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High fungal selectivity for algal symbionts in the genus *Bryoria*

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Abstract: In this study we examined photobiont identity, diversity and selectivity in the genus *Bryoria*. We focused on *B. fremontii* and section *Implexae* in order to determine whether secondary chemistry is correlated with photobiont identity. DNA from two loci for photobionts and three loci for mycobionts was sequenced for both parsimony and Bayesian phylogenetic analyses. A comparison of photobiont and mycobiont phylogenies reveals that most *Bryoria* species associate exclusively with lineages of the *Trebouxia simplex* group; only *B. smithii* was associated with a different photobiont. We conclude that most *Bryoria* species included in our study are highly selective in their choice of algal partners and that the presence/concentration of different secondary compounds does not correlate with photobiont identity either in section *Implexae* or in *B. fremontii*.

Key words: lichens, photobiont, secondary chemistry, selectivity, *Trebouxia simplex*

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Introduction

Lichens are symbiotic associations of heterotrophic fungi and photoautotrophic green algae and/or cyanobacteria. In these close associations, interactions between the symbionts have resulted in highly specialized structures such as dual asexual propagules and secondary metabolites present only in the lichenized state (Hawksworth 1988). Lichenization occurs only when a fungal partner, the mycobiont, meets a compatible photosynthetic partner, the photobiont (Ahmadjian 1993). Lichen-forming fungi have often been shown to be highly discriminating in their selection of photobiont species, both in algal (Rambold *et al.* 1998; Beck *et al.* 2002; Yahr *et al.* 2004) and in cyanobacterial associations (Stenroos *et al.* 2006; Myllys *et al.* 2007). In some cases, however, the same lichen fungus can associate with different photobiont species, that is one mycobiont can either form morphologically identical thalli with different photobiont species or genotypes (Friedl & Büdel 2008; Piercey-Normore 2006), or two or more photobionts can co-exist in a single thallus (Casano *et al.* 2011). Likewise, even in cases where a given mycobiont has been shown to be selective with respect to a given photobiont, that same photobiont might be shared among unrelated fungi belonging to the same lichen community (Helms *et al.* 2001; Piercey-Normore & DePriest 2001; Rikkinen *et al.* 2002; O'Brien *et al.* 2005; Myllys *et al.* 2007).

It has been suggested that some modes of dispersal in lichens may be better adapted than others to maintain symbiotic associations from one generation to the next (Nelsen & Gargas 2008; Wornik & Grube 2010). In sexually reproducing lichens, the fungal partner produces meiotic spores that need to encounter a compatible photobiont to re-establish the symbiosis. Such lichen systems offer an opportunity for horizontal transfer of the algal or cyanobiont partner. Suitable partners could be obtained by several means, including recruitment from the vegetative

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propagules (soredia, isidia, thallus fragments) of other lichens (Beck *et al.* 1998), association with free-living algae or cyanobacteria (Mukhtar *et al.* 1994; Sanders & Lücking 2002; Hedenås *et al.* 2007), and parasitization of another lichen (Friedl 1987). By contrast, asexually reproducing lichens reproduce by means of vegetative propagules that contain both partners, usually in the form of soredia or isidia. In principle, such lichens might be expected to retain the same photobiont from one generation to the next, potentially resulting in a high level of specificity in both symbiotic partners (Nelsen & Gargas 2008). Continuous association between the symbionts may lead to co-speciation (i.e. synchronous speciation events in different taxa), and even to parallel cladogenesis (i.e. correlated evolution along lineages), which does not necessarily involve concomitant speciation (Futuyma 1998). Recent studies, however, have shown that dispersal from vegetative propagules is not invariably linked to retention of the same photobiont even throughout a single lichen life cycle, much less over evolutionary time scales (Nelsen & Gargas 2008; Wornik & Grube 2010).

Bryoria Brodo & D. Hawksw., with some 80 described species, is a lichenized euascomycete genus in the family *Parmeliaceae* (http://www.indexfungorum.org). It has a mainly circumpolar distribution in the Northern Hemisphere, though some species occur in mountainous regions of the Southern Hemisphere (Brodo & Hawksworth 1977). *Bryoria* is a fruticose genus, easily recognized by its fine, hair-like, pendent or shrubby, grey, brown or black stems, which are repeatedly branched. Most species reproduce mainly asexually by soredia or thallus fragmentation, whereas sexual states are rare or even unknown in some species. In the absence of fruiting bodies, species delimitation has traditionally been based on morphological characters such as branching pattern, presence and type of soralia, presence and form of pseudocyphellae, thallus colour, as well as chemical characters, that is secondary metabolic compounds produced by the lichen thallus (Brodo & Hawskworth 1977; Krog 1980). In a recent phylogenetic study on *Bryoria*, however, Myllys *et al.* (2011) found that both morpho- and chemospecies concepts are problematic, especially in section *Implexae* (Gyeln.) Brodo & D. Hawksw. where none of the currently recognized species are discriminated using existing genetic markers, *Bryoria glabra* (Motyka) Brodo & D. Hawksw. being the only exception. Furthermore, in their study on *Bryoria fremontii* (Tuck.) Brodo & D. Hawksw., Velmala *et al.* (2009) showed that the concentration of vulpinic acid, a secondary substance unique for this taxon in *Bryoria*, is not correlated with the phylogeny of the mycobiont. While this suggests that the mycobiont may not be decisive in this regard, it leaves open the question as to whether a correlation could exist between the production of specific secondary compounds and the identity of the photobiont.

The objective of this study was to examine photobiont identity, diversity and selectivity in the genus *Bryoria* using photobiont ITS and COX2 DNA sequence data. We compared photobiont and mycobiont phylogenies to examine patterns of selectivity and specificity among the symbionts, and more specifically to determine whether photobiont identity correlates with the occurrence of specific secondary metabolites.

Materials and Methods

Taxon selection

Eighty-one specimens from 16 different *Bryoria* species were included in our analyses (Table 1). For the most part, the material used in this study formed the basis of an earlier study by Myllys *et al.* (2011). Taxon selection focused on *Bryoria fremontii* and section *Implexae sensu* Myllys *et al.* (2011). To examine whether lichen substances have a role in photobiont choice, we included additional specimens from section *Implexae*. In Myllys *et al.* (2011), section *Implexae* was amended, in comparison with its previous circumscription, to include most members of section *Bryoria*. The following six species from section *Implexae* were included in this study: *B. capillaris* (Ach.) Brodo & D. Hawksw., *B. fuscescens* (Gyeln.) Brodo & D. Hawksw., *B. glabra*, *B. implexa* (Hoffm.) Brodo & D. Hawksw. (including five chemotypes, see Table 1 and Holien 1989), *B. lanestris* (Ach.) Brodo & D. Hawksw., and *B. subcana* (Nyl. ex Stizenb.) Brodo & D. Hawksw. Multiple samples of each taxon were used to capture potential geographical variation.

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TABLE 1. *List of taxa, voucher information, GenBank accession numbers and secondary chemistry.*

			GenBank accession numbers					
Taxon	ID	Voucher specimen	Fungal ITS	Fungal GAPDH	Fungal mtSSU	Algal ITS	Algal COX2	Chemistry
Bryoria americana	L199	Canada, BC, *Goward* 02-165 (UBC)	HQ402678	HQ402606	HQ402637	**KJ576640**	**KJ599485**	fum, cfum, (pro)
B. americana	S69	Finland, *Velmala* 63, *Halonen & Keihäs* (H)	HQ402677	HQ402605	HQ402636	**KJ576639**	**KJ599486**	fum
B. americana	S329	Canada, Newfoundland, *Ahti* 67654 (H)	HQ402682	HQ402599	HQ402641	**KJ576693**	**KJ599487**	fum
B. bicolor	S23	Finland, *Velmala* 24, *Halonen & Myllys* (H)	HQ402689	HQ417113	HQ402644	**KJ576642**	**KJ599488**	bar, pso, fum
B. bicolor	L183	Finland, *Kuusinen* 1063 & *Lampinen* (H)	HQ402691	HQ402612	HQ402645	**KJ576641**	–	No TLC
B. capillaris	L141	Finland, *Haikonen* 22228 (H)	FJ668493	FJ668399	FJ668427	**KJ576671**	**KJ599489**	bar, (ale)
B. capillaris	L211	Finland, *Myllys* 485 (H)	GQ996287	GQ996259	GQ996331	**KJ576678**	**KJ599490**	bar, ale
B. capillaris	S192	Spain, Tenerife, *Keihäs* s.n. (OULU)	GQ996289	GQ996261	GQ996321	**KJ576689**	**KJ599491**	bar, ale, atr, (fum)
B. capillaris	L210	Canada, BC, *Goward* 05-18 (UBC)	**KJ576714**	**KJ599467**	**KJ599553**	**KJ576643**	**KJ599492**	bar, (ale)
B. capillaris	L209	Canada, BC, *Goward* 05-19 (UBC)	GQ996281	GQ996253	GQ996311	**KJ576677**	**KJ599493**	bar, ale
B. capillaris	S394	USA, Oregon, *Spribille* 29879 (hb. Spribille)	**KJ576727**	**KJ599480**	**KJ599564**	**KJ576697**	**KJ599494**	bar, ale
B. fremontii	L205	Canada, BC, *Goward* 05-04 (UBC)	FJ668503	FJ668408	FJ668436	**KJ576646**	**KJ599495**	no substances
B. fremontii	S13	Finland, *Velmala* 13b, *Halonen & Myllys* (H)	FJ668498	FJ668404	FJ668432	**KJ576644**	**KJ599496**	no substances
B. fremontii	L208	Canada, *Goward* 05-16 (UBC)	FJ668504	FJ668409	FJ668437	**KJ576645**	**KJ599497**	no substances
B. fremontii	S33	Finland, *Velmala* 32, *Halonen & Myllys* (H)	FJ668512	FJ668417	FJ668445	**KJ576703**	**KJ599498**	vul
B. fremontii	L236	Canada, BC, *Wright* 2005-16 (UBC)	FJ668513	FJ668418	FJ668446	**KJ576702**	**KJ599499**	vul, (atr)
B. fremontii	S327	Canada, BC, *Crawford* 25C				**KJ576713**	**KJ599542**	vul, nsti
B. fremontii	S320	Canada, BC, *Crawford* 22B				**KJ576711**	**KJ599543**	vul, nsti
B. fremontii	S215	Canada, BC, *Goward* 07-02-0003 (UBC)				**KJ576710**	**KJ599544**	vul
B. fremontii	S324	Canada, BC, *Crawford* 43A				**KJ576712**	**KJ599545**	vul, nsti
B. fremontii	S37	Finland, *Velmala* 35b, *Halonen & Myllys* (H)				**KJ576709**	**KJ599546**	no substances
B. fremontii	S328	Canada, BC, *Crawford* 74				**KJ576707**	**KJ599547**	vul
B. fremontii	S319	Canada, BC, *Crawford* 52A				**KJ576706**	**KJ599548**	vul
B. fremontii	S236b	Canada, BC, *Goward* 07-02-0025 (UBC)				**KJ576705**	**KJ599549**	vul
B. fremontii	S229a	Canada, BC, *Goward* 07-02-0018 (UBC)				**KJ576704**	**KJ599550**	vul
B. fremontii	L260	Finland, *Kääntönen* 229/01				**KJ576708**	–	vul
B. furcellata	L138	Canada, Manitoba, *Ahti* 63217 (H)	HQ402721	HQ402602	HQ402666	**KJ576647**	–	fum, (pro), (cfum)
B. furcellata	L147	Finland, *Haikonen* 22770 (H)	HQ402722	HQ402627	HQ402667	**KJ576700**	–	fum, pro, cfum
B. furcellata	L148	Finland, *Haikonen* 22571 (H)	HQ402723	HQ402628	HQ402668	KJ576672	–	fum, pro, cfum
B. furcellata	S31	Finland, *Velmala* 31a, *Halonen & Myllys* (H)	**KJ576731**	**KJ599484**	**KJ599568**	**KJ576701**	–	fum, pro, cfum
B. furcellata	S275	Canada, Alberta, *Colberg & Prokopetz* (UBC)	HQ402724	HQ402629	HQ402669	**KJ576692**	–	no substances
B. fuscescens	S24	Finland, *Velmala* 25, *Halonen & Myllys* (H)	**KJ576715**	**KJ599468**	**KJ599554**	**KJ576649**	**KJ599501**	fum, pro
B. fuscescens	L149	Finland, *Stjernberg* s.n. (H)	GQ996290	GQ996262	GQ996322	**KJ576673**	**KJ599502**	fum, pro, cfum
B. fuscescens	S267	Canada, Alberta, *Kamin* 033 (UBC)	**KJ576716**	**KJ599469**	–	**KJ576691**	**KJ599503**	fum, (pro), (cfum)
B. fuscescens	S277a	Canada, Alberta, *Kamin* 037 (UBC)	**KJ576718**	**KJ599471**	–	–	–	fum, pro, cfum
B. fuscescens	S56	Finland, *Velmala* 51 & *Halonen* (H)	GQ996291	GQ996263	GQ996332	**KJ576648**	**KJ599504**	fum, pro, cfum
B. fuscescens	S272	Canada, Alberta, *Adams* 0089 & Hall (UBC)	**KJ576717**	**KJ599470**	**KJ599555**	–	–	fum, (pro), (cfum)
B. glabra	L186	Finland, Kuusamo, *Halonen* s.n. (OULU)	FJ668494	FJ668400	FJ668428	**KJ576650**	**KJ599505**	no substances
B. glabra	L203	Canada, BC, *Goward* 05-22 (UBC)	HQ402725	HQ402630	HQ402670	**KJ576652**	**KJ599506**	no substances
B. glabra	L198b	Canada, BC, *Goward* 05-26 (UBC)	**KJ576730**	**KJ599483**	**KJ599567**	**KJ576651**	**KJ599507**	no substances

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TABLE 1. *Continued*

Taxon	ID	Voucher specimen	GenBank accession numbers					Chemistry
			Fungal ITS	Fungal GAPDH	Fungal mtSSU	Algal ITS	Algal COX2	
Bryoria implexa chemotype 1	S22	Finland, *Velmala* 23, *Halonen & Myllys* (H)	GQ996294	GQ996266	GQ996315	**KJ576656**	**KJ599508**	pso, (atr)
B. implexa chemotype 1	S39	Finland, *Velmala* 37, *Halonen & Myllys* (H)	GQ996293	GQ996265	GQ996323	**KJ576653**	**KJ599509**	pso, (gyr)
B. implexa chemotype 1	S36	Finland, *Velmala* 35a, *Halonen & Myllys* (H)	**KJ576719**	**KJ599472**	**KJ599556**	**KJ576684**	**KJ599510**	pso
B. implexa chemotype 2	L274	Norway, *Holien* 10177 (TRH)	GQ996296	GQ996268	GQ996325	**KJ576682**	**KJ599511**	nsti
B. implexa chemotype 2	S128	Sweden, *Högnabba* 593 (H)	**KJ576720**	**KJ599473**	**KJ599557**	**KJ576685**	**KJ599512**	nsti, cnsti, atr
B. implexa chemotype 2	L244a	Iran, East Azarbaijan, *Sohrabi* 4656 (H)	GQ996295	GQ996267	GQ996324	**KJ576654**	**KJ599520**	nsti, (atr)
B. implexa chemotype 2	S387	USA, Alaska, *Berg* 3242-A (UBC)	**KJ576726**	**KJ599477**	**KJ599561**	**KJ576696**	**KJ599526**	nsti, (cnsti)
B. implexa chemotype 2	S386	USA, Alaska, *Berg* 3242 (UBC)	**KJ576725**	**KJ599478**	**KJ599562**	**KJ576695**	**KJ599527**	nsti, (cnsti)
B. implexa chemotype 2	L240b	Canada, BC, *Goward* 05-31 (UBC)	GQ996282	GQ996254	GQ996309	**KJ576679**	**KJ599528**	(nsti)
B. implexa chemotype 3	S10	Finland, *Velmala* 11b, *Halonen & Myllys* (H)	GQ996297	GQ996269	GQ996314	**KJ576655**	**KJ599513**	gyr
B. implexa chemotype 3	S62	Finland, *Velmala* 57, *Myllys & Puolasmaa* (H)	**KJ576721**	**KJ599474**	**KJ599558**	**KJ576660**	**KJ599514**	gyr, (atr)
B. implexa chemotype 3	L272	Norway, *Holien* 10039 (TRH)	GQ996299	GQ996271	GQ996335	**KJ576681**	**KJ599515**	gyr
B. implexa chemotype 3	S395a	USA, Alaska, *Jovan* s.n. (KLGO-50787)	**KJ576728**	**KJ599481**	**KJ599565**	**KJ576698**	**KJ599500**	gyr, unknowns
B. implexa chemotype 4	S57	Finland, *Velmala* 52 & *Halonen* (H)	**KJ576722**	**KJ599475**	**KJ599559**	**KJ576658**	**KJ599516**	fum, pro, cfum, (atr)
B. implexa chemotype 4	L160	Sweden, *Rydberg* s.n. (UPS)	GQ996300	GQ996272	GQ996327	**KJ576674**	**KJ599517**	fum, pro, cfum, (atr)
B. implexa chemotype 4	S166	Russia, Perm, *Melekhin* 10123 (H)	GQ996308	GQ996273	GQ996317	**KJ576688**	**KJ599518**	fum, (pro), (cfum), atr
B. implexa chemotype 5	S45	Finland, *Velmala* 43a, *Halonen & Myllys* (H)	GQ996302	GQ996275	GQ996328	**KJ576659**	**KJ599519**	no substances
B. implexa chemotype 5	S47	Finland, *Velmala* 44, *Halonen & Myllys* (H)	**KJ576723**	**KJ599476**	**KJ599560**	**KJ576657**	**KJ599536**	no substances
B. implexa chemotype 5	S164	Russia, Perm, *Ateeva* 5055 (H)	GQ996285	GQ996257	GQ996316	**KJ576687**	**KJ599535**	(atr)
B. lanestris	L232	Denmark, Greenland, *Hansen* Lich. Greenl. Exs. 946 (H)	GQ996304	GQ996277	GQ996329	**KJ576661**	**KJ599521**	(atr)
B. lanestris	S274	Canada, Alberta, *Kamin* 016 (UBC)	GQ996303	GQ996276	GQ996333	–	–	no substances
B. lanestris	S256	Canada, Alberta, *Adams* 0076B *& Hall* (UBC)	GQ996307	GQ996280	GQ996318	–	–	(fum)
B. nadvornikiana	L161	Sweden, *Hermansson* 14179 (UPS)	HQ402719	HQ402625	HQ402664	**KJ576663**	**KJ599522**	bar, fum
B. nadvornikiana	S79	Finland, *Velmala* 73, *Halonen & Keihäs* (H)	HQ402718	HQ402624	HQ402663	**KJ576662**	**KJ599523**	bar, (ale), fum, (cfum), (atr)
B. nadvornikiana	L245	Iran, East-Azarbaijan, *Sohrabi* 4510 (H)	HQ402720	HQ402626	HQ402665	**KJ576680**	**KJ599524**	bar
B. nitidula	L163	Sweden, *Granbo* s.n. (UPS)	HQ402713	HQ402621	HQ402658	**KJ576664**	**KJ599525**	no TLC
B. nitidula	S121	Denmark, Greenland, *Högnabba* 752 (H)	HQ402711	HQ402619	HQ402656	–	–	fum, (pro), (cfum)
B. nitidula	S123	Denmark, Greenland, *Högnabba* 818 (H)	HQ402712	HQ402620	HQ402657	–	–	fum, (pro), (cfum)
B. simplicior	L296	Norway, *Holien* 10328 (TRH)	HQ402717	HQ402623	HQ402662	**KJ576683**	**KJ599529**	fatty acids
B. simplicior	S30b	Finland, *Velmala* 30, *Halonen & Myllys* (H)	HQ402714	HQ402622	HQ402659	**KJ576666**	**KJ599530**	fatty acids
B. simplicior	L231b	Russia, Sakha Republic, *Ahti* 61399 (H)	HQ402716	HQ402601	HQ402661	**KJ576665**	**KJ599531**	no substances
B. smithii	S65	Finland, *Velmala* 60, *Myllys & Puolasmaa* (H)	HQ402684	HQ402609	HQ402642	**KJ576667**	**KJ599532**	no substances
B. smithii	L174	India, *Tibell* 23319 (UPS)	HQ402685	HQ402610	HQ402643	KJ576675	–	unknown

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TABLE 1. *Continued*

Taxon	ID	Voucher specimen	GenBank accession numbers					Chemistry
			Fungal ITS	Fungal GAPDH	Fungal mtSSU	Algal ITS	Algal COX2	
Bryoria sp.	L206	Canada, BC, *Goward* 05-05 (UBC)	GQ996283	GQ996255	GQ996310	KJ576676	KJ599533	no substances
B. sp.	S239a	Canada, BC, *Goward* 07-02-0028 (UBC)	GQ996284	GQ996256	GQ996313	KJ576690	KJ599534	no substances
B. sp.	S384	USA, Alaska, *Berg* 3082 (UBC)	**KJ576724**	**KJ599479**	**KJ599563**	**KJ576694**	**KJ599537**	no substances
B. subcana	L189	Finland, *Halonen* s.n. (OULU)	GQ996305	GQ996278	GQ996312	**KJ576668**	**KJ599538**	fum, (pro), (cfum)
B. subcana	S157	Russia, Perm, *Schajachmetova* 18.9 (H)	GQ996306	GQ996279	GQ996336	**KJ576686**	**KJ599541**	fum, pro, atr
B. subcana	S396	Norway, *Holien* 11496 (TRH)	**KJ576729**	**KJ599482**	**KJ599566**	**KJ576699**	**KJ599539**	fum
B. tenuis	L164	Sweden, *Hermansson* 12855d (UPS)	HQ402695	HQ402616	HQ402649	**KJ576669**	–	fum
B. tenuis	S70	Finland, *Velmala* 64, *Halonen & Keihäs* (H)	HQ402694	HQ402615	HQ402648	**KJ576670**	**KJ599540**	fum
Gowardia arctica	L169	Canada, Nunavut, *Mattsson* 5142 (UPS)	EU282505	EU282521	–			ale
Pseudephebe pubescens	L221	USA, Alaska, *Ahti* 63704 (H)	HQ402676	HQ402604	HQ402635			nsti
Trebouxia impressa from *Parmelina tiliacea*						AJ007388		
Uncultured *Trebouxia* photobiont						EU715045		
T. jamesii from *Letharia vulpina*						FJ170763		
T. asymmetrica from *Diploschistes diacapsis*						AJ249565		
T. decolorans from *Xanthoria parietina*						AJ969555		
T. jamesii 'vulpinae' from *Letharia vulpina*						AF242457		
T. simplex from *Lecanora conizaeoides*						AJ511352		
T. jamesii from *Pseudevernia furfuracea*						AF242459		
T. jamesii from *Flavocetraria nivalis*						AY444766		
T. jamesii from *Umbilicaria antarctica*						AJ431574		
T. jamesii from *Flavocetraria nivalis*						AY444768		
T. simplex from *Lecanora conizaeoides*						AJ511351		
T. jamesii from *Vulpicida juniperinus*	HL480P	Finland, *Myllys* s.n. (H)				**KJ599551**		
T. jamesii from *Vulpicida pinastri*	HL481P	Finland, *Myllys* s.n. (H)				**KJ599552**		

Accession numbers marked in bold are new for this study. Abbreviations of secondary metabolites: ale = alectorialic acid, atr = atranorin, bar = barbatolic a., cfum = confumarprotocetraric a., cnsti = connorstictic a., fum = fumarprotocetraric a., gyr = gyrophoric a., nsti = norstictic a., pro = protocetraric a., pso = psoromic a., vul = vulpinic a., ( ) = present in small amounts.

Bryoria fremontii was used to determine whether metabolite concentration, here vulpinic acid, might correlate with photobiont phylogeny. In an earlier study, Velmala *et al.* (2009) detected only minor genetic variation within this species, prompting us to limit our mycobiont sampling to only four thalli, each representative of a single subclade. For photobiont sampling, we included an additional ten specimens, divided evenly between thalli that lack vulpinic acid (except in soralia and apothecia) and thalli in which this yellow pigment occurs throughout (= former *B. tortuosa* (G. Merr.) Brodo & D. Hawksw.; see Brodo & Hawksworth 1977; Velmala *et al.* 2009).

Photobiont ITS sequences acquired from *Bryoria* specimens were compared with 12 *Trebouxia* Puymaly sequences obtained from GenBank to find out the possible identities of photobionts of the genus *Bryoria*. We also included two *Trebouxia* sequences from the vulpinic acid-containing species *Vulpicida juniperinus* (L.) J.-E. Mattsson & M. J. Lai and *V. pinastri* (Scop.) J.-E. Mattsson & M. J. Lai; both sequences are original in this study. *Gowardia arctica* Halonen *et al.* and *Pseudephebe pubescens* (L.) M. Choisy were selected as outgroup species for the mycobiont analyses. After testing several outgroup candidates for the photobiont ITS analysis, we selected *Trebouxia impressa* Ahmadjian because it did not group among any of the ingroup taxa in our preliminary analyses. In the combined photobiont ITS and COX2 analysis, only photobiont sequences from *Bryoria* were used since there were no matching COX2 sequences available in GenBank from the *Trebouxia* sequences used as a reference in the ITS analysis. Based on the ITS photobiont analysis, we used the photobiont of *B. smithii* (Du Rietz) Brodo & D. Hawksw. as an outgroup in the combined ITS and COX2 analysis.

Molecular techniques

Total genomic DNA was extracted from dried lichen thalli using Qiagen's DNeasy Blood & Tissue Kit following the protocol described in Myllys *et al.* (2011). Both photobiont and mycobiont sequences were obtained from the same DNA extractions. A total of 162 mycobiont sequences (Table 1) were obtained from the previous studies of Velmala *et al.* (2009) and Myllys *et al.* (2011). For 18 additional specimens, three DNA regions were amplified and sequenced: 1) internal transcribed spacer regions of the nuclear ribosomal DNA (ITS), 2) partial sequence from the protein-coding gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 3) partial sequence of the small subunit of the mitochondrial ribosomal DNA (mtSSU). PCR amplification was carried out with primers described by Myllys *et al.* (2011) and under conditions as described in Velmala *et al.* (2009).

For the photobiont analyses, the internal transcribed spacer regions of the nuclear ribosomal DNA (ITS) and part of the cytochrome oxidase subunit 2 gene (COX2) were amplified and sequenced. The PCR amplification of the ITS region was carried out with primers ITS1AKL (Dahlkild *et al.* 2001) and ITS4 (White *et al.* 1990), for COX2 new primers, COXIIf2 (ttaacgcctaacgagggaac) and COXIIr (atacgaaatcccgttcctga), were designed based on ten COX2 sequences published in Fernández-Mendoza *et al.* (2011) and using the primer design software Primer3Web version 3.0.0 (Rozen & Skaletsky 2000). PCR amplification was performed in 25 μl reaction volumes using PuRe Taq Ready-To-Go PCR beads (GE Healthcare) under the following conditions for ITS: initial denaturation of 5 min at 95°C followed by five cycles of 30 s at 95°C, 30 s at 58°C and 1 min at 72°C. For the remaining 30 cycles, the annealing temperature was decreased to 56°C. PCR ended with a final extension of 7 min at 72°C. For COX2, the initial annealing temperature was 57°C, decreasing to 55°C, and the number of cycles was 35 instead of 30.

PCR products were run on a 1% agarose gel stained with ethidium bromide and visualized under UV light. Purification of the PCR products was performed using GE Healthcare's illustra GFX™ PCR DNA and Gel Band Purification Kit following the manufacturer's protocol and eluted with 30 μl of elution buffer. Sequencing was carried out as described in Myllys *et al.* (2011). The same primers used for PCR were also used for sequencing in all samples. Sequences were assembled with SeqMan II 4.00 (DNASTAR). All photobiont sequences obtained in this study were subjected to a BLAST search (Altschul *et al.* 1990) to confirm that they really were algal sequences and to find out the identity of the algal species.

Sequence alignment and phylogenetic analyses

Sequences of each DNA region were aligned with MUSCLE v3.7 (Edgar 2004), located at CSC – IT Center for Science (http://www.csc.fi/english), using default parameters. The matrices obtained were edited manually and the three mycobiont matrices were combined into a concatenated matrix in MacClade 4.08 (Maddison & Maddison 2005). From the mycobiont matrix, the hypervariable region of the mtSSU corresponding to positions 808–860 of HQ402637 GenBank accession (Table 1) was removed. The photobiont ITS and COX2 gene regions were first analyzed separately and then combined into a concatenated matrix. All photobiont and mycobiont data sets were subjected to maximum parsimony analysis and Bayesian phylogenetic analysis. Parsimony analyses were performed in TNT v1.1 (Goloboff *et al.* 2008) using a traditional search of 100 replicates with random addition of sequences and TBR branch swapping. Ten trees were saved for each replicate and gaps were treated as missing data. Node support was estimated using the bootstrapping method with 1000 replicates.

For Bayesian analyses, a suitable substitution model was selected by calculating AIC (Akaike Information Criterion) scores in jModelTest2 v2.1.1 (Guindon & Gascuel 2003; Darriba *et al.* 2012). Models with the lowest AIC scores were selected for the analyses. The substitution model of the ITS region was estimated separately for each partition. For the mycobiont, data model GTR+G was used for ITS1, ITS2, mtSSU and GAPDH gene regions and the model K80 was used for 5.8S. The model GTR+G was also used for the photobiont ITS1 and ITS2 regions, and GTR for the 5.8S. Model HKY was selected for the COX2 gene region. Bayesian analyses were then conducted for all four data sets using MrBayes v3.2.1 (Huelsenbeck & Ronquist

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2001). For the mycobiont data, two parallel runs of 10 000 000 generations were performed using four chains and sampling every 500th tree. The temperature parameter was set to 0·05. After the analysis, 5000 samples per run were discarded as burn-in. For the separate and combined analysis of photobiont ITS and COX2 data, two parallel runs of 2 000 000 generations were performed, also using four chains but sampling every 100th tree. Again, a default burn-in fraction of 25% was used after the analysis and the number of discarded samples was 5000 per run. The program Tracer v1.5 (Rambaut *et al.* 2013) was used for all four data sets to check that the runs had reached convergence.

Results

Mycobiont data

Fifty-two new sequences were generated for this study. The combined alignment of mycobiont ITS, mtSSU and GAPDH gene regions consisted of 2346 characters, of which 435 were variable and 93 parsimony-informative. The ITS alignment comprised 481 characters, of which 150 were variable and 73 parsimony-informative. The mtSSU alignment on the other hand included 927 characters, of which 67 were variable and none parsimony-informative. The GAPDH alignment was 938 characters long and consisted of 218 variable characters, of which 20 were parsimony-informative.

The parsimony analysis of combined mycobiont ITS, mtSSU and GAPDH data of 71 taxa and 2346 characters produced 7 equally parsimonious trees, of which a strict consensus tree is shown in Figure 1. All *Bryoria* sections observed in Myllys *et al.* (2011), that is, sections *Americanae* Myllys & Velmala, *Bryoria*, *Divaricatae* (DR.) Brodo & D. Hawksw., *Implexae* and *Tortuosae* (Bystr.) Brodo & D. Hawksw., also appeared as strongly supported monophyletic groups in our analysis. Within section *Implexae*, *B. glabra* is the only monophyletic species. All other taxa in the section are unresolved and are divided into two subclades, referred to here as the North American subclade and the European subclade (see also Myllys *et al.* 2011), although the latter subclade also includes some North American specimens. The Bayesian phylogeny of the combined mycobiont data is consistent with the parsimony phylogeny (Bayesian phylogeny not shown).

Photobiont data

A total of 141 new *Bryoria* photobiont sequences were produced for this study. The photobiont ITS alignment consisted of 709 characters, of which 233 were variable and 56 parsimony-informative. The combined alignment of photobiont ITS and COX2 gene regions comprised 1074 characters, of which 191 were variable and 46 parsimony-informative. The ITS portion of the alignment consisted of 685 characters, of which 186 were variable and 43 parsimony-informative, whereas the COX2 alignment was 389 characters long and had five variable characters, of which three were parsimony-informative.

The parsimony analysis of photobiont ITS data of 89 taxa and 709 characters produced 49 equally parsimonious trees, of which a strict consensus tree is shown in Figure 2. In the ITS parsimony phylogeny, seven clades with moderate to high bootstrap support are distinguished. Clade I consists of the photobionts of *B. smithii* as well as *Trebouxia asymmetrica* Friedl & Gärtner and *T. decolorans* Ahmadjian. Clade II contains photobionts of *B. bicolor* (Ehrh.) Brodo & D. Hawksw. and *B. tenuis* (Å. E. Dahl) Brodo & D. Hawksw. In Clade III are photobionts of *B. furcellata* (Fr.) Brodo & D. Hawksw., together with two *T. simplex* Tscherm.-Woess sequences obtained from *Lecanora conizaeoides* Nyl. ex Cromb. Clade IV has only two sequences: photobionts of one *B. americana* (Motyka) Holien specimen and one *B. fremontii* specimen. In Clade V are photobionts of six *B. fremontii* specimens and one *T. jamesii* (Hildreth & Ahmadjian) Gärtner sequence obtained from *Letharia lupina* Altermann & Goward ined. Clade VI consists of photobionts of eight *B. fremontii* specimens and one *T. jamesii* sequence from *Letharia vulpina* (L.) Hue. Photobionts of vulpinic acid-containing *B. fremontii* specimens did not form a distinct clade in either of the photobiont phylogenies, but instead

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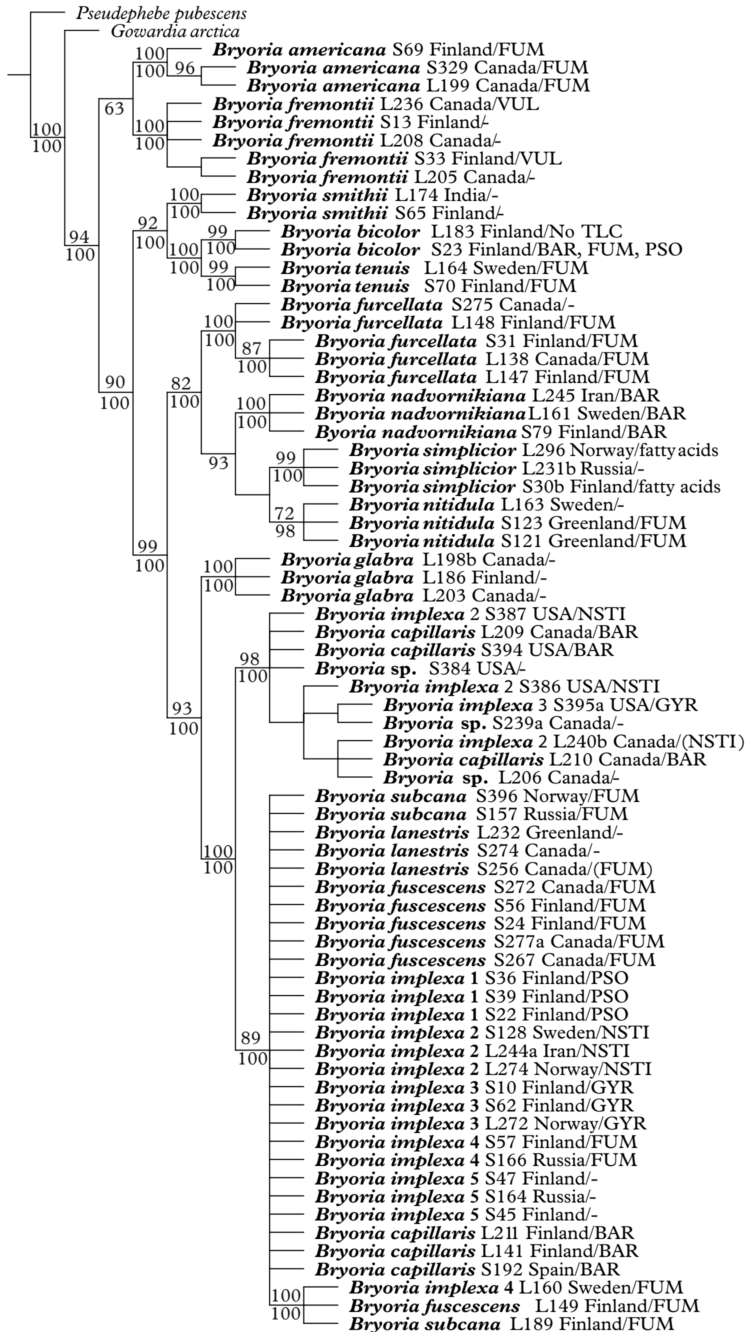

FIG. 1. Phylogenetic relationships among *Bryoria* species. Strict consensus tree obtained from ITS, mtSSU and GAPDH data. Bootstrap support values are shown above nodes and Bayesian posterior probability values are shown below nodes. Abbreviations of secondary metabolites: BAR = barbatolic acid, FUM = fumarprotocetraric a., GYR = gyrophoric a., NSTI = norstictic a., PSO = psoromic a., VUL = vulpinic a., - = no substances. Only main diagnostic secondary substances are shown.

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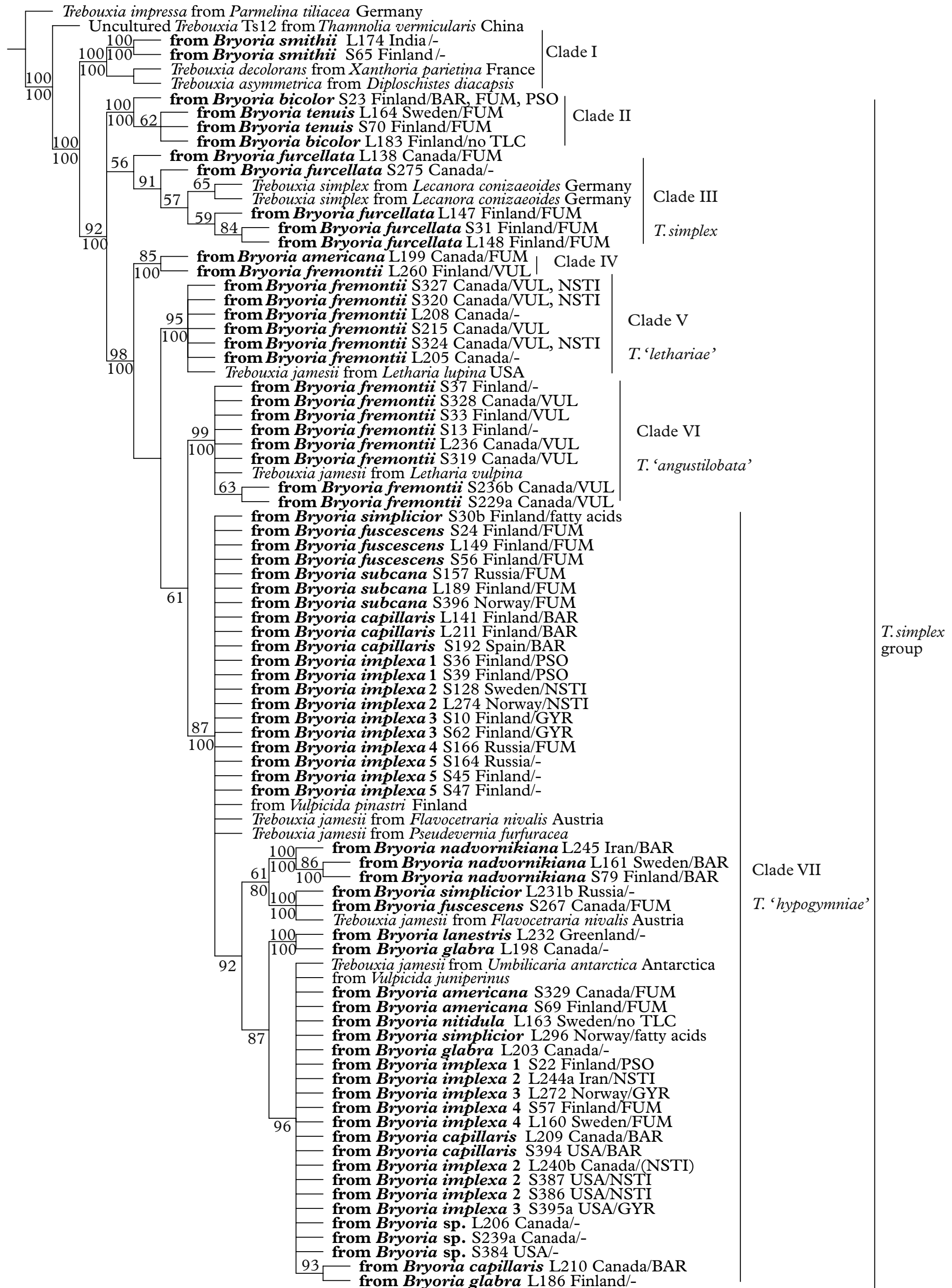

FIG. 2. Strict consensus tree of ITS data obtained from photobionts in *Bryoria* and from reference sequences obtained from GenBank. Bootstrap support values are shown above nodes and Bayesian posterior probability values are shown below nodes.

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were evenly distributed among the three clades containing *B. fremontii* specimens. Clade VII contains all photobionts of species in section *Implexae*, as well as the photobionts of *B. nadvornikiana* (Gyeln.) Brodo & D. Hawksw., *B. nitidula* (Th. Fr.) Brodo & D. Hawksw., *B. simplicior* (Vain.) Brodo & D. Hawksw. and two *B. americana* specimens. The phylogenetic relationships of photobionts inside this clade remain mostly unresolved with the exception of photobionts of *B. nadvornikiana*, which form a strongly supported small monophyletic subclade. Interestingly, all North American specimens of section *Implexae* fell into one subclade inside Clade VII. Also in Clade VII are photobiont sequences from *Flavocetraria nivalis* (L.) Kärnefelt & A. Thell, *Pseudevernia furfuracea* (L.) Zopf, *Umbilicaria antarctica* Frey & I. M. Lamb, *Vulpicida juniperinus* and *V. pinastri*. The parsimony analysis of COX2 data of 66 taxa and 389 characters produced one tree (phylogeny not shown). COX2 contained less variation than ITS, with only three parsimony-informative sites, and this was also evident in the phylogeny as only *B. nadvornikiana* distinguished as a monophyletic species.

The parsimony analysis of combined photobiont ITS and COX2 data of 75 taxa and 1074 characters produced 30 equally parsimonious trees, of which a strict consensus tree is shown in Figure 3. The combined ITS and COX2 phylogeny included only photobiont sequences from *Bryoria* but was otherwise mostly consistent with the ITS phylogeny, with the exception of *B. americana* L199 specimen and species *B. fremontii*, *B. bicolor*, *B. tenuis* and *B. furcellata*. In the ITS phylogeny, photobionts of *B. fremontii* formed three clades (Clades IV, V and VI, Fig. 2), and one of these clades (Clade IV) included the photobiont from specimen L199 of *B. americana*. However, in the combined ITS and COX2 phylogeny, photobionts of all *B. fremontii* specimens and the photobiont of *B. americana* specimen L199 grouped together, although this clade was not supported. Specimens of *B. bicolor* and *B. tenuis* formed a distinct clade in the ITS phylogeny (Clade II, Fig. 2) and grouped together with *B. fucellata* in the combined ITS and COX2 phylogeny, but this clade was also not supported. The Bayesian phylogenies of ITS, COX2 and combined ITS and COX2 data were consistent with corresponding parsimony phylogenies (Bayesian phylogenies not shown).

Discussion

Photobiont identity and selectivity

A major finding of this study is that the photobionts of all but one *Bryoria* species subjected to ITS and COX2 sequence analyses can be assigned to the *Trebouxia simplex* group [also referred to as *Trebouxia jamesii*; see Opanowicz & Grube (2004) and Doering & Piercey-Normore (2009) for discussion]. Previous studies have shown that this group consists of several phylogenetic species, many of which, however, have not been formally described due to a lack of cultured strains (Kroken & Taylor 2000; Piercey-Normore 2006; Hauck *et al.* 2007). Below we discuss the possible identities of photobionts in the genus *Bryoria* while acknowledging the taxonomic uncertainty of many of these phylogenetic species. Only in the case of *B. bicolor* and *B. tenuis* (Clade II, Fig. 2) and *B. americana* and *B. fremontii* appearing in Clade IV (Fig. 2) do the identities of the photobionts remain unclear, owing to a lack of GenBank reference sequences.

In the study of Hauck *et al.* (2007), *Lecanora conizaeoides* was found to associate with *Trebouxia simplex*. In our study, photobionts of *B. furcellata* grouped with the photobionts of *L. conizaeoides* (Clade III, Fig. 2) sequenced by Hauck *et al.* (2007), and we suggest that these most probably represent *T. simplex* as well.

Photobionts of all but one specimen of *Bryoria fremontii* grouped with either *T. jamesii* from *Letharia 'lupina'* (Clade V, Fig. 2) or with *T. jamesii* from *Letharia vulpina* (Clade VI, Fig. 2). Kroken & Taylor (2000) referred to these two algal clades as *T. jamesii 'letharii'* and *T. jamesii 'vulpinae'* respectively, and suggested they be regarded as separate phylogenetic species. Based on similarities in chloroplast morphology, the latter most likely

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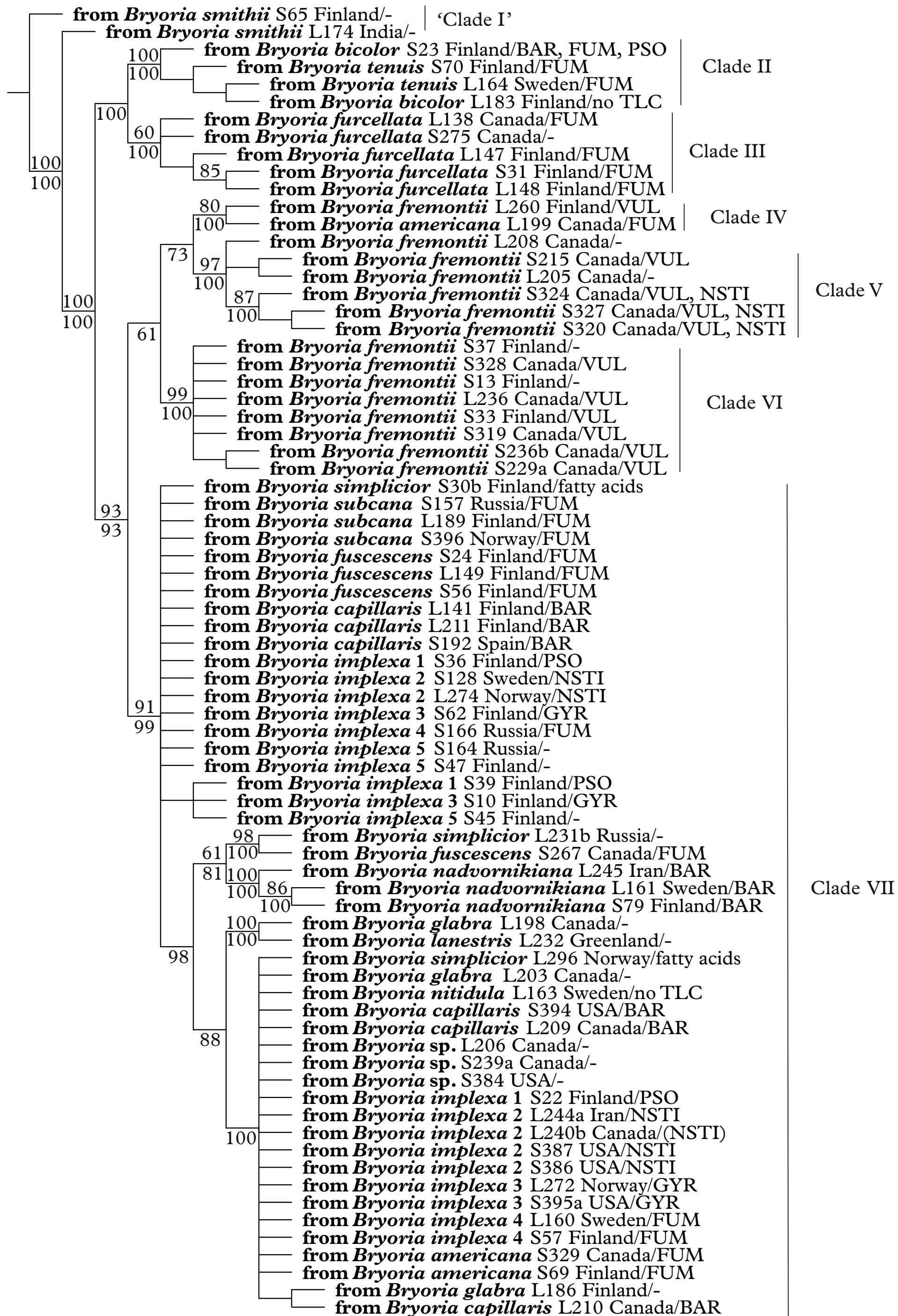

FIG. 3. Strict consensus tree of combined ITS and COX2 data obtained from photobionts in *Bryoria*. Bootstrap support values are shown above nodes and Bayesian posterior probability values are shown below nodes.

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represents *T. jamesii* subsp. *angustilobata* A. Beck. Hauck *et al.* (2007) provisionally named *T. jamesii* '*letharii*' as *T. lethariae* and proposed that the name *T. angustilobata* be used in place of *T. jamesii* subsp. *angustilobata*. However, they refrained from formally describing either species, owing to a lack of cultured strains.

All photobionts from section *Implexae*, as well as the photobionts of *B. nadvornikiana*, *B. nitidula*, *B. simplicior* and two *B. americana* specimens, are closely related to the photobionts of *Pseudevernia furfuracea*, *Umbilicaria antarctica*, *Flavocetraria nivalis*, *Vulpicida juniperinus* and *V. pinastri* (Clade VII, Fig. 2). Judging from the low level of sequence divergence, these photobionts probably represent a single algal lineage for which Hauck *et al.* (2007) introduced the name *Trebouxia hypogymniae* Hauck & Friedl ined. However, as pointed out by Hauck *et al.* (2007), a formal description of the species is not possible until the samples used in the analyses have been cultured. In the meantime, it is interesting to note phylogenetic structure within Clade VII (Fig. 2), where the photobionts of *B. nadvornikiana* formed a strongly supported subclade both in the ITS and in the combined ITS and COX2 phylogenies. In addition, the photobionts of the North American specimens were found only in one subclade.

The photobiont of *B. smithii* appears to be unique within *Bryoria* in being more closely related to *T. asymmetrica* and *T. decolorans* than to any of the lineages in the *T. simplex* group (Clade I, Fig. 2). However, the exact identity of this alga remains unsolved. *Bryoria smithii* is an oceanic species distributed in South-East Asia, the Himalayas, and central and northern Europe (Hawksworth 1972; Jørgensen 1972), with an outlier in the mountains of Hawaii (Smith 1984). In Fennoscandia, the species is rare and confined to suboceanic localities. Our sample, based on two specimens from widely disjunct portions of the species range, is consistent with the hypothesis that this species may associate with the same photobiont throughout its range. One of the reasons for the current distribution pattern of the species could be the association with a photobiont adapted to oceanic habitats. However, this needs to be investigated further with a broader sampling of specimens including photobiont sequences from other lichen species and from other areas.

Most of the species examined, which appeared as distinct species in the mycobiont phylogeny (i.e. *B. bicolor*, *B. furcellata*, *B. nadvornikiana*, *B. simplicior*, *B. smithii* and *B. tenuis*), appear to be highly selective with regard to their photobiont. Likewise, all members of section *Implexae* examined appear to associate with a single photobiont lineage. We caution, however, that these findings must be treated with care as in most cases they are based on only a small number of sequences. The only exceptions to this pattern were *B. americana* and *B. fremontii*, which associate with two and three lineages of the *T. simplex* group, respectively. Even if most *Bryoria* species appeared as highly selective towards their photobionts, few of the photobionts were found to be selective. Only *B. smithii* associated with a specific photobiont not found in other *Bryoria* species. Furthermore, in most cases *Bryoria* shared its photobionts with other genera in the *Parmeliaceae* such as *Pseudevernia*, *Flavocetraria* and *Letharia*, as well as with genera in the *Umbilicariaceae* and *Lecanoraceae*. Our visual inspection of the phylogenies revealed little evidence of parallel cladogenesis between the symbionts. The only exceptions are *B. bicolor* and *B. tenuis*, which are closely allied (Fig. 1) and most probably share the same photobiont. The recent studies of Nelsen & Gargas (2008) and Wornik & Grube (2010) found no correlation between algal and fungal phylogenies in spite of joint dispersal and concluded that sorediate species do not necessarily maintain the symbiotic partner, but may obtain their photobionts from vegetative propagules of other individuals of the same species, or even from other lichen species or from free-living algae. In contrast to these studies, our results suggest that the same photobiont lineage is maintained over consecutive generations in *Bryoria*. As already mentioned, the *Bryoria* species included in this study reproduce predominantly asexually from soredia or from thallus fragments, which

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could be one of the reasons for the high levels of fungal selectivity observed. However, it must be noted that there are also other factors beyond co-dispersal which may explain the high fungal selectivity. Irrespective of the dispersal mode, the mycobiont may achieve higher fitness by increasing specialization in the selection of a photobiont. Although production of ascomata is rare in most of the species sampled, even rare sexual reproductive events would reduce the level of selectivity that was observed in some species (*B. americana* and *B. fremontii*) in our study.

Photobiont identity and secondary chemistry

In section *Implexae*, secondary chemistry plays a major role in species delimitation and the presence of a specific secondary compound may be the only character separating one species from another. However, this chemospecies concept is controversial and has led different authors to treat these taxa at different taxonomic ranks. Brodo & Hawksworth (1977) treated *Bryoria pseudofuscescens* (which contains norstictic acid), *B. friabilis* (gyrophoric acid) and *B. implexa* (psoromic acid) as separate species, whereas Holien (1989) found little morphological variation and considered them to be conspecific. Our results are in agreement with the recent phylogenetic analysis of *Bryoria* by Myllys *et al.* (2011), and show that the genetic diversity in section *Implexae* is extremely low, with only *B. glabra* appearing as a distinct species (see Fig. 1). Instead, the results suggest that North American *B. capillaris* and *B. implexa* are genetically distinct from European *B. capillaris* and *B. implexa*, respectively. Within these two subclades genetic variation is non-existent, suggesting that all the species inside them might be conspecific. In the light of these results, it is not surprising that species from this section all associated with the same photobiont, *Trebouxia hypogymniae* Hauck & Friedl ined.

Only a few studies have examined the role of the *in vivo* photobiont in the production of lichen secondary metabolites. For instance, Blaha *et al.* (2006) examined photobiont diversity in *Lecanora rupicola* (L.) Zahlbr. and found no correlation between different chemotypes and the associated photobiont. In this study, we investigated whether chemical diversity within *Bryoria* section *Implexae* correlates with photobiont identity. According to our results, this does not seem to be the case. Otherwise, individuals with different chemistries would have formed their own separate clades in the photobiont phylogeny. Similarly, our study did not find any correlation between the presence/concentration of vulpinic acid and photobiont identity, as photobionts of *B. fremontii* specimens containing this substance did not form their own clade in the photobiont phylogeny. As discussed by Hauck *et al.* (2007), most lichen species associating with *T. simplex* and *T. hypogymniae* Hauck & Friedl ined. are found on acidic substrata. They suggested that the substratum rather than the chemistry of the lichen would explain the photobiont identity. The occurrence of the same photobiont lineages in taxonomically diverse but ecologically similar lichens has also been reported for *Asterochloris*-associating species (Peksa & Škaloud 2011).

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