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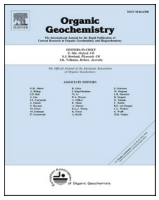

Aliphatic hydrocarbons in the lichen class Lecanoromycetes and their potential use as chemotaxonomic indicators and biomarkers

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ABSTRACT

Lichens are found in a wide range of terrestrial habitats and have important roles in terrestrial ecosystems. However, because lichens are easily decomposed and are rarely preserved as fossils, their paleoenvironmental ecology and evolutionary history remain a mystery. We performed lipid analyses on 29 lichen samples belonging to Lecanoromycetes, the largest class of lichens, from several locations in Japan, to determine their potential for use as taxonomic tools and biomarkers. We found that the lichens contained aliphatic hydrocarbons, including *n*-alkanes, alkenes, and long-chain branched alkanes, fernenes, diploptene, and hop-21-ene. Lichens with a green algal photobiont (photosynthetic symbiotic algae) contained 1,8-heptadecadiene or 6,9-heptadecadiene and 8- and 7-heptadecene, whereas lichens with cyanobacteria as a photobiont did not contain the heptadecadienes but did contain octadecene, nonadecene and nonadecadiene. These differences in characteristics could be attributed to phylogenetic differences in the photobionts that comprised the lichens, indicating that the alkene composition could be used for lichen chemotaxonomy. Although additional research is needed to confirm that this signal gets preserved in sedimentary archives, our results suggest a previously unknown origin for the C_{17}–C_{19} alkenes in sediments and imply that these components could be used to reconstruct the past composition of lichens.

1. Introduction

Lichens are obligate symbioses between fungi (mycobiont) and photosynthetic microalgae, including green algae and cyanobacteria (photobiont). Lichenization, in which fungi establish a symbiotic relationship with algae, is one of the most important fungal lifestyles. There are more than 19,000 species of lichenized fungi, accounting for one in five recognized fungal species (Feuerer and Hawksworth, 2007; Lücking et al., 2016). Approximately 85% of lichens contain green algal photobionts (chlorolichens), 10% contain cyanobacterial photobionts (cyanolichens), and approximately 4% contain both simultaneously (tripartite lichens) (Honegger, 2008). Lichens are found in a wide variety of terrestrial habitats and play important roles in terrestrial ecosystems (Elbert et al., 2012; Asplund and Wardle, 2017; Harris et al., 2018). The earliest known lichen fossils date back to the Early Devonian period (415 Ma) (Honegger et al., 2013a, 2013b), but the exact date of the first appearance of lichens is uncertain (e.g., Nelsen et al., 2020). Reports of lichen fossils are extremely rare because their remains are easily decomposed; consequently, most studies of their evolutionary history and response to the paleoenvironment have relied on a molecular clock approach (Printzen and Lumbsch, 2000; Gaya et al., 2015; Kraichak et al., 2015; Huang et al., 2019; Nelsen et al., 2020).

Several studies have attempted to reconstruct the paleoclimatic responses of lichens by employing biomarkers. Various components of lichen lipids have been reported, including *n*-alkanes, alkanols, alkanoic acids, neutral monosaccharides, depsides, depsidones, dibenzofurans, anthraquinones, carotenoids, steroids, and terpenoids (Huneck and Yoshimura, 1996). Certain compounds such as *n*-alkanes and alkanoic acids have been considered as 'lichen substances', for use in the chemical classification of lichens (e.g., Zygadlo et al., 1993; Sassaki et al., 2001; Santos et al., 2015). Several studies have reported that lichens contain abundant *n*-alkenes (Corbier and Teisseire, 1974; Gavin et al., 1978; Solberg, 1986, 1987; Ikeda et al., 2018, 2021), but little has been noted regarding their composition. Through the analytical measurement of *n*-alkanes, *n*-alkanols, and *n*-alkanoic acids in Scottish peat samples and peat-forming plants, Ficken et al. (1998) demonstrated that the lichen contribution to past biomass may have been greater than that estimated by the fossil record. Jia et al. (2008) used the composition of neutral monosaccharides in peat cores to reconstruct the variations in vegetation of the major peat-forming plants, including lichens.

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However, neutral monosaccharides are susceptible to microbial degradation (e.g., Amon et al., 2001), and there are many selective compositional changes of each monosaccharide, as indicated by the different degradation orders reported in previous studies (Hedges et al., 1985; Opsahl and Benner, 1999; Panagiotopoulos and Sempéré, 2007).

In this study, we comprehensively analyzed aliphatic compounds, especially *n*-alkenes, in samples of lichen belonging to Lecanoromycetes and examined their potential for use as biomarkers and as a chemotaxonomic tool.

2. Samples and methods

2.1. Samples

The 29 samples analyzed contained 27 species of lichens from the class Lecanoromycetes (Orders Lecanorales, 16 species; Teloschistales, one species; Caliciales, four species; Peltigerales, six species) (Table 1). These specimens were collected from several locations in Japan (Supplementary Table S1 lists the collection sites and dates). Lichen species were identified based on morphological and biochemical techniques. Chemical spot tests with paraphenylenediamine, potassium hydroxide, and calcium hypochlorite were used to determine the biochemical characteristics. A microcrystallization technique (Asahina and Shibata, 1954; Huneck and Yoshimura, 1996) was also used to identify some specimens. These characteristics were compared to the descriptions of the species provided by Yoshimura (1994) and Yamamoto (2017). The nomenclature used followed the AlgaeBase (Guiry and Guiry, 2020) for symbiotic algae, and the MycoBank (https://www.mycobank.org/) for the lichenized fungi. Following collection, the samples were stored at -30 °C in the laboratory. At the time of analysis, any non-lichen debris impurities were removed with tweezers, and the samples were washed with distilled water and lyophilized.

2.2. Extraction and fractionation

Lipid extraction and separation were performed according to Sawada et al. (2013) and Nakamura et al. (2015). To extract the free compounds, crushed samples were steeped with methanol and a mixture of methanol/dichloromethane (1:1, v/v) overnight. Tetracosane-d_{50} was added to the crude extract as an internal standard. After adding distilled water, the combined extracts were shaken and centrifuged to separate the neutral lipids into the organic solvent layer, while water-soluble compounds were separated into the aqueous layer. The organic solvent layer was siphoned off and passed through an anhydrous Na_2SO_4 column. The eluted neutral lipids were dried in a rotary evaporator, re-dissolved in *n*-hexane, and then passed through a 95% activated silica gel column. The aliphatic hydrocarbon fraction was eluted with *n*-hexane and analyzed by gas chromatography–mass spectrometry (GC–MS).

2.3. Lipid assignment and quantification

Lipids were identified by GC–MS using an Agilent 7890B GC instrument equipped with a 30 m × 0.25 mm i.d. DB-5HT fused silica column (Agilent, Santa Clara, CA, USA) directly coupled to an Agilent 5977A MSD quadruple mass spectrometer (electron voltage, 70 eV; scan range, *m/z* 50–650 in 1.3 s). The oven temperature was programmed as follows: 50 °C (held for 4 min) to 310 °C at 4 °C/min (held for 20 min). The injection temperature was 310 °C and the instrument was run in

Table 1
Mycobint and photobiont classification of lichens used in the experiment.

Samples	Classification	Photobiont		
		Type	Type (classification)	Reference
Platismatia interrupta	Lecanoromycetes Lecanorales Parmeliaceae	Green algae	*Trebouxia* sp.	[1]; [2]; [3]; [4]
Flavoparmelia caperata [a]	Lecanoromycetes Lecanorales Parmeliaceae	Green algae	*Trebouxia* sp.	[5]
Parmotrema clavuliferum	Lecanoromycetes Lecanorales Parmeliaceae	Green algae	*Trebouxia* sp.	[6]
Usnea bismolliuscula [a]	Lecanoromycetes Lecanorales Parmeliaceae	Green algae	*Trebouxia* sp.	[7]
Usnea rubrotincta	Lecanoromycetes Lecanorales Parmeliaceae	Green algae	*Trebouxia* sp.	[7]
Usnea mutabilis	Lecanoromycetes Lecanorales Parmeliaceae	Green algae	*Trebouxia* sp.	[7]
Cladonia scabriuscula [a,b]	Lecanoromycetes Lecanorales Cladoniaceae	Green algae	*Asterochloris* sp.	[8]; [9]; [10]
Cladonia vulcani [b]	Lecanoromycetes Lecanorales Cladoniaceae	Green algae	*Asterochloris* sp.	[8]; [9]; [10]
Cladonia fruticulosa	Lecanoromycetes Lecanorales Cladoniaceae	Green algae	*Asterochloris* sp.	[8]; [9]; [10]
Cladonia stellaris	Lecanoromycetes Lecanorales Cladoniaceae	Green algae	*Asterochloris* sp.	[8]; [9]; [10]
Cladonia cryptochlorophaea	Lecanoromycetes Lecanorales Cladoniaceae	Green algae	*Asterochloris* sp.	[8]; [9]; [10]
Cladonia ramulosa	Lecanoromycetes Lecanorales Cladoniaceae	Green algae	*Asterochloris* sp.	[8]; [9]; [10]
Cladonia rangiferina	Lecanoromycetes Lecanorales Cladoniaceae	Green algae	*Asterochloris* sp.	[8]; [9]; [10]
Stereocaulon japonicum	Lecanoromycetes Lecanorales Stereocaulaceae	Green algae	*Asterochloris* sp.	[11]
Ramalina sinensis	Lecanoromycetes Lecanorales Ramalinaceae	Green algae	*Trebouxia* sp.	[12]
Ramalina yasudae [a]	Lecanoromycetes Lecanorales Ramalinaceae	Green algae	*Trebouxia* sp.	[12]
Xanthoria mandschurica **1** [a,b]	Lecanoromycetes Teloschistales Teloschistaceae	Green algae	*Trebouxia* sp.	[3]; [13]
Xanthoria mandschurica **2**	Lecanoromycetes Teloschistales Teloschistaceae	Green algae	*Trebouxia* sp.	[3]; [13]
Pyxine endochrysina	Lecanoromycetes Caliciales Caliciaceae	Green algae	*Trebouxia* sp.	[3]
Heterodermia subascendens	Lecanoromycetes Caliciales Physciaceae	Green algae	*Trebouxia* sp.	[3]
Heteroder7mia obscurata	Lecanoromycetes Caliciales Physciaceae	Green algae	*Trebouxia* sp.	[14]
Anaptychia isidiza	Lecanoromycetes Caliciales Physciaceae	Green algae	*Trebouxia* sp.	[15]
Lobaria spathulata [a,c]	Lecanoromycetes Peltigerales Peltigeraceae	Green algae, Cyanobacteria	*Symbiochloris* sp., *Nostoc* sp.	[3]; [16]; [17]; [18]
Lobaria orientalis **1** [c]	Lecanoromycetes Peltigerales Peltigeraceae	Green algae, Cyanobacteria	*Symbiochloris* sp., *Nostoc* sp.	[3]; [16]; [17]; [18]
Lobaria orientalis **2** [c]	Lecanoromycetes Peltigerales Peltigeraceae	Green algae, Cyanobacteria	*Symbiochloris* sp., *Nostoc* sp.	[3]; [16]; [17]; [18]
Lobaria tuberculata [c]	Lecanoromycetes Peltigerales Peltigeraceae	Green algae, Cyanobacteria	*Symbiochloris* sp., *Nostoc* sp.	[3]; [16]; [17]; [18]
Peltigera degenii [a]	Lecanoromycetes Peltigerales Peltigeraceae	Cyanobacteria	*Nostoc* sp.	[3]; [19]; [20]; [21]
Collema furfuraceum [a]	Lecanoromycetes Peltigerales Collemataceae	Cyanobacteria	*Nostoc* sp.	[3]; [22]
Collema complanatum	Lecanoromycetes Peltigerales Collemataceae	Cyanobacteria	*Nostoc* sp.	[3]; [22]

[1]: Czeczuga & Czeczuga-Semeniuk, 2002; [2]: Miadlikowska et al., 2006; [3]: Miadlikowska et al., 2014; [4]: Palmqvist & Dahlman, 2006; [5]: Beck et al., 1998; [6]: Leavitt et al., 2015; [7]: Rafat et al., 2015; [8] Moya et al., 2015; [9]: Pino-Bodas & Stenroos, 2021; [10]: Škaloud & Peksa, 2010; [11]: Peksa & Škaloud, 2011; [12]: Voytsekhovich & Beck, 2016; [13]: Nyati et al., 2014; [14]: Xu et al., 2022; [15]: Dahlkild et al., 2001; [16]: Cornejo & Scheidegger, 2015; [17]: Dal Grande et al., 2014; [18]: Škaloud et al., 2016; [19]: Myllys et al., 2007; [20]: O'Brien et al., 2005; [21]: Rikkinen et al., 2002; [22]: Otálora et al., 2010.

[a] Ikeda et al., 2021.

[b] Ikeda et al., 2018.

[c] Green algae as the primary photobiont with localized cyanobacteria in cephalodia.

splitless mode with helium as the carrier gas. Alkanes and alkenes were quantified using a GC-2025 GC-flame ionization detector (Shimadzu, Kyoto, Japan) equipped with the same DB-5HT column as that used for the GC–MS analysis. The injection temperature, temperature program, and carrier gas used were identical to those used in the GC–MS analysis. The precision of the measurements was calculated for some samples based on triplicate or quadruplicate analyses of the same lichen samples (Supplementary Tables S2 and S3).

2.4. Statistical analysis

The statistical analyses and data visualization were performed in R version 4.2.0 (R Core Team, 2022). The optimal number of clusters was determined with the fpc package (Hennig, 2020), and a clustered heatmap was created using the pheatmap package (Kolde, 2019).

3. Results and discussion

3.1. Composition and concentrations of aliphatic compounds

3.1.1. n-Alkanes

n-Alkanes with carbon numbers ranging between C_{13} and C_{42} were detected in all lichen samples (Fig. 1, Fig. S1, Table 2, Table S2). The average carbon preference index (CPI) of the lichen samples was 11.2, with an odd carbon number dominance (Table 2). However, the carbon number of the most prominent *n*-alkane varied (Fig. 2, Fig. S2). In some samples, medium long-chain *n*-alkanes (C_{21}–C_{23}) were predominant, whereas a bimodal distribution between medium and long-chain *n*-alkanes ($\geq C_{24}$) was observed in others. Among the short-chain *n*-alkanes, there were also samples with a prominent *n*-C_{17} peak. The *n*-C_{17} alkane was frequently detected in the cyanobacteria symbiotic genera *Lobaria*, *Peltigera*, and *Collema*, which may be attributed to the symbiotic alga *Nostoc* sp. Many cyanobacteria produce the *n*-C_{17} alkane (Gelpi et al., 1970; Ladygina et al., 2006; Coates et al., 2014), and particularly the genus *Nostoc* (e.g., Liu et al., 2013; Coates et al., 2014). Based on

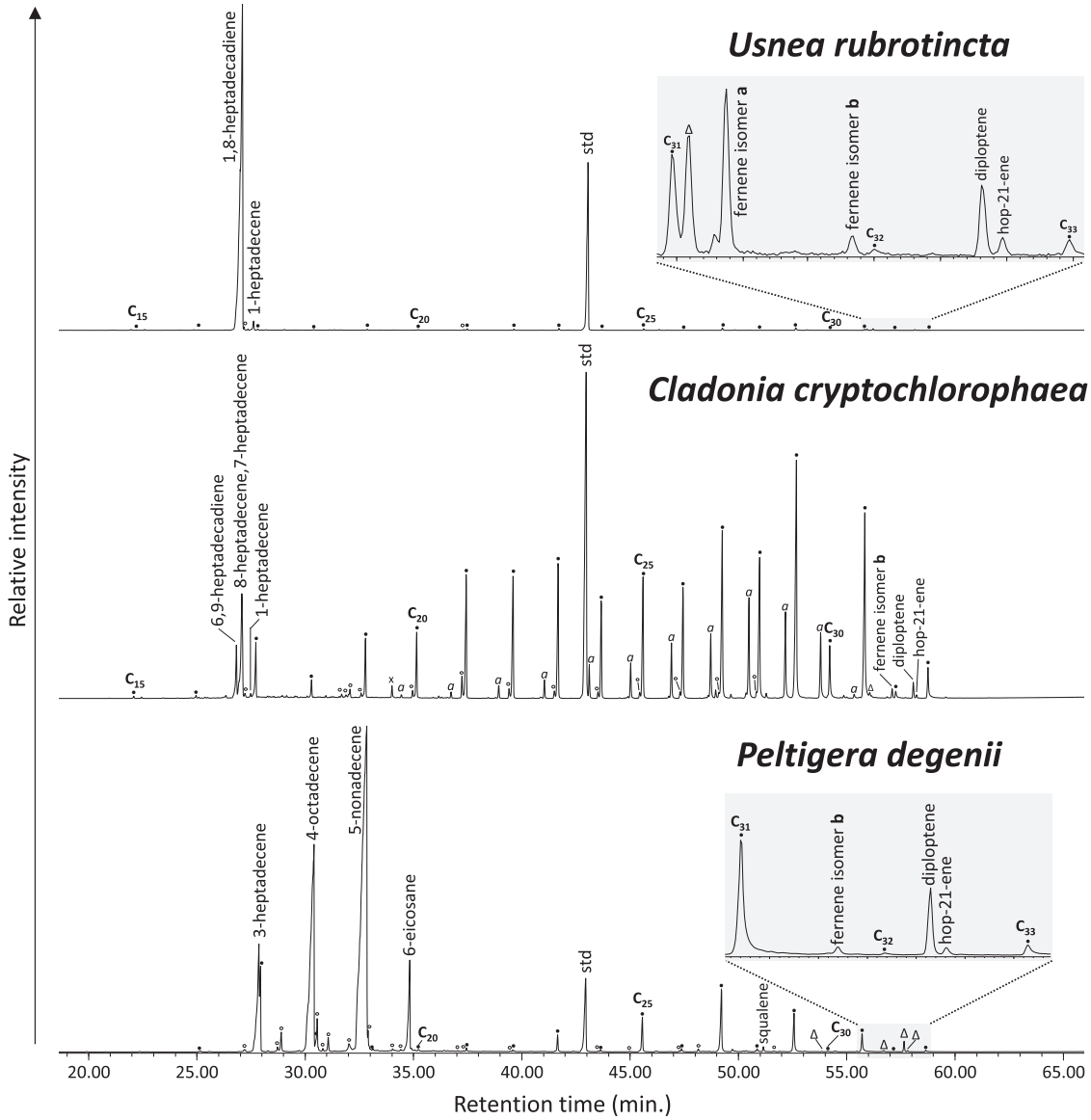

Fig. 1. Total ion chromatograms showing the distribution of aliphatic hydrocarbons detected in *Usnea rubrotincta*, *Cladonia cryptochlorophaea*, and *Peltigera degenii* (after Ikeda et al., 2021). std, standard (tetracosane-d_{50}); •, *n*-alkanes; ○, *n*-alkenes with unknown double bond positions; *a*, *anteiso*-alkanes; x, contamination; Δ, hopanoid.

Table 2
Compositions and concentrations of aliphatic hydrocarbons in lichen samples.

Samples	*n*-Alkane conc. (μg/g dry wt.)	*n*-Alkane ACL	CPI (24–32)	range	Alkene conc. (μg/g dry wt.)	Alkene main components	Branched alkane *anteiso*-alkanes	Squalene	compound **c**
Platismatia interrupta	40.75	25.93	13.07	C_{15}-C_{33}	45.51	1,8-$C_{17:2}$	n.d.		
Flavoparmelia caperata [a]	12.61	27.69	9.16	C_{15}-C_{33}	73.37	1,8-$C_{17:2}$	n.d.	x	x
Parmotrema clavuliferum	5.71	27.88	10.00	C_{17}-C_{31}	60.19	1,8-$C_{17:2}$	n.d.	x	xx
Usnea bismolliuscula [a]	4.73	28.38	6.61	C_{16}-C_{33}	62.4	1,8-$C_{17:2}$	n.d.	x	x
Usnea rubrotincta	2.05	26.96	6.32	C_{13}-C_{33}	74.27	1,8-$C_{17:2}$	n.d.		
Usnea mutabilis	3.02	26.16	4.61	C_{13}-C_{33}	102.84	1,8-$C_{17:2}$	n.d.		
Cladonia scabriuscula [a,b]	19.77	28.51	4.67	C_{14}-C_{33}	23.52	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	C_{28}-C_{30}	x	
Cladonia vulcani [b]	5.55	26.80	8.50	C_{14}-C_{31}	33.82	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	n.d.	x	
Cladonia fruticulosa	31.74	27.80	3.44	C_{14}-C_{36}	20.87	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	C_{26}-C_{30}		
Cladonia stellaris	12.4	30.07	9.86	C_{14}-C_{33}	32.37	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	C_{25}-C_{30}		
Cladonia cryptochlorophaea	94.01	28.56	2.49	C_{14}-C_{33}	15.07	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	C_{20}-C_{31}		
Cladonia ramulosa	40.62	27.43	2.06	C_{14}-C_{33}	18.69	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	C_{20}-C_{31}		
Cladonia rangiferina	29.14	29.71	10.00	C_{15}-C_{33}	31.51	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	C_{22}-C_{30}		
Stereocaulon japonicum	74.2	29.09	7.57	C_{14}-C_{33}	37.31	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	C_{28}, C_{30}	x	x
Ramalina sinensis	29.83	27.38	14.84	C_{16}-C_{33}	18.14	1,8-$C_{17:2}$	n.d.	x	xx
Ramalina yasudae [a]	6.92	26.88	10.32	C_{16}-C_{31}	32.18	1,8-$C_{17:2}$	C_{28}	x	x
Xanthoria mandschurica **1** [a,b]	25.59	26.57	3.56	C_{14}-C_{42}	42.29	1,8-$C_{17:2}$	n.d.	x	
Xanthoria mandschurica **2**	66.84	23.85	3.38	C_{14}-C_{33}	159.67	1,8-$C_{17:2}$	n.d.		
Pyxine endochrysina	74.61	29.27	9.17	C_{13}-C_{33}	44.95	1,8-$C_{17:2}$	C_{28}, C_{30}		x
Heterodermia subascendens	60.05	28.55	11.69	C_{15}-C_{33}	31.22	1,8-$C_{17:2}$	n.d.	x	
Heterodermia obscurata	14.12	26.61	25.69	C_{16}-C_{31}	34.85	1,8-$C_{17:2}$	n.d.	x	
Anaptychia isidiza	45.39	28.96	5.08	C_{13}-C_{33}	22.95	1,8-$C_{17:2}$	n.d.		
Lobaria spathulata [a]	21.87	26.39	11.35	C_{15}-C_{31}	94.49	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$, 5-$C_{19:1}$	n.d.	x	xx
Lobaria orientalis **1**	15.67	23.00	NA	C_{14}-C_{23}	84.6	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	n.d.		xx
Lobaria orientalis **2**	9.86	27.71	13.27	C_{14}-C_{33}	41.95	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	n.d.		xx
Lobaria tuberculata	18.24	27.38	4.60	C_{15}-C_{33}	75.14	6,9-$C_{17:2}$, 8-, 7-$C_{17:1}$	n.d.		xx

(*continued on next page*)

Table 2 (*continued*)

Samples	*n*-Alkane conc. (μg/g dry wt.)	*n*-Alkane ACL	CPI (24–32)	range	Alkene conc. (μg/g dry wt.)	Alkene main components	Branched alkane *anteiso*-alkanes	Squalene	compound c
Peltigera degenii [a]	65.47	27.26	23.08	C_{15}-C_{33}	307.79	4-$C_{18:1}$, 5-$C_{19:1}$	n.d.	x	
Collema furfuraceum [a]	243.77	28.96	8.54	C_{13}-C_{33}	417.74	1-$C_{18:1}$	n.d.		
Collema complanatum	199.35	25.34	11.75	C_{15}-C_{29}	646.17	1-$C_{18:1}$, $C_{19:2}$	C_{31}	x	

ACL =(23[C_{23}] + 25[C_{25}] + 27[C_{27}] + 29[C_{29}] + 31[C_{31}] + 33[C_{33}])/([C_{23}] + [C_{25}] + [C_{27}] + [C_{29}] + [C_{31}] + [C_{33}]) [C_x]: concentrations of *n*-C_x alkanes (Poynter & Eglinton, 1990; Zhang et al., 2004)
CPI = 2([C_{25}] + [C_{27}] + [C_{29}] + [C_{31}])/([C_{24}] + [C_{26}] + [C_{28}] + [C_{30}]) + ([C_{26}] + [C_{28}] + [C_{30}] + [C_{32}])], [C_x]: concentrations of *n*-C_x alkanes (Bray & Evans, 1961)
x: detected; xx: abundant.
[a] Ikeda et al., 2021.
[b] Ikeda et al., 2018.

separate cultures of the photobiont and mycobiont of *Xanthoria parietina*, Torres et al. (2003) reported that the medium- to long-chain *n*-alkanes are primarily derived from fungi, with the *n*-C_{17} alkane content being insignificant. The composition of short-chain *n*-alkanes in lichens is primarily influenced by the differences in photobiont species. In our samples, the genera *Peltigera* and *Collema*, which contain cyanobacteria as their exclusive photosymbiont, had higher relative abundances of the C_{17} *n*-alkane compared to lichen from the genus *Lobaria*, which has green algae as its primary photosymbiont and secondary photobiont cyanobacteria restricted to small structures (cephalodia) that occur sporadically within their tissues. These results suggest that these structures were introduced by the symbiotic *Nostoc* sp.

The differences in the distribution of long-chain *n*-alkanes may be influenced not only by mycobionts but also by environmental factors. According to Huang et al. (2012), the $\delta^{13}C$ values of *n*-alkanes in some lichen samples from Dajiuhu and Qizimei, China, indicated that the > C_{23} *n*-alkane homologs were derived from mycobionts, with a small contribution from photobionts. They also demonstrated that lichen-derived *n*-alkanes exhibited two distinct patterns depending on their environment, either a predominance of *n*-C_{29} alkanes or a bimodal distribution of *n*-C_{23} and *n*-C_{29} alkanes. Similarly, Piervittori et al. (1996) reported that the *n*-alkane composition of lichens varied depending on the environmental variability. In our study, differing distribution patterns were observed among samples collected at the same time and location, indicating that differences in multiple parameters, such as interspecific chemotaxonomy and the microenvironment, may influence the *n*-alkane composition.

3.1.2. *n-Alkenes*

In our study, lichens were found to contain C_{16}–C_{29} alkenes (Fig. 1, Fig. S1, Tables 2 and S3). The structural identification of the major alkenes was previously addressed by Ikeda et al. (2021). In most samples, 1,8-heptadecadiene or 6,9-heptadecadiene and 8- and 7-heptadecene were the most prominent alkenes in the aliphatic fraction (Fig. 1, Fig. S1, Table 2). In contrast, 4-octadecene and 5-nonadecene were the most abundant alkenes in the genus *Peltigera*, while 1-octadecene and the $C_{19:2}$ alkene were the most abundant alkenes in the genus *Collema*. Although there are few reports of alkenes in lichens, 1,8-heptadecadiene has been widely detected in the Parmeliaceae species *Evernia prunastri* and *Cetraria islandica* (Corbier and Teisseire, 1974; Gavin et al., 1978; Solberg, 1986). Solberg (1987) additionally detected heptadecadiene and heptadecene in four Parmeliaceae lichens: *Cetraria delisei*, *Lobaria pulmonaria*, *Stereocaulon tomentosum*, and *Usnea hirta*. As discussed in Section 3.1.1, the *n*-alkane compositions of lichens are susceptible to environmental influences. Similarly, a clear seasonal variation in the fatty acid composition of lichens has also been reported (e.g., Dembitsky et al., 1994a, b; Piervittori et al., 1995). In our study, the heptadecadiene and heptadecene compositions in *Xanthoria mandschurica* and *Lobaria orientalis* samples collected at two different sites were similar, although the concentrations of these compounds differed substantially between the two sites. These results indicate that the alkene composition of lichens may be more affected by taxonomic variations than environmental effects.

3.1.3. *Branched alkanes*

Long-chain (C_{20}–C_{31}) *anteiso*-(3-methyl)-alkanes were identified in several lichens (Fig. 1, Fig. S1, Fig. S3, Table 2). The *anteiso*-alkanes were most prevalent in the genus *Cladonia*, while *Stereocaulon japonicum*, *Ramalina yasudae*, *Pyxine endochrysina*, and *Collema complanatum* also contained *anteiso*-alkanes with various chain lengths. Long-chain *anteiso*-alkanes have previously been detected only in the lichens *Ramalina intermediella* and *Siphula ceratites* (Gaskell et al., 1973; Huang et al., 2012). We found a wider occurrence of the compounds among seven species of the genus *Cladonia*. Huang et al. (2012) reported that *R. intermediella* collected from Qizimei Mountain, China, contained C_{24}–C_{32} *anteiso*-alkanes and C_{27}, C_{29}, and C_{31} *iso*-alkanes. In addition,

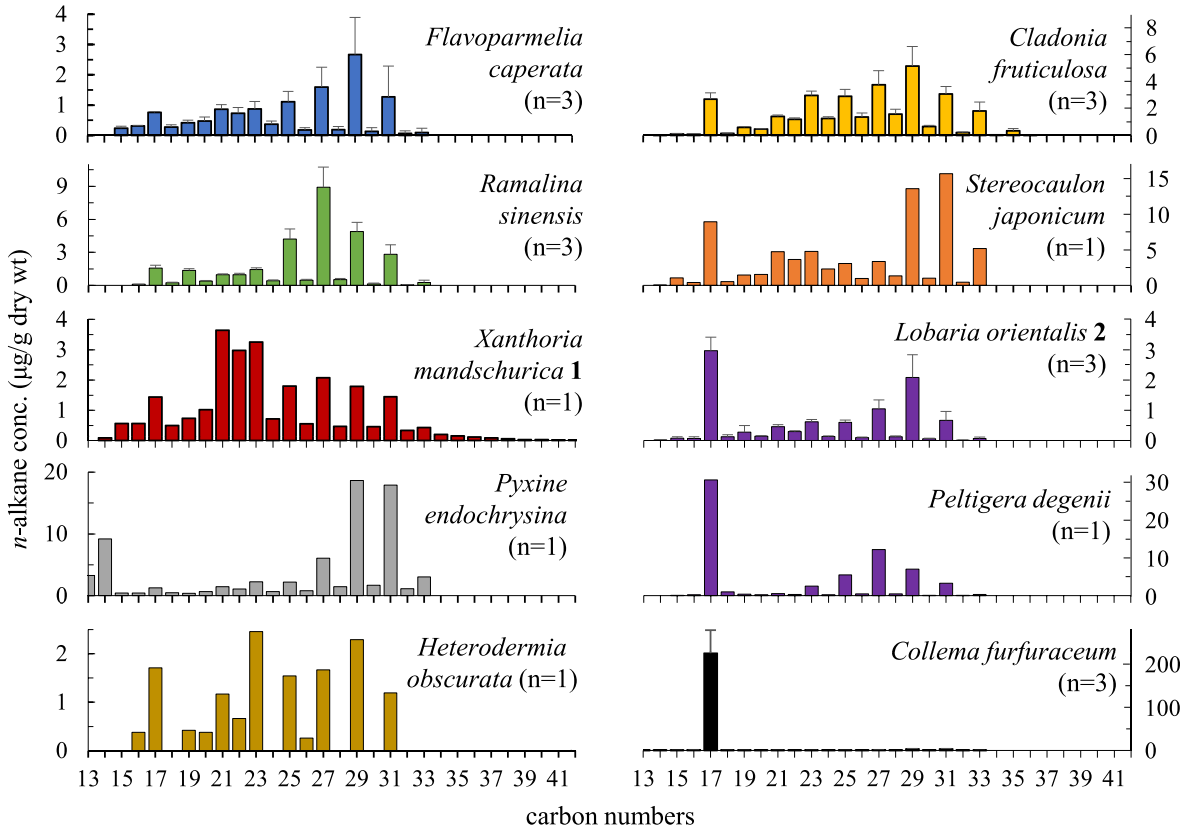

Fig. 2. *n*-Alkane distributions in lichens showing the averaged distribution values with the mean standard deviation of each sample. See also Fig. S2 and Table S2 for all data.

they also detected long-chain branched alkanes in other lichens from Qizimei, but neither *anteiso*-alkanes nor *iso*-alkanes were detected in *R. intermediella* collected from the Dajiuhu wetland, China. The previous findings suggest that these branched alkanes may be produced under specific environmental conditions, but our findings suggest that the influence of species variations rather than regional (environmental) differences may be more strongly associated with *anteiso*-alkane production. Long-chain *anteiso*-alkanes and *iso*-(2-methyl) alkanes have been detected in lacustrine sediments (Fukushima et al., 1996, 2005), Antarctic rock (Matsumoto et al., 1992), coal and ancient sediments (Chaffee et al., 1986; Summons et al., 1988; Cheng et al., 2019), insect wax (Blomquist and Kearney, 1976; Nelson et al., 1981; Bernier et al., 1998), higher plants (Maffei, 1994; Rogge et al., 1994; Kavouras et al., 1998; Reddy et al., 2000; Grice et al., 2008; Pautler et al., 2010; Huang et al., 2011; He et al., 2015a), urban aerosol (Rogge et al., 1994; Kavouras et al., 1998; Bi et al., 2005), and modern and Holocene microorganism mats (Shiea et al., 1990; Kenig et al., 1995; He et al., 2015b).

Since the 2000s, attention has focused on the role and variety of a third symbiont, the internal bacterial community, within lichen symbioses (e.g., Cardinale et al., 2006; Grube and Berg, 2009). Bacteria are typical producers of *iso*- and *anteiso*-fatty acids (Kaneda, 1991), and it is possible that specific bacteria or bacterial symbioses synthesize these long-chain *anteiso*-alkanes in lichens. The origin of long-chain *anteiso*-alkanes is unclear, but some higher plants also produce them (e.g., Grice et al., 2008). Remarkably, Matsumoto et al. (1992) detected C_{20}–C_{30} *anteiso*-alkanes in lichen-dominated microbial communities, which were derived from unidentified heterotrophic bacteria or symbiotic processes in distinct microbial communities, growing on Antarctic rocks. Alphaproteobacteria comprise the majority of the internal bacterial communities in lichens (Cardinale et al., 2006, 2008; Grube and Berg, 2009; Bates et al., 2011; Pankratov, 2018). In our study, *Cladonia* lichens were the most prominent producers of the long-chain *anteiso*-alkanes, while some of the other lichens contained these compounds as a minor constituent. It is therefore possible that internal bacterial communities in lichens are responsible for the production of long-chain *anteiso*-alkanes, although the relationship of these bacteria to the mycobionts or photobionts is still unknown.

Other branched alkanes, a squalene, and a compound **c**, were detected in some samples. Solberg (1987) reported the detection of squalene in several lichens, as well as a prominent polyunsaturated branched hydrocarbon in *L. pulmonaria* with an estimated C_{27} homologue. We tentatively identified compound **c** (Fig. S1, Fig. S3, Table 2) as a polyunsaturated compound with a similar cleavage pattern to the C_{27} polyunsaturated branched hydrocarbon reported by Solberg (1987). As in Solberg (1987), this compound was found to be prominent in the genus *Lobaria*, and it was also detected in some other lichen species (Fig. S1, Table 2).

3.1.4. Other apolar compounds

A series of hopanoids was detected in the lichen samples (Fig. 1, Fig. S1, Fig. S3, Table 2). Diploptene and its related compound hop-21-ene have been identified in soil, peat, lake, and marine sediments, and are produced by various types of bacteria, including cyanobacteria (Brassell et al., 1980; Brassell and Eglinton, 1981; Volkman et al., 1986; Venkatesan, 1988; van Winden et al., 2012; Méjanelle et al., 2017). This compound has also been found in ferns and mosses (Ageta and Arai, 1983; Toyota et al., 1998; Huang et al., 2010; Li et al., 2022). Li et al. (2022) reported that the diploptene may be provided by the coexisting heterotrophic bacteria, methanotrophs, or methylotrophs on the basis of the carbon isotope ratios of the diploptene and long-chain *n*-alkanes of the epiphytic bryophytes. It is possible that the lichen symbiotic bacteria are involved in the production of hopanoids because these compounds have been detected prominently not only in cyanolichens but also in

chlorolichens.

Fernenes were also detected as a major terpenoid in all lichen samples (Fig. 1, Fig. S1). All samples contained varying amounts of two fernene isomers, and some samples contained a trace amount of fernadiene, but with a low reliability of identification (Fig. S3). Fernene has been found in terrestrial plants, such as ferns (Ageta and Arai, 1983), gymnosperm *Podocarpus* species (Silva et al., 1972), and extinct pteridosperms (seed ferns) (Paull et al., 1998). However, some bacteria also produce these compounds (Volkman et al., 1986; Douka et al., 2001). It was therefore presumed that the internal bacterial communities in lichens synthesized the fernene compounds as well as diploptene.

3.2. Taxonomic variability of aliphatic components

Variations in the photobionts, rather than the mycobionts, were likely responsible for many of the differences in the aliphatic hydrocarbon compositions. The predominant alkene in species belonging to the orders Lecanorales, Caliciales, and Teloschistales was 1,8-heptadecadiene (with the exception of the families Cladoniaceae and Stereocaulaceae). Furthermore, 6,9-heptadecadiene was abundant in species belonging to the families Cladoniaceae and Stereocaulaceae in the order Lecanorales, and the genus *Lobaria* in the family Peltigeraceae (Peltigerales). These 6,9-heptadecadiene-producing species were also distinguished by their high concentrations of 8- and 7-heptadecenes. In contrast, the species belonging to Collemataceae and *Peltigera* in the family Peltigeraceae (Peltigerales) were distinguished by their high abundances of C_{18} and C_{19} alkenes and lack of heptadecadienes. Biosynthetic pathways that produce C_{17}–C_{21} alkenes are present in cyanobacteria and eukaryotic microalgae (Coates et al., 2014; Sorigué et al., 2016). Comparing the alkene composition of lichens with the photobiont taxonomies revealed that the C_{17} alkadienes were abundant in lichens with only green algae photobionts, whereas the C_{18} and C_{19} alkenes were more prevalent in lichens with only cyanobacterial photobionts. *Lobaria* species, which can harbor both green algae and cyanobacteria, exhibited a combination of the characteristics of both groups, containing both the C_{17} alkadiene and C_{19} alkene (5-nonadecene). It was therefore presumed that the differences in alkene composition were attributable to taxonomic variations among symbiotic algae. Furthermore, although the photobiont in the majority of chlorolichens was *Trebouxia*, *Asterochloris* sp. was the photobiont of *Cladonia* and *Stereocaulon* species (Škaloud and Peksa, 2010; Peksa and Škaloud, 2011; Moya et al., 2015; Pino-Bodas and Stenroos, 2021). We therefore hypothesized that the 1,8-heptadecadiene was derived from *Trebouxia*, and the 6,9-heptadecadiene, and 8- and 7-heptadecenes were derived from *Asterochloris*. Previous studies (Dembitsky et al., 1994a, b; Piervittori et al., 1995, 1996; Reis et al., 2005) have found that seasonality and habitats could influence the alkanoic acid and *n*-alkane composition of lichen lipids. However, it is unlikely that the alkene composition varied significantly due to environmental conditions, although our samples were collected from an uncontrolled environment.

We performed a cluster analysis to determine whether the composition of lichen aliphatic hydrocarbons could be used for lichen chemotaxonomy. The cluster analysis using only the *n*-alkene composition, or *n*-alkane and *n*-alkene composition, of each sample, resulted in clusters that reflected the differences among symbiotic algae (Fig. 3, Fig. S5). In contrast, the cluster analysis using the *n*-alkane composition of each sample did not permit a good interpretation of the cohesiveness of each cluster (Fig. S4). The major difference between the two clusters

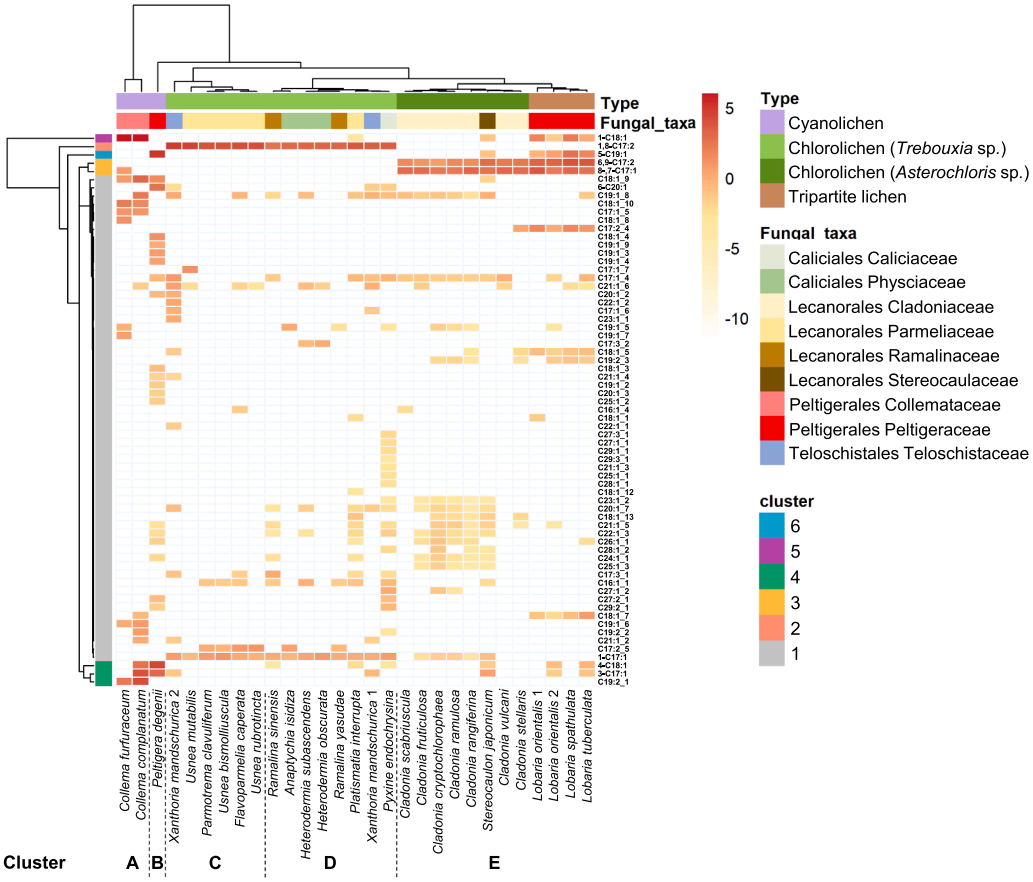

Fig. 3. Heatmap of the $\log_e$-transformed alkene concentration in lichens. Species (columns) and alkenes (rows) are clustered based on the Euclidean distance and Ward D2 minimum variance clustering. For convenience, $\log_e(0)$, i.e., not detected, is indicated on the heatmap with a value one order of magnitude smaller than the smallest value.

of lichens with only *Trebouxia* as a symbiont may be due to the 1,8-heptadecadiene concentration. Cluster 'C' had high concentrations of 1,8-heptadecadiene (145.74–56.68 μg/g), whereas Cluster 'D' had relatively low concentrations (41.93–14.81 μg/g), indicating a difference between these two clusters. The amounts of the alkene produced by the mycobiont may have been influenced by other factors, given that two *X. mandschurica* samples from different collection sites were classified into distinct clusters despite containing identical symbiotic algae. The presence or absence of the two heptadecadienes and the major C_{17}–C_{19} alkenes was a major cause of the differences between these clusters. Consequently, the differences in the alkene composition of the lichen samples was the primary factor reflecting the differences in lichen taxa, particularly the photobiont, rather than environmental factors.

3.3. Potential biomarkers of lichen

Huang et al. (2012) proposed that long-chain *anteiso*-alkanes could be used as a biomarker for lichens. We further confirmed the presence of long-chain *anteiso*-alkanes in lichens. These compounds were characteristic for some lichens, especially the genus *Cladonia*, which is an important lichen in polar peatlands and tundra regions (e.g., Payette and Delwaide, 2018), and could be used as a biomarker to evaluate lichen assemblages in peatlands and tundra. Our data showed that chlorolichens produce a substantial amount of alkadiene. Numerous studies have used the alkenes, particularly long-chain alkenes ($>C_{20}$), preserved in sediments as biomarkers of terrestrial higher plants or algae for paleoenvironmental reconstructions (Matsumoto et al., 1990; Jaffé and Hausmann, 1995; Zhang et al., 2004, 2007; Theissen et al., 2005; de Mesmay et al., 2007; van Bree et al., 2014). In contrast, the origin of short- to medium-chain alkenes has not been well documented (Matsumoto et al., 1990; Cardoso et al., 1983; Cranwell et al., 1987; Yongdong et al., 2015; Kaiser et al., 2016). There are few reports of the alkene composition of lichens, but as described in Section 3.1.2, lichens with green algae commonly produce heptadecadiene. In addition, Matsumoto et al. (1992) discovered heptadecadiene in Antarctic rocks colonized by living and fossil cryptoendolithic lichens and fungi. Because heptadecadiene has been detected prominently in lichen in some studies (Corbier and Teisseire, 1974; Gavin et al., 1978; Solberg, 1986, 1987), we propose that chlorolichens universally produce heptadecadienes.

We assumed that the alkenes were produced in lichens by an algal photobiont or as a result of symbiosis with certain species of photobiont because the alkene composition was attributed to phylogenetic differences in the symbiotic algae. Green algae that are symbiotic with lichens, such as *Trebouxia* and *Asterochloris*, have adapted to the terrestrial environment and can occupy a wide variety of habitats as a result of their symbiosis with lichens. Laboratory experiments have shown that these algae have a heterotrophic tendency toward fungi (Ahmadjian, 1993). These green algae, which make up the majority of lichens, are rarely found in nature and are thought to have escaped from damaged lichen thalli (Ahmadjian, 1988). In conjunction with our results, these compounds may serve not only as chemotaxonomic markers of lichens, but also as potential biomarkers in environments and sediments. Indeed, the detection of 6,9-heptadecadiene in boreal peat (Faubert et al., 2011) and long-chain *anteiso*-alkanes in peat samples (Huang et al., 2014) suggests the promise of these compounds as valuable biomarkers in environments where lichens have a significant impact. However, further research is needed to demonstrate fully their utility for correlating lichen inputs with these compounds in sediments.

4. Conclusions

We performed lipid analyses on 29 samples of lichen belonging to the order Lecanorales collected in Japan. The samples contained alkenes, branched alkanes, and hopanoids in addition to *n*-alkanes. Alkenes were widely detected in all samples, indicating that their composition may have been strongly influenced by the differences in the photosynthetic organisms that make up the lichens. Although there are a limited number of alkene compositions reported for lichens, the compositions identified here were similar to those reported previously, suggesting that these compounds are common to many lichens. The feasibility of using these compounds as biomarkers in sediments warrants further investigation, but they may be useful to trace lichen fingerprints, especially in peat and boreal sediments with high lichen contributions. In addition, the origin of short- to medium-chain homologues in sediments has not been well documented, but our study provides an example of the origin of these compounds and presents a potential new biomarker for determining the past constituents of lichens.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2023.104588.

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