancha	205	LS483216	LS483108
P. detersa	H	*Ahti* 64412F		Russia, Sakha Republic		EF582761	
P. detersa	GenBank					AF224372	
P. detersa	Esslinger	*Esslinger* 14682		USA, Ontario		AY368115	
P. distorta	Malíček	*Malíček* 7935	2012	Macedonia, Galičica NP	106	LS483167	LS483074
P. distorta	PRC	*Staro178*	2015	Sweden, Skillingaryd	178	LS483199	LS483098
P. distorta	PRC	*Staro179*	2015	Sweden, Värnamo	179	LS483200	LS483099
P. distorta	Malíček	*Malíček* 10469	2017	Spain, Andalusia	199	LS483213	LS483105
P. distorta	GenBank			Spain, Cáceres		DQ862486	
P. distorta	GenBank			Spain		KC559093	
P. distorta	GenBank			United Kingdom		FR799275	
P. distorta	GenBank			United Kingdom		FR799274	
P. enteroxantha	UCR	*Knudsen* 1014KK12	2013	USA, California	92	LS483160	LS483067
P. enteroxantha	PRC	*Svoboda* 1666	2009	Albania, Permet	112	LS483169	LS483076
P. enteroxantha	Hollinger	JPH 15780	2016	USA, California	146	LS483184	
P. enteroxantha	Hollinger	JPH 18711	2016	USA, Nevada	149	LS483186	
P. enteroxantha	Malíček	*Malíček* 10471	2017	Spain, Castilla La Mancha	201	LS483214	LS483106
P. grisea	Malíček	*Malíček* 7419	2014	Italy, Sicily, Cesaro	6	LS483113	LS483033
P. grisea	PRC	*Staro65*	2015	Czech Rep., Moravský Krumlov	65	LS483142	LS483050
P. grisea	PRC	*Staro183*	2016	Morocco, Imlil	183	LS483202	
P. grisea	PRC	*Staro186*	2016	Morocco, Imlil	186	LS483205	
P. grisea	PRC	*Staro192*	2016	Morocco, Imlil	192	LS483208	
P. grisea	GenBank	MAF-Lich 9895		Spain, Ciudad Real		DQ862488	
P. grisea	GenBank	*Cubero* (MAF 9788)		Spain, Aragón		AY368128	
P. grisea	GenBank	*Cubero* (MAF 9787)		Spain, Avila		AY368126	
P. grisea	GenBank	*Dornes* 112e		Germany, Kressbronn		AF540524	
P. grisea	GenBank	MAF-Lich 17760		Spain		KC559094	
P. grisea	GenBank	*Cubero* (MAF 9786)		Austria, Graz		AY368127	
P. isidiomuscigena	PRC	*Svoboda* 2708	2016	Canada, British Columbia	132	LS483173	
P. isidiomuscigena	PRC	*Svoboda* 2710	2016	Canada, British Columbia	134	LS483174	LS483080
P. isidiomuscigena	PRC	*Svoboda* 2711	2016	Canada, British Columbia	135	LS483175	LS483081
P. isidiomuscigena	Hollinger	JPH 11331	2016	USA, Nevada	143	LS483181	LS483086
P. isidiomuscigena	Hollinger	JPH 13774	2016	USA, Oregon	144	LS483182	LS483087

(*Continued*)

Table 1. (*Continued.*)

Species	Herbarium	Collection	Collection date	Location	DNA no.	ITS rDNA	TEF1-α
P. isidiomuscigena	Hollinger	JPH 11822	2016	USA, Nevada	145	LS483183	LS483088
P. muscigena	PRC	*Svoboda* 927	2004	Czech Rep., CHKO Pálava	3	LS483110	LS483031
P. muscigena	Malíček	*Malíček* 6940	2014	Czech Rep., CHKO Pálava	4	LS483111	
P. muscigena	PRC	*Svoboda* 2611	2014	Austria, Innsbruck	8	LS483114	
P. muscigena	Malíček	*Malíček* 2335	2009	Austria, Tirol	10	LS483115	LS483034
P. muscigena	Malíček	*Malíček* 5750	2012	Romania, Transylvania	12	LS483116	LS483035
P. muscigena	Malíček	*Malíček* 4133	2011	Macedonia, Tetovo	13	LS483117	LS483036
P. muscigena	Malíček	*Malíček* 3248	2010	Slovakia, Belianské Tatry	14	LS483118	LS483037
P. muscigena	PRA	*Palice* 18071	2014	Slovakia, Poprad	15	LS483119	LS483038
P. muscigena	PRC	*Staro*17	2014	Morocco, Imlil	17	LS483120	
P. muscigena	PRC	*Staro20*	2014	Slovakia, Nízké Tatry	20	LS483122	LS483040
P. muscigena	PRC	*Staro*21	2014	Slovakia, Nízké Tatry	21	LS483123	
P. muscigena	PRC	*Staro*22	2014	Slovakia, Nízké Tatry	22	LS483124	
P. muscigena	PRC	*Staro*23	2014	Slovakia, Nízké Tatry	23	LS483125	
P. muscigena	PRC	*Staro*24	2014	Slovakia, Spišské Podhradie	24	LS483126	
P. muscigena	GZU	*Hafellner* 67703	2006	Austria, Oberösterreich	29	LS483127	
P. muscigena	GZU	*Hafellner* 76894	2007	Austria, Kärnten	30	LS483128	
P. muscigena	GZU	*Hafellner* 72915	2008	Austria, Vorarlberg	31	LS483129	
P. muscigena	GZU	*Hafellner* 78906	2008	Austria, Vorarlberg	32	LS483130	
P. muscigena	GZU	*Obermayer*, Dupla Graecensia Lich. 930	2012	Austria, Steiermark	34	LS483131	LS483041
P. muscigena	GZU	*Hafellner* 75363	2007	Albania, Malësi e Madhe	36	LS483132	
P. muscigena	GZU	*Atanassova* 150803	2005	Bulgaria, Rila mountain	37	LS483133	
P. muscigena	GZU	*Myerhofer* 492	2010	Kosovo, Prokletije, Hajla	38	LS483134	LS483042
P. muscigena	GZU	*Hafellner* 79410	2009	Germany, Bayern	41	LS483135	LS483043
P. muscigena	GZU	*Muggia*	2012	Switzerland, Canto Ticino	42	LS483136	LS483044
P. muscigena	GZU	*Hafellner* 79946	2010	USA, Alaska	44	LS483137	LS483045
P. muscigena	H	*Hansen*, Lich. Groenl. Exs. 1027	2007	Greenland, Kap Morris Jesup	47	LS483138	LS483046
P. muscigena	H	*Veli Haikonen* 27979	2010	Finland, Kil, Muonio	48	LS483139	LS483047
P. muscigena	H	*Juha Pykälä* 39242	2010	Finland, Ks, Kuusamo	53	LS483140	LS483048
P. muscigena	H	*Juha Pykälä* 35611	2010	Finland, Ks, Kuusamo	54	LS483141	LS483049
P. muscigena	BP	*Lökös*	2008	France, Mercantrour	69	LS483145	
P. muscigena	UBC	*Björk* 38167	2014	Canada, Nunavut	70	LS483146	LS483053
P. muscigena	UBC	*Björk* 36039	2014	Canada, Nunavut	72	LS483147	LS483054
P. muscigena	UBC	*Björk* 28389	2012	Canada, Nunavut	73	LS483148	LS483055
P. muscigena	UBC	*Björk* 34371	2014	Canada, British Columbia	75	LS483149	LS483056
P. muscigena	UBC	*Goward* 08-04a	2008	Canada, British Columbia	76	LS483150	LS483057
P. muscigena	UBC	*Björk* 28855	2012	Canada, Nunavut	78	LS483151	LS483058
P. muscigena	UBC	*Björk* 38216	2014	Canada, Nunavut	80	LS483152	LS483059
P. muscigena	UBC	*Björk* 29329	2012	Canada, Nunavut	81	LS483153	LS483060
P. muscigena	UBC	*Björk* 32022	2013	Canada, British Columbia	82	LS483154	LS483061
P. muscigena	UBC	*Björk* 31996	2013	Canada, British Columbia	83	LS483155	LS483062

(*Continued*)

Table 1. (*Continued.*)

Species	Herbarium	Collection	Collection date	Location	DNA no.	ITS rDNA	TEF1-α
P. muscigena	UBC	*Goward* 12-143	2012	Canada, Alberta	84	LS483156	LS483063
P. muscigena	UBC	*Björk* 21320	2010	USA, Idaho	85	LS483157	LS483064
P. muscigena	UBC	*Björk* 22626	2014	Canada, British Columbia	87	LS483158	LS483065
P. muscigena	PRC	*Staro*88	2015	Czech Rep., Hrubý Jeseník	88	LS483159	LS483066
P. muscigena	PRA	*Vondrák* 14138	2015	Russia, Dagestan	94	LS483161	LS483068
P. muscigena	PRA	*Vondrák* 14137	2015	Russia, Dagestan	95	LS483162	LS483069
P. muscigena	Uhlík P.	*Sokolov*	2015	Svalbard, Pyramiden	98	LS483164	LS483072
P. muscigena	PRA	*Palice* 9461	2005	Norway, Sør-Trøndelag	129	LS483172	LS483079
P. muscigena	PRC	*Svoboda* 2712	2016	Canada, British Columbia	136	LS483176	LS483082
P. muscigena	PRC	*Svoboda* 2713	2016	Canada, British Columbia	137	LS483177	LS483083
P. muscigena	PRC	*Svoboda* 2714	2016	Canada, British Columbia	138	LS483178	
P. muscigena	PRC	*Svoboda* 2716	2016	Canada, British Columbia	140	LS483179	LS483084
P. muscigena	PRC	*Svoboda* 2717	2016	Canada, British Columbia	141	LS483180	LS483085
P. muscigena	Hollinger	JPH 11834	2016	USA, Nevada	154	LS483189	LS483092
P. muscigena	Hollinger	NN 3182	2016	USA, California	155	LS483190	LS483093
P. muscigena	Hollinger	JPH 14233	2016	Canada, British Columbia	156	LS483191	
P. muscigena	Hollinger	JPH 11445	2016	USA, Nevada	157	LS483192	LS483094
P. muscigena	PRC	*Staro*164	2017	Czech Republic, Pálava	164	LS483196	LS483095
P. muscigena	PRC	*Staro*167	2017	Czech Republic, Pálava	167	LS483197	LS483096
P. muscigena	PRC	*Staro*173	2017	Czech Republic, Pálava	173	LS483198	LS483097
P. muscigena	PRC	*Staro*182	2016	Svalbard, Longyearbyen	182	LS483201	LS483100
P. muscigena	PRC	*Staro*196	2017	Kosovo, Restelice	196	LS483210	LS483102
P. muscigena	PRC	*Staro*197	2017	Serbia, Kopaniok NP	197	LS483211	LS483103
P. muscigena	Malíček	*Malíček* 10468	2017	Spain, Sierra Nevada NP	198	LS483212	LS483104
P. muscigena var. bayeri	PRA	*Palice* 14851	2011	Czech Rep., Praha	5	LS483112	LS483032
P. muscigena var. bayeri	PRC	*Staro*66	2015	Czech Rep., Praha	66	LS483143	LS483051
P. muscigena var. bayeri	PRC	*Staro*67	2015	Czech Rep., Praha	67	LS483144	LS483052
P. perisidiosa	PRC	*Staro*18	2014	Morocco, Imlil	18	LS483121	LS483039
P. perisidiosa	PRC	*Svoboda* 2590	2006	France, Corsica	99	LS483165	
P. perisidiosa	PRC	*Svoboda* 1654	2009	Montenegro, Bielašica NP	113	LS483170	LS483077
P. perisidiosa	Hollinger	JPH 10711a	2016	USA, Nevada	158	LS483193	
P. perisidiosa	Hollinger	JPH 11821	2016	USA, Nevada	159	LS483194	
P. perisidiosa	Hollinger	JPH 15778	2016	USA, California	161	LS483195	
P. perisidiosa	PRC	*Staro*184	2016	Morocco, Imlil	184	LS483203	
P. perisidiosa	PRC	*Staro*185	2016	Morocco, Imlil	185	LS483204	
P. perisidiosa	PRC	*Staro*188	2016	Morocco, Imlil	188	LS483206	LS483101
P. perisidiosa	PRC	*Staro*189	2016	Morocco, Imlil	189	LS483207	
P. perisidiosa	PRC	*Staro*193	2016	Morocco, Imlil	193	LS483209	
P. perisidiosa	Malíček	*Malíček* 10472	2017	Spain, Castilla La Mancha	202	LS483215	LS483107
P. perisidiosa	GenBank	*Esslinger* 15399		USA, North Dakota		AY368142	
P. perisidiosa	GenBank			Germany, München		AF540525	
P. perisidiosa	GenBank	*Cubero* (MAF 9784)		Spain, Madrid		AY368140	

(*Continued*)

Table 1. (*Continued.*)

Species	Herbarium	Collection	Collection date	Location	DNA no.	ITS rDNA	TEF1-α
P. perisidiosa	GenBank	*Cubero* (MAF 9801)		Spain, Avila			AY368141
P. rossica	PRA	*Vondrák* 14139	2015	Russia, Dagestan	96	LS483163	LS483070
P. rossica	PRA	*Vondrák* 14140	2015	Russia, Dagestan	97		LS483071
P. rossica	H	*Urbanavichus* 019		Russia, Bashkortostan		EF594741	
P. venusta	Malíček	*Malíček* 7593	2012	Italy, Sicily	107	LS483168	LS483075
P. venusta	PRC	*Svoboda* 1657	2009	Albania, Shkoder	116	LS483171	LS483078
P. venusta	Malíček	*Malíček* 10477	2017	Spain, Andalusia	206	LS483217	LS483109

Sequence alignments

The final dataset consisted of 271 newly generated sequences from this study and 20 sequences obtained from GenBank (Table 1). Sequences were subjected to BLAST searches to confirm their identities. Only high quality sequences were used for phylogenetic analyses. Sequences were manually edited using BioEdit 7.2.5 (Hall 1999) and FinchTV 1.4.0 (Geospiza Inc., Seattle, WA, USA). Sequences were automatically aligned with MEGA7 using the MUSCLE algorithm (Kumar *et al.* 2016). All new sequences were deposited in GenBank (Table 1).

Phylogenetic analyses

We analyzed three datasets: ITS rDNA, mtSSU rDNA and TEF1-α. The number of variable and parsimony-informative sites is summarized in Table 3. We did not use a combined dataset because ITS rDNA and TEF1-α regions showed different evolutionary histories based on the ILD test ($P = 0.002$) performed using PAUP v. 4.0b10 (Swofford 2002). Tree graphics were created using the program FigTree v1.3. (http://tree.bio.ed.ac.uk/software/figtree/). Phylogenetic analyses were performed by Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist *et al.* 2012) and maximum likelihood analysis (ML) was performed using the software MEGA7 (Kumar *et al.* 2016). The best-fit substitution model for each gene was determined using the Bayesian information criterion (BIC) in jModelTest v. 2.1.5 (Darriba *et al.* 2012) for BI analyses. BIC was TrNef + G for all datasets. Substitution models for ML analyses were K2 + I, T92 and K2 + G, respectively and the analyses ran 1000 replicates for branch support. For the BI analyses, we performed two independent runs of 5 000 000 generations, each with four incrementally heated simultaneous Markov chains and the first 25% of samples discarded as burn-in; the remaining trees were used to compute a 50% majority-rule consensus tree with posterior probabilities as Bayesian branch support. The average standard deviation of split frequencies estimating convergence reached the level of 0.004, 0.07 and 0.006 at the end of the analysis of ITS rDNA, mtSSU rDNA and TEF1-α, respectively.

We used the closely related genus *Anaptychia* as outgroup for all analyses.

Haplotype network analysis

Haplotype networks for ITS and TEF1-α were inferred with the program PopART (Leigh & Bryant 2015). We used TCS network analysis for haplotype relationship assessment and visualized geographical range and the presence of any secondary substances in these networks (PopART; Leigh & Bryant 2015).

Results

TLC analysis

We analyzed 253 herbarium specimens of *Physconia* and detected a new secondary metabolite that is present in the majority of specimens (in 138 of 234 *P. muscigena* specimens, 8 of 9 *P. muscigena* var. *bayeri* and in 5 of 9 *P. isidiomuscigena* specimens). The exact chemical structure is unknown and it is neither a fatty acid nor a terpenoid. The substance does not match any of the commonly used TLC standards in lichenology (Orange *et al.* 2010). We did not detect secalonic acid A.

Molecular analyses

Phylogenetic reconstruction shows large genetic variation in the *Physconia muscigena* group in the ITS rDNA and TEF1-α datasets. The *P. muscigena* group is well supported as a monophyletic clade. *Physconia muscigena* var. *bayeri* and *P. isidiomuscigena* appear together with *P. muscigena* (Figs 2 & 3). These three taxa cannot be distinguished on the basis of ITS rDNA, mtSSU rDNA and TEF1-α sequences.

We observed some differences in the topology of the gene trees. There were also differences in statistical support of some nodes when comparing the results of the ML and BI analysis of each dataset. These differences were visualized in the DensiTree - ITS rDNA + TEF1-α dataset (Bouckaert 2010) (Fig. 4). The combination where *P. muscigena*, *P. muscigena* var. *bayeri* and *P. isidiomuscigena* were put together showed more relevant topology (Fig. 4A).

These three taxa have been delimited based on morphology, chemistry and distribution. However, neither the presence of specific secondary metabolites nor geographical patterns correlate with the topology of the phylogenetic tree. *Physconia muscigena* var. *bayeri* has no distinct differences in morphology to the nominal variety and does not contain different chemical substances. The only other distinction reported is its distribution in lowlands. However, it is now clear that *P. muscigena* also commonly grows in temperate lowlands (see Supplementary Material Table S1, available online). *Physconia isidiomuscigena* can be distinguished from *P. muscigena* by the presence of sorediate-isidiate propagules on its upper surface but, in our gene trees, this morphological trait appears ungrouped in various termini, not in a monophyletic

Table 2. Loci used for molecular analyses, with corresponding primer sequences and literature references.

Locus	Primer	Position	Primer DNA sequence (5′–3′)	References
ITS rDNA	ITS 1F	forward	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns 1993
	ITS 4	reverse	TCCTCCGCTTATTGATATGC	White *et al.* 1990
mtSSU	SSU 1	forward	AGCAGTGAGGAATATTGGTC	Zoller *et al.* 1999
	SSU 3	reverse	ATGTGGCACGTCTAT	Zoller *et al.* 1999
TEF1-α	EF 983	forward	GCYCCYGGHCAYCGTGAYTTYAT	Carbone & Kohn 1999
	EF 2218	reverse	ATGACACCRACRGCRACRGTYTG	Carbone & Kohn 1999
	fph	forward	TCTSCTKGCCTTYACYCTGG	Present study
	rph	reverse	GCATGCAATGTGGGCRGT	Present study

Table 3. Characterization of sequence datasets used in the molecular analyses.

DNA region	No. of sequences	No. of nucleotide sites	No. of parsimony informative sites	No. of variable sites
ITS rDNA	135	448	84	153
mtSSU rDNA	84	359	5	19
TEF1-α	80	538	73	174

clade. Likewise, geography and ecological characteristics do not segregate in our gene trees.

In this study, the TEF1-α region was used for the first time in the genus *Physconia*. Our results show rather low variability of nucleotide sequences compared to the ITS region, but they do successfully separate individual species. On the other hand, the mtSSU marker showed low interspecies variability (Table 3) and was not suitable to resolve species boundaries. Therefore, we did not use the mtSSU marker in the subsequent molecular analyses. Further results show that *P. perisidiosa*/*P. venusta* were not supported as separate species based on ITS rDNA and TEF1-α sequences. The same has been shown in the *P. detersa*/*P. distorta* clade (Figs 2, 3 & 4).

The sampled material of *Physconia muscigena* includes 19 different ITS haplotypes in 71 samples (Figs 5 & 6). The network consists of one main clade containing 37 haplotypes, and 18 minor clades that contain 1–6 sequences. The TEF1-α network showed 15 different haplotypes in 62 samples, with the main clade containing 40 haplotypes (Supplementary Material Figs S1 & S2). In the main clade, there are haplotypes with mixed geographical distributions and a presence or absence of secondary metabolites (see above). In both cases, haplotype structures could not be explained on the basis of secondary metabolites or geography (Figs 5 & 6, Supplementary Material Figs S1 & S2). Some haplotypes of *P. isidiomuscigena* are nested within the ancestral clade *P. muscigena*.

Discussion

Genetic variation of Physconia muscigena

Our results show large genetic variation variability in the *P. muscigena* complex (Figs 2 & 3). This variability does not match the geographical distribution of analyzed samples, in contrast to, for example, *Biatora helvola* (Printzen *et al.* 1999), *Bryoria fremontii* (Velmala *et al.* 2009), *Ramalina menziesii* (Sork & Werth 2014) or *Parmelina tiliacea* (Núñez-Zapata *et al.* 2015). Authors of these studies found separated molecular lineages that correlated to portions of the geographical distribution of the species. Furthermore, in these studies ecology and secondary metabolite characters also did not segregate into clades on the gene trees. The factors that regulate the mode of reproduction and production of secondary metabolites in lichen individuals remain unknown.

Some studies have shown that geographical patterns and molecular markers cannot be used for delimiting species complexes/pairs which differ only in reproduction modes (Myllys *et al.* 2001; Articus *et al.* 2002; Messuti *et al.* 2016).

The *Physconia muscigena* group contains 19 distinct ITS haplotypes of 71 samples (Figs 5 & 6). We found members of identical haplotypes from different geographical regions (Europe and Canada) together in the ancestral clade (Fig. 5). Some minor clades contain only a single sequence. We observed the same situation in the case of the TEF1-α network (Supplementary Material Figs S1 & S2, available online). Printzen *et al.* (2003) found similar results for *Cavernularia hultenii* (*Parmeliaceae*), where ancestral clades contained haplotypes from different geographical regions. Their dataset contained 49 different haplotypes across 62 populations with two main clades. The authors explained the extant disjunction of *C. hultenii* by fragmentation of a formerly coherent distribution with long-distance dispersal and recurrent diaspore exchange. This fragmentation caused incomplete removal of ancestral haplotypes from the post-fragmentation and post-expansion areas by slow genetic drift (Printzen *et al.* 2003). In the case of *P. muscigena*, after the last glacial period the species' geographical range could have expanded into newly ice-free treeless areas with calcium-rich bare soils. With subsequent progressive climate warming during the continuing post-glacial period, suitable habitats diminished in area and *P. muscigena* now survives only in fragmented refugia. This could result in the reduction of sexual reproduction and formation of fragmented isolated populations not connected by

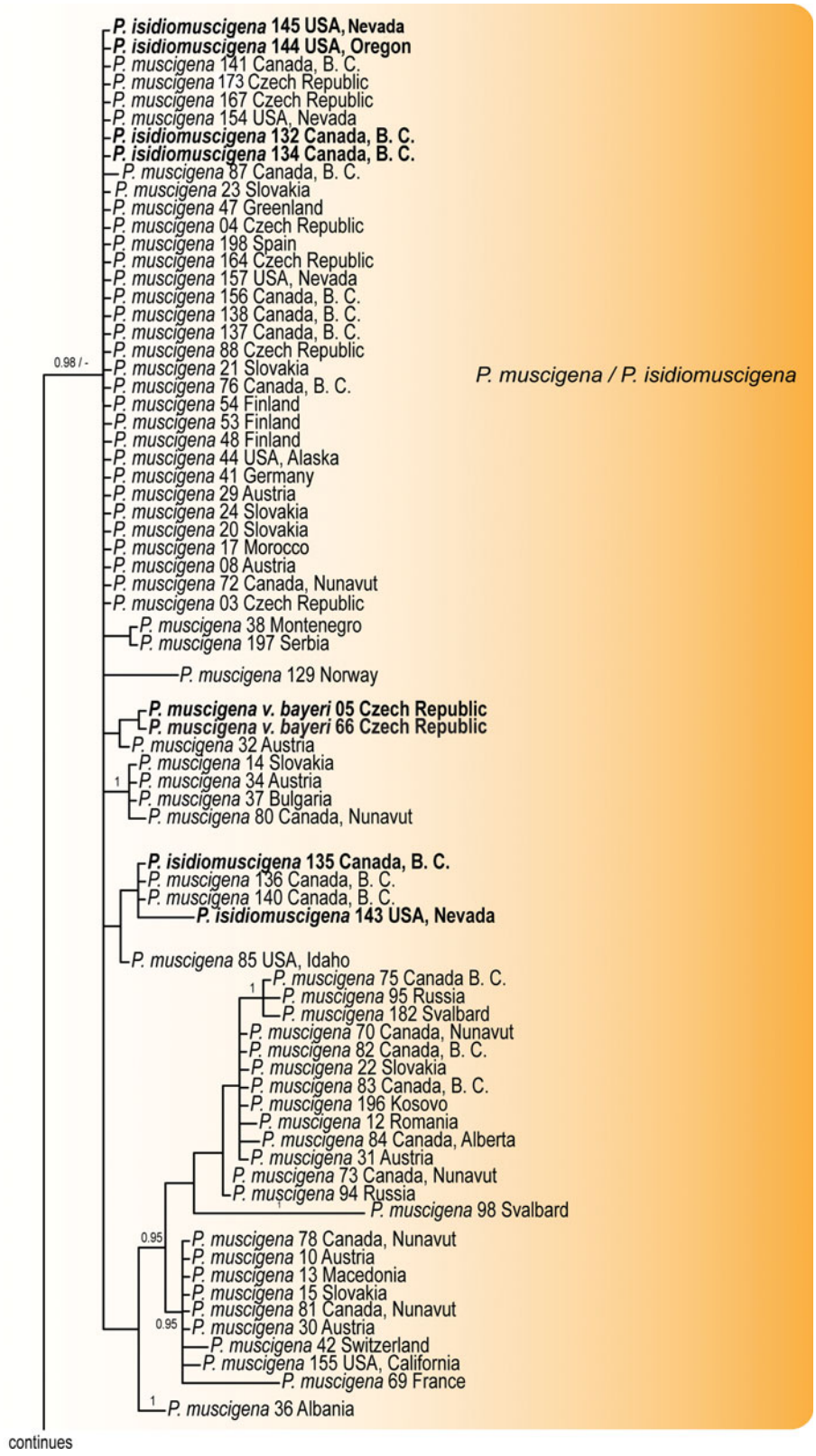

Fig. 2. Majority-rule consensus tree produced by the Bayesian (BI) and maximum likelihood (ML) analyses of the ITS rDNA sequences of *Physconia* species. Support values (BI/ML) are given above the branches. *Physconia muscigena* var. *bayeri* and *P. isidiomuscigena* sequences are in bold. Information for the sequences used are given in Table 1 and Supplementary Table S1 (available online). The tree is rooted with *Anaptychia ciliaris*. In colour online.

long-distance dispersal (Zoller *et al.* 1999). Hence we think it is possible that previously widely distributed haplotypes of *P. muscigena* occurring in the Northern Hemisphere could be found in small isolated populations persisting across the species' geographical range. Simultaneously, some haplotypes could perish while others expand geographically. Genetic drift and/or shifting climatic conditions could cause the changes in the frequency of sexual and vegetative reproduction, which is, in the case of *P. muscigena*, towards vegetative reproduction. Further analyses of haplotypes from the Southern Hemisphere could help elucidate

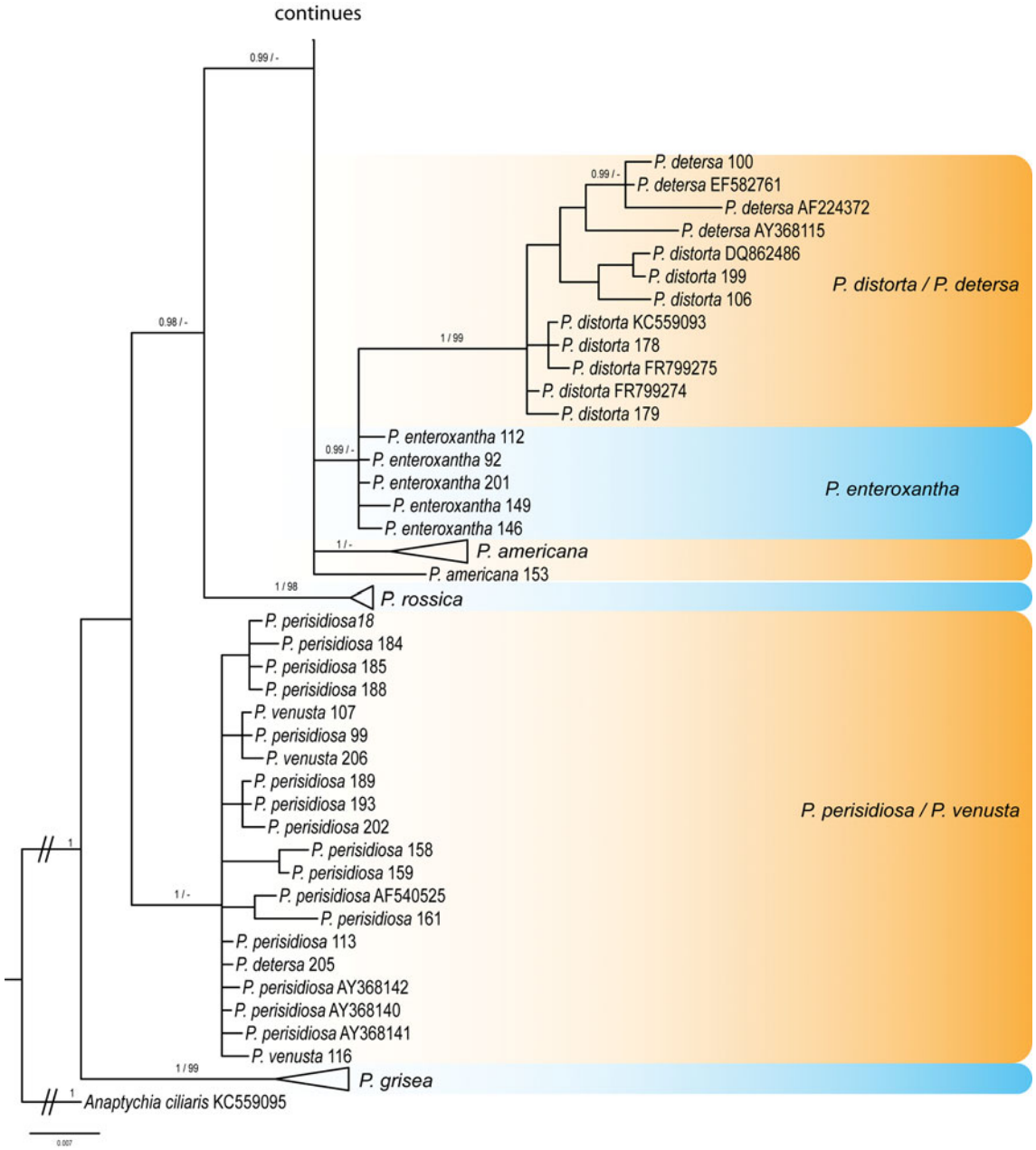

Fig. 2.

global patterns in genetic structure that are not clear in the current, more limited dataset.

Phylogenetic position of Physconia isidiomuscigena

Physconia isidiomuscigena differs from *P. muscigena* by the production of sorediate-isidiate propagules (Esslinger 2000). There are no other morphological or anatomical differences present, and we also did not find any molecular difference. Our samples of *P. isidiomuscigena* did not form a separate monophyletic clade (Figs 2 & 3). The existence of two forms of one species differing by their reproductive strategy is not unknown in lichens; for example, *Peltigera didactyla* may have sorediate and apotheciate thalli, and non-sorediate apotheciate thalli are commonly intermixed among sorediate sterile thalli within populations (Goffinet *et al.* 2003). Another example is *Pseudocyphellaria pilosella* (Messuti *et al.* 2016) which has sorediate as well as apotheciate forms that usually lack soredia. Tehler (1982) asserted that the sterile forms in these species pairs should not be regarded as species in the strict sense but rather as asexual clones developed from a mother species with the potential for both sexual and asexual propagation. On the other hand, some authors consider different types of reproduction to be taxonomically important; we therefore recombine *P. isidiomuscigena* as a variety of *P. muscigena* (see below).

Physconia muscigena var. isidiomuscigena (Essl.) Starosta & D. Svoboda comb. et stat. nov.

MycoBank No.: MB 830984

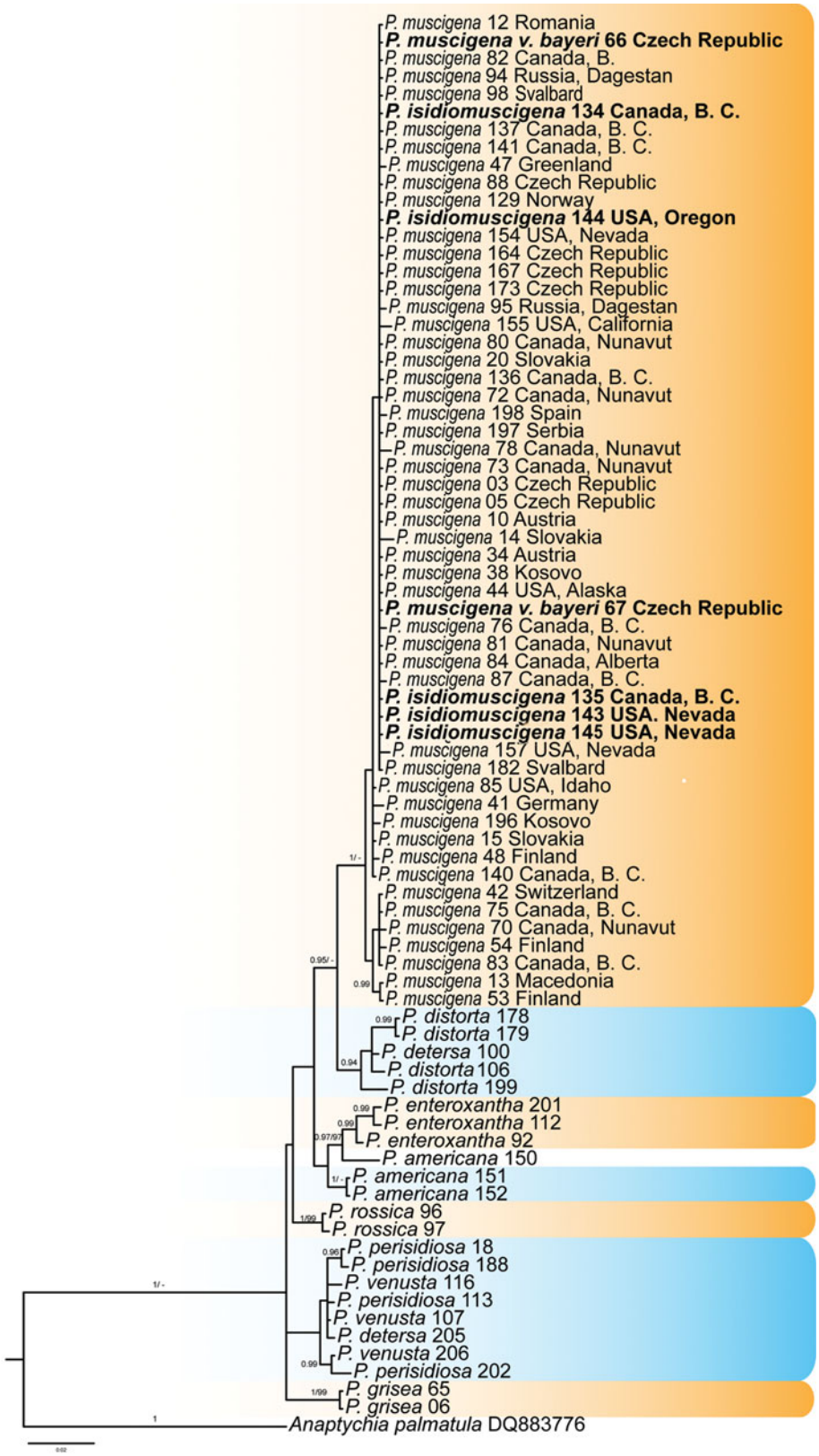

Fig. 3. Majority-rule consensus trees produced by the Bayesian (BI) and maximum likelihood (ML) analyses of the TEF1-α sequences of *Physconia* species. Support values (BI/ML) are given above the branches. *Physconia muscigena* var. *bayeri* and *P. isidiomuscigena* sequences are in bold. Information for the sequences used are given in Table 1 and Supplementary Table S1 (available online). The tree is rooted with *Anaptychia palmatula*. In colour online.

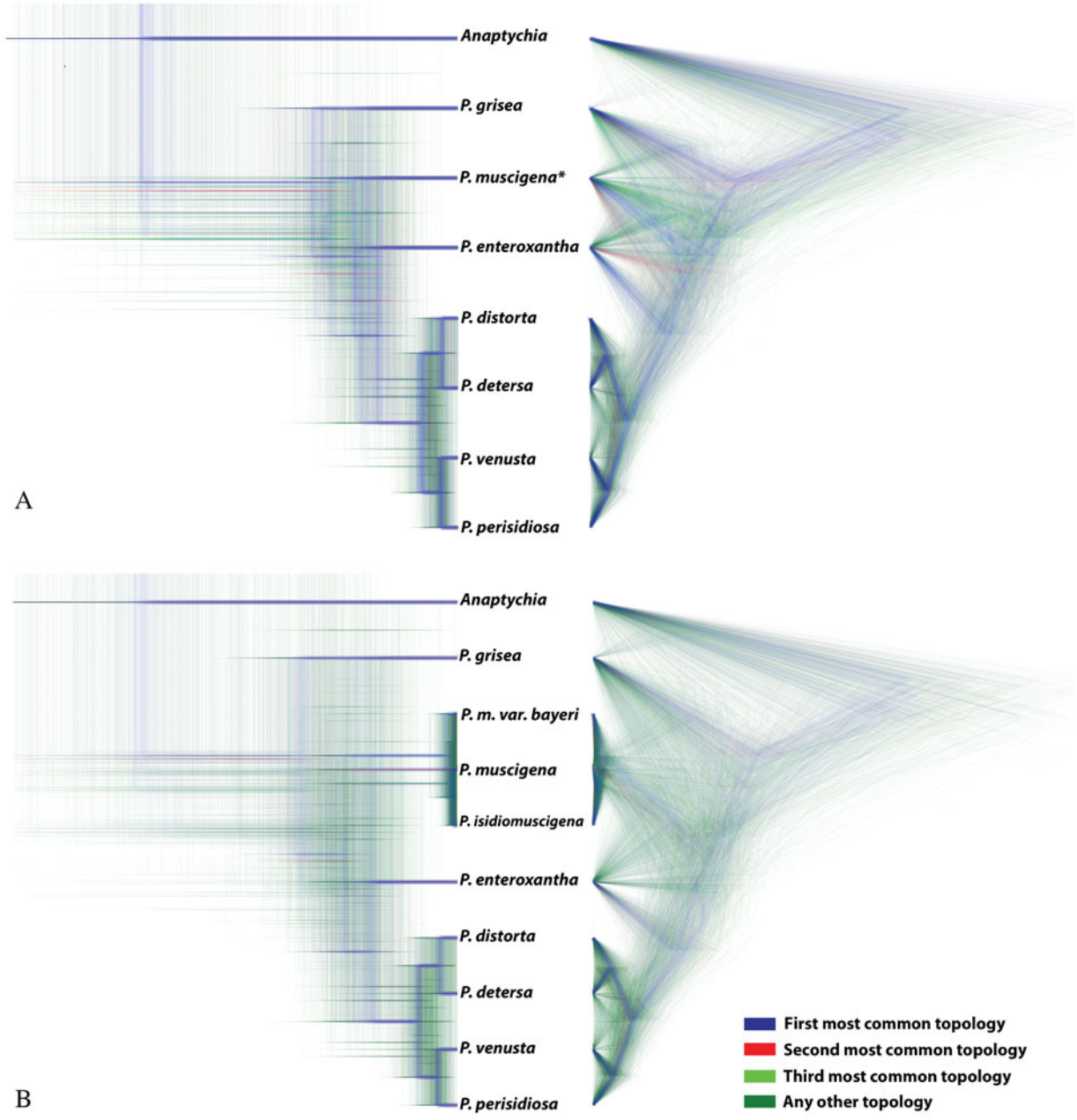

Fig. 4. Species tree inferred with *BEAST visualized using DensiTree (Bouckaert 2010): ITS rDNA + TEF1-α dataset. All trees created in the analysis are displayed (burn-in 25%). There are five clearly distinguishable clades, with large uncertainty of the topologies within the one 4-leaf clade of closely related species (*distorta-detersa-venusta-perisidiosa*). Analysis showed a different topology when *P. muscigena** (including *var. bayeri* and *P. isidiomuscigena*) was used as one species (A), and when *P. muscigena*, *P. muscigena* var. *bayeri* and *P. isidiomuscigena* were included as different species (B).

Physconia isidiomuscigena Essl., *Bull. Calif. Lichen Soc.* **7**, 5 (2000); type: USA, Arizona, Coconino Co., Grand Canyon National Park, *Nash* 30843 (ASU—holotype; TLE—isotype).

Phylogenetic position of P. muscigena *var.* bayeri

In our analysis, the ITS rDNA and TEF1-α sequences of *Physconia muscigena* var. *bayeri* grouped together with other *P. muscigena* sequences, but did not form a well-supported isolated clade (see Figs 2 & 3).

In the original description of *P. muscigena* var. *bayeri*, Nádvorník (1947) noted the yellow reaction of the medulla in KOH without identifying the substance responsible for the reaction. Otte *et al.* (2002) attributed the yellow reaction to the presence of secalonic acid A. This substance is also known to be present in several other *Physconia* species. Otte *et al.* (2002) did not describe in detail the method used to identify the substance. In this study we examined 262 *Physconia muscigena/isidiomuscigena* specimens from 25 countries using TLC and we did not detect secalonic acid A in any of the specimens studied. Other treatments also do not mention its presence (Moberg 1987, 2002; Andreev *et al.* 2008). Therefore, we assume that previous records of this acid in the thalli of *P. muscigena* were based on occasional observations only and that the substance is of rare and sporadic occurrence.

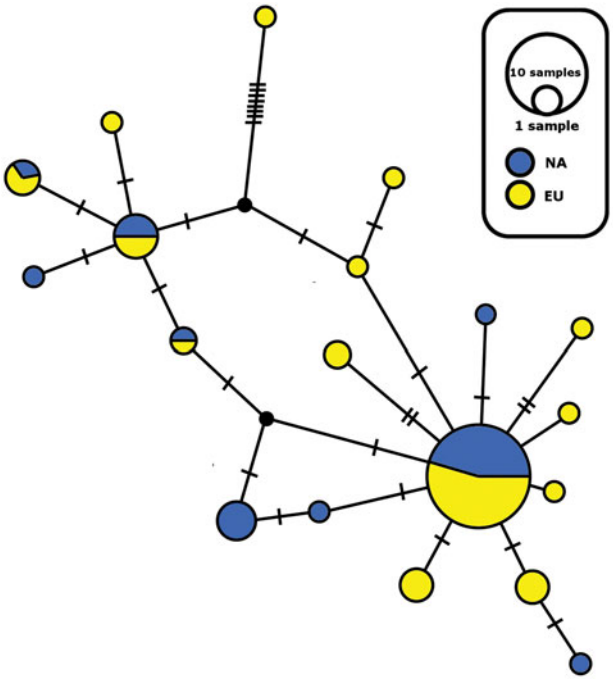

Fig. 5. Haplotype ITS network for *Physconia muscigena*; distribution of North American (NA) and European (EU) haplotypes. The size of the circles is approximately proportional to the number of sampled sequences of that haplotype, with the largest circle representing the ancestral clade. Perpendicular lines show mutation steps. In colour online.

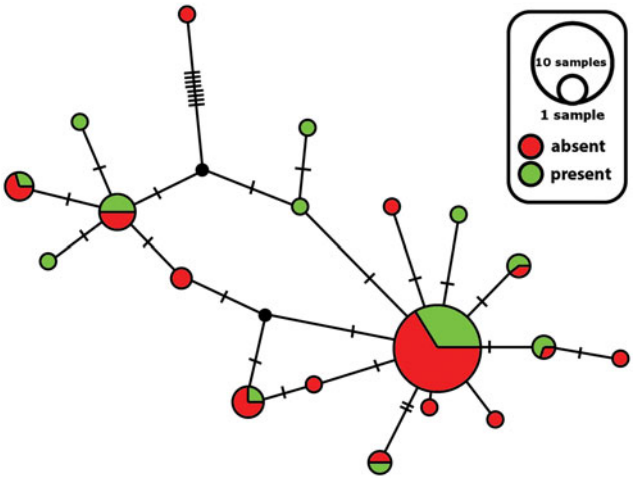

Fig. 6. Haplotype ITS network for *Physconia muscigena*; presence of secondary metabolites in thallus. The size of the circles is approximately proportional to the number of sampled sequences of that haplotype, with the largest circle representing the ancestral clade. Perpendicular lines show mutation steps. In colour online.

We did not find any morphological or chemical differences between *P. muscigena* and *P. muscigena* var. *bayeri*, and because in our gene trees specimens of the two taxa do not form distinct groups, we synonymize *P. muscigena* var. *bayeri* with *P. muscigena*. Nádvorník (1947) used the yellow reaction of the medulla in KOH to distinguish var. *bayeri* from *P. muscigena*, although *P. muscigena* can have a positive reaction in some populations (Esslinger 2002). As we could not verify this yellow reaction of var. *bayeri* (including in the Nádvorník reference collections) using TLC, we do not consider this difference taxonomically relevant.

Physconia muscigena (Ach.) Poelt

Nova Hedwigia **9**(1–4), 30 (1965).—*Physconia muscigena* var. *muscigena* (Ach.) Poelt, *Nova Hedwigia* **9**(1–4), 30 (1965).—*Parmelia muscigena* Ach., *Lich. Univ.*, 472 (1810); type: H-ACH 1406A (lectotype, designated by Moberg 1977).

Physconia muscigena var. *bayeri* (Nádv.) Poelt, *Nova Hedwigia* **9**(1–4), 30 (1965).—*Physcia muscigena* var. *bayeri* (Nádv.) Poelt, *Mitteleuropäische Flechten IV*, 279 (1957).—*Physcia bayeri* Nádv., *Studia Botanica Čechoslovaca VIII*, 124 (1947); type: PRC 4596 (MBT 389440, neotype designated here; MB354287), leg. by Z. Černohorský 1931, det. by J. Nádvorník.

Note. The type specimens mentioned by Nádvorník (Praha-Nová Ves et Motol (Bayer, Servít!)) were not found in any herbarium (PRC, PRM or BRA) where Nádvorník's collections are deposited. Therefore, we chose the well-developed specimen from the same locality in Prague, which was determined by J. Nádvorník himself, as a neotype. The collections from Nádvorník's *Physciaceae* exsiccati (Dec. 2, No. 18) could be considered as topotypes.

Our investigations of *P. muscigena* and related species did not contain samples from the Southern Hemisphere. Including additional populations throughout the distributional range of these taxa would probably provide further biogeographical insights and could help to disentangle phylogenetic relationships among the species studied. In addition, employing next generation sequencing methods could shed light on population structure (RAD-Seq, SNP, SSR).

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