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Multifaceted characterization of a *Lemanea fluviatilis* population (Batrachospermales, Rhodophyta) from a glacial stream in the south–eastern Alps

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Abstract: The aim of this study was a combined and multifaceted characterization (morphological, molecular, lipid, pigment, and ecological data) of a *Lemanea* (freshwater red alga) population from the south–eastern Alps, exploring its adaptive strategies to the montane habitat, (turbulent, very–cold glacial stream with extremely low–conductivity). Although the thalli were small (only up to 1 cm), the morphology was within the current circumscription of *Lemanea fluviatilis*. The molecular data placed this population within a clade of specimens identified as *L. fluviatilis* and *L. fucina*. This *L. fluviatilis* population was determined to possess lipid classes, especially phosphatidylcholine and monogalactosyldiacylglycerol with high unsaturation index (UI) and long acyl chains, which are typical adaptations for maintaining adequate membrane fluidity and consequently all the metabolic processes associated to the plasma membrane. The carotenoids profile revealed that, besides α /β–carotene, there are significant amounts of zeaxanthin and lutein. This study further demonstrated that red algae are a rich source of important food web ω–3 fatty acids and may play an important role in the diets of grazers. *L. fluviatilis* is reported from one of the highest elevations (2,170 m a.s.l.) known for the genus *Lemanea* and this species. This study confirms the presence of *L. fluviatilis* in a cold, unpolluted, turbulent stream and this type of stream may be its preferred habitat.

Key words: *Lemanea fluviatilis*, freshwater, red alga, glacial stream, pigment analysis, lipidomics, adaptive mechanisms

Introduction

Within the freshwater order Batrachospermales, there are four genera, *Lemanea, Paralemanea, Psilosiphon* and *Petrohua*, with a unique pseudoparenchymatous tubular gametophytic thallus construction (Entwisle et al. 2009). This thallus morphology appears to have evolved three times in this order with *Lemanea* and *Paralemanea* being sister taxa and both *Psilosiphon* and *Petrohua* being distantly related in other clades of the Batrachospermales phylogeny (Vis et al. 2007). The genus *Lemanea* is easily distinguished from these other three pseudoparenchymatous tubular genera by the following suite of characters: a central axis lacking internal cortical filaments, T– /or L–shaped ray cells closely abutting the outer cortex, and spermatangia present in discrete patches on the nodes (Vis & Sheath 1992). *Lemanea* appears to be widely distributed in boreal and temperate regions of North America (e.g., Vis & Sheath 1992), and in fast–flowing streams in Europe (e.g., Eloranta & Kwandrans 2007; Kučera et al. 2008; Eloranta et al. 2011, 2016). It has been frequently reported from India, but appears to be localized to Manipur state (Ganesan et al. 2015). In addition, this genus has been infrequently reported from China (Xie et al. 2004). *L. fluviatilis* has been frequently reported from Europe, North America and India (Vis & Sheath 1992; Eloranta et al. 2011; Ganesan et al. 2015 and the references therein).

Lemanea is known from a wide range of stream habitats from lowlands to mountains in both North America and Europe (Vis & Sheath 1992; Kučera

et al. 2008). However, this genus appears to be more prevalent in cooler, faster flowing streams (Sheath & Hambrook 1990; Eloranta & Kwandrans 2007; Kučera et al. 2008; Eloranta et al. 2016). There is scatter data in the literature regarding stream chemistry including nutrients (Sheath & Hambrook 1990 and the references therein; Vis & Sheath 1992; Carmona et al. 2011. In Austria, it is considered indicative of high–altitude streams with low nutrient content (Pipp & Rott 1994; Rott et al. 1999). Likewise, *Lemanea* species have been incorporated as an indicator of low nutrient streams in other European countries (e.g., Rott & Schneider 2014).

Previous research on *Lemanea* has covered a variety of applied and basic topics. There have been a few applied studies of *Lemanea* in India ranging from use as an herbal remedy, diabetes treatment and biofuel production (Ganesan et al. 2015 table 2 and the references therein). Much of the basic *Lemanea* research has focused on the systematics, biogeography and potential use in biomonitoring (Harding & Whitton 1981; Vis & Sheath 1992; Eloranta et al. 2011). There have been a few studies of the ecophysiology of this genus primarily relating temperature and current velocity to chlorophyll a, dry mass and carbon assimilation as measures of vigor and growth (Thirb & Benson–Evans 1982, 1984, 1985). To our knowledge, there have been no studies regarding the lipid content of this taxon and how this may relate to thermal niche. However, the effects of temperature on fatty acids composition patterns have been addressed by some studies on micro– (e.g., Fuschino et al. 2011; Flaim et al. 2012; Leblond et al. 2015) and macroalgae (e.g., Becker et al. 2010).

The primary goal of the present study was to characterize a high–mountain population of *Lemanea* from a glacial stream in the south–eastern Alps by means of a multifaceted approach (genetics, morphological, membrane lipid and pigment analyses, and ecology), and relating these to potential adaptive mechanisms of this taxon to its glacial–stream habitat.

Materials and Methods

Study site. The *Lemanea* specimens were sampled at an elevation of 2170 m a.s.l., in the south–eastern Alps (Chiese stream at Levade, in the upper part of the Fumo Valley in the Adamello batholith, 46° 07′ 23″ N, 10° 33′ 54″ E), in a glacier–fed, turbid and turbulent, well–oxygenated and cold (5.86 °C, August 28th 2014 noon) stream. This stream flows over holocrystalline bedrock in the Adamello–Brenta Nature Park (Figs 1a–c).

Hydrochemical characterization. Water sampling was conducted using polyethylene bottles, which had previously been cleaned with ultrapure water and superpure nitric acid (1%). Water temperature, pH, and specific conductivity were assessed in the field using a HYDROLAB H20 multiprobe datasonde. Detailed hydrochemical characteristics of the stream including major ions, nutrients, trace elements and metals were investigated following standard procedures and methods (APHA 2000). Metals were analyzed by means of ICP–OES (Optima 5300 Perkin Elmer Corp.). The ions, Na^+, Ca^{2+}, Mg^{2+}, Cl^- and SO_4^{2-} were measured using ionic chromatography (ICS 1500 Dionex Corp.). The nutrients ($N–NO_3^-$, $N–NH_4^+$, TN, TP and SRP) were by molecular absorption spectrometry and silicates as SiO_2 by the molybdosilicate method (APHA 2000; Cantonati et al. 2011).

Sampled algal materials. A stream reach about 20 m in length was inspected with an aquascope to identify the most suitable sampling points and to gain some information on the distribution of the species. Specimens included in this study were collected from rocks using forceps and placed in 100–ml sterile clean polyethylene (PET) bottles for transport. The specimens were transported on ice to the laboratory for further studies. In the lab, the specimens were cleaned using distilled water to remove epiphytes and debris. This step was verified by microscopic examination. Subsequently, the specimens were divided into four portions. The first portion was dried in silica desiccant for DNA extraction, the second portion was fixed in 4% (v/v) formaldehyde solution for morphological identification, the third portion that was well–cleaned was immediately used for lipidomics and bioorganic screening, and the fourth portion was stored as a voucher specimen in the Museo delle Scienze–MUSE, Trento, Italy, and the Phycology Unit NO. 341 in the Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt. For morphological identification and to visualize chromoplast autofluorescence, specimens were examined using both Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) in Italy and BEL® photonics biological microscope (Italy) in Egypt. Morphometric diagnostic features were measured and photographed using Axiocam and Canon Powershot G12 digital cameras. A total of 20 measurements were made for the taxonomically important morphometric features: thallus height, diameter without and with the spermatangial papillae, and the length of internodes. Specimens were morphologically identified using the relevant literature: Vis & Sheath (1992), Sheath & Sherwood (2011) and Eloranta et al. (2011). Photomicrographs were arranged into plates using Adobe Photoshop (version CS 4, Adobe Systems Inc.).

Molecular data generation. For DNA extraction, thalli were ground in liquid N_2 and extracted using Nucleospin Plant Genomic DNA kit (Macherey–Nagel) according to the manufacturer's protocol. The *rbc*L gene was PCR amplified using the primer set (F160 & rbcLR; Vis et al. 1998) and Extaq Polymerase system (Clontech Laboratories Inc.) in the thermocycler conditions described in Keil et al. (2015). PCR product was purified using UltraClean PCR Clean–up DNA Purification Kit (Mo Bio Laboratories). The PCR product was sequenced using an ABI 3100 Genetic Analyzer (Applied Biosystems); the amplification primers and two internal primers (F650, R897.lem) were used to completely sequence the sense and anti–sense strand. Sequences were compiled in Sequencer 5.2.4 (GeneCodes Corp.). In order to explore the phylogenetic affinities of the new sequence, all *rbc*L sequences available in GenBank for *Lemanea* (34) and *Paralemanea* (14) were obtained (GenBank accessed 25 March 2016). Two sequences were excluded AF029153 due to sequence quality and DQ523257 as it appeared to be a *Batrachospermum* and not a *Lemanea* sequence. Additional sequences of *Ba-*

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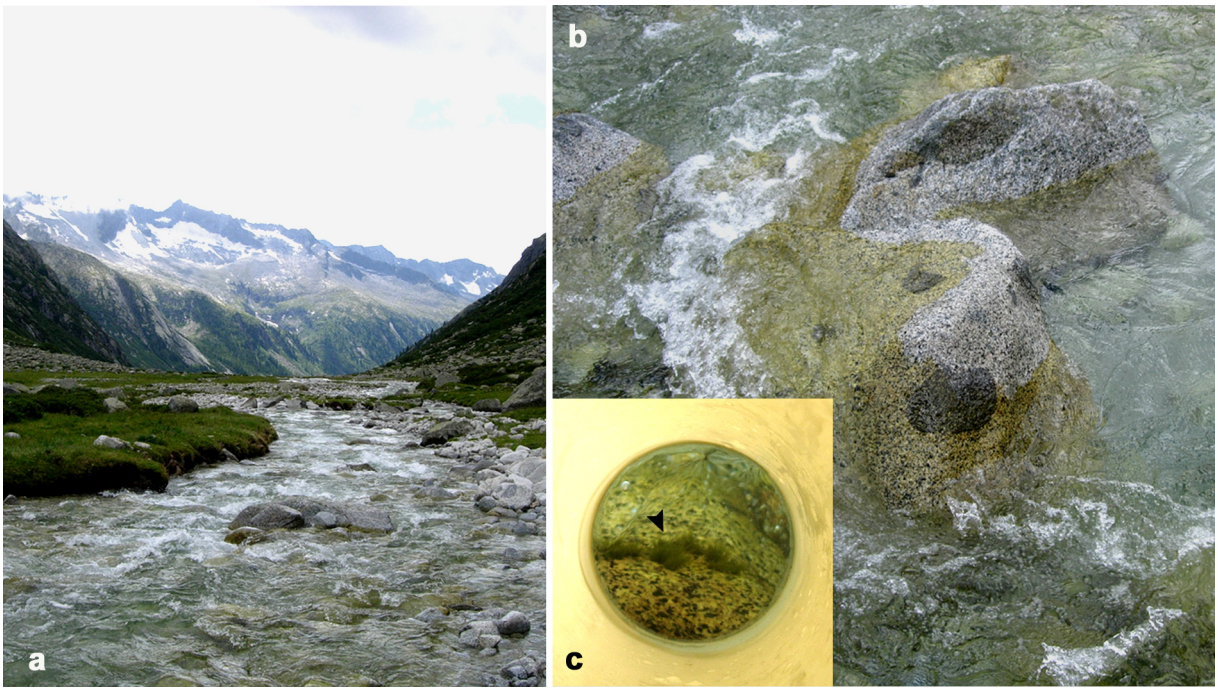

Fig. 1. *Lemanea fluviatilis* sampling site: (a) Chiese Stream at Levade in the Fumo Valley (Adamello–Brenta Nature Park, south–eastern Alps); (b) large boulders overflown by turbulent, swift, and cold water; (c) *L. fluviatilis* tufts on a boulder in the stream viewed using an aquascope.

trachospermum gelatinosum (L) DC, *Sirodotia delicatula* Skuja, *S. huillensis* (Welwitsch ex West & G.S.West) Skuja, *S. suecica* Kylin and *Tuomeya americana* (Kützing) Papenfuss were utilized as outgroup sequences as these taxa have been shown to be most closely related to *Lemanea* and *Paralemanea* in previous studies of the Batrachospermales (Vis et al. 1998; Entwisle et al. 2009). If sequences were longer than 1282 bp, they were trimmed to that length before further analyses since most sequences were 1282 or shorter. The new sequence from this study was aligned with the previously published GenBank sequences using Muscle (Edgar 2004) as implemented in Geneious Pro version 8.1.5 (Biomatters, Ltd., New Zealand, Kearse et al. 2012). The phylogenetic placement of the specimen was explored using Bayesian Inference (BI) Analysis in Mr.Bayes v.3.2.6 (Ronquist et al. 2012) and Maximum Likelihood (ML) Analysis in PhyML (Guindon & Gascuel 2003) as implemented in Geneious Pro version 9.1.2. For both analyses, the general time reversible model was implemented with gamma for the BI and both gamma and invariant sites estimated by the program for ML. The BI analysis was run for 1,100,000 generations with a burn–in of 100,000. The ML analysis was run for 1000 generations using a parsimony–inferred starting tree with 1000 bootstrap replicates using a random starting tree. The new *rbc*L sequence was submitted to GenBank (KU343187).

Lipid and pigment analyses. Total lipids were extracted by a slightly modified Folch method (Folch et al. 1957). Briefly, cell clusters were collected into 15 ml glass tubes, re–suspended in 10 ml of chloroform/methanol 2:1 (v/v), sonicated for 15 min in an ultrasonic bath (Sonorex Super, Bandelin electronics, Berlin, Germany), and centrifuged at 3000× *g* for 10 min at room temperature to separate the organic phase (bottom layer). For each cell pellet, the extraction procedure was repeated three times. All the organic phases were collected, filtered by using glass filters under vacuum, and reduced to dryness on a rotary evaporation (Büchi Labortechnik AG, Flawil, Switzerland) to obtain crude lipid extracts. Extracts were re–suspended in 300 µl of methanol/chloroform 9:1 (v/v).

Crude lipid extracts were subjected to Reverse Phase Liquid Chromatography–Electrospray Ionization–Ion Trap–Mass Spectrometry analyses (RPLC–ESI–IT–MS). Under this chromatographic setup, lipid molecular species were separated primarily according to the hydrophobicity of their acyl chains. Details are reported in Guella et al. (2003). Hydrophilic Interaction LIquid Chromatography (HILIC) was also used to establish the membrane lipid class composition based on the different polarity of lipids head groups (Anesi & Guella 2015).

Each lipid molecular species was quantified with respect to the total area of all lipid species belonging to the same class (e.g., relative quantification of $MGDG_x$ was performed with respect to total area of MGDG). The unsaturation index (UI) and the average chain length (ACL) were calculated for each lipid class, using the formulas

$UI_{class\ y} = \Sigma$ (relative area $lipid_x$ * double bond number of $lipid_x$) and

$ACL_{class\ y} = \Sigma$ (relative area $lipid_x$ * acyl chain length of $lipid_x$)

where $lipid_x$ represents each single molecular species belonging to the *y* lipid class, respectively.

Pigments (chlorophylls and carotenoids) were simultaneously analyzed in the same chromatographic conditions through a Photo–Diode–Array detector (PDA) operating at 470 nm (carotenoids) and 660 nm (chlorophylls). Chromatographic peaks were identified by comparing retention times and on-line spectra (UV–Vis and positive and negative–ion ESI–MS spectra) against known standards (Frassanito et al. 2005).

Results

Ecological features

The *Lemanea* population was thriving on the downstream edge of large boulders overflown by swift currents (>1 $m.s^{-1}$). The thalli were in tufts or lawns on the boulders. In the stream reach examined the species colonized larger boulders in strong currents.

Stream chemistry including the primary ions, nutrients, trace elements, and metals' concentrations are listed in Table 1. Despite the extremely low conductivity (9 $\mu S.cm^{-1}$), the water was only very–slightly acidic (pH 6.2). Nutrient concentrations were very low, especially phosphorus (S.R.P. 3 $\mu g.l^{-1}$). Trace elements and metals were measured in parts per trillion. The only elements having notable concentrations were Al, Fe, U, Ti, Zn due to the lithological characteristics of the bedrock.

Morphological Characterization

The gametophyte thalli were small (up to 1 cm long), olive green, primarily unbranched and in growing in dense tufts (Figs 2a,b). The base of the thalli are stalked and the spermatangia in rusty–brown discrete patches (Figs 2c,d). The thalli are pseudoparenchymatous, tubular, 150–500 µm in diameter without the spermatangial papillae and 200–600 µm in diameter with the spermatangial zones, with the surface covered in numerous hair cells (Figs 2e–g). Internodes 0.25–0.35 mm long. In addition, the carposporophytes can be seen to project inwardly into the hollow centre (Figs 2f,g). The outer thallus is composed of small cells with several, parietal, disc–shaped chromoplast that can be easily seen in autofluorescence (Figs 2d,h,i). In cross–section, it is evident that much of the inner thallus is hollow with no cortical filaments filling the center and ray cells abutting the larger cells of the inside of the tube (Figs 2 f,g,j,k).

Both the qualitative and quantitative characteristics of the population were assessed and the most recent key to European *Lemanea* species consulted (Eloranta et al. 2011). The diagnostic characteristics in the key showed the specimens to be *Lemanea fluviatilis*. However, the feature thallus height (6–30 cm long) in the description was longer than the population study. Given that thallus length may vary considerably and all other characteristics were within the ranges provided, the population was assigned to *Lemanea fluviatilis* on the basis of morphology.

Molecular data analysis

An examination of sequence similarity showed that the sequence from this population was most closely related (99.1%, similar or 11 bp different) to a group of GenBank sequences (AY575163, AY575164, AY575167, AY575170). Both the BI and ML analyses produced trees with similar topologies such that only the BI tree with both the posterior probabilities and ML bootstrap values is shown (Fig. 3). The sequence from this study was sister to a clade containing sequences labelled as *Lemanea fluviatilis* (9), *L. fucina* (5) and *Lemanea* sp. (1) and its inclusion with the clade was well support (1.0/89). Like the sequence from the current study, all within this clade were collected from various European locations. *L. borealis* was shown to be sister to this clade but the support was low (0.73/–). In the current analysis, *L. fluviatilis*, *L. fucina* and *L. borealis* were all shown to be paraphyletic.

Pigments and lipids

The carotenoids profile of this alga contains significant amounts ofα /β–carotene, zeaxanthin and lutein – (Fig. 4a). Among chlorophylls (Fig. 4b), chlorophyll *a* is dominant; the absence of chlorophyllide–*a* (loss of phytyl chain, not detected) and the very low amount of phaeophytin–*a* (loss of the magnesium ion, about 1%)

Table 1. Physical and chemical characteristics of Chiese stream inhabited by *Lemanea fluviatilis.*

Variable	Chiese Stream at Levade
Temperature (°C)	5.9
Conductivity ($\mu S.cm^{-1}$)	9
pH	6.2
$N–NO_3^-$ ($\mu g.l^{-1}$)	136
$N–NH_4^+$ ($\mu g.l^{-1}$)	9
TN ($\mu g.l^{-1}$)	219
TP ($\mu g.l^{-1}$)	6
S.R.P. ($\mu g.l^{-1}$)	3
SiO_2 ($mg.l^{-1}$)	2.4
Na^+ ($mg.l^{-1}$)	0.70
Ca^{2+} ($mg.l^{-1}$)	1.1
Mg^{2+} ($mg.l^{-1}$)	0.13
SO_4^{2-} ($mg.l^{-1}$)	0.6
Cl^- ($mg.l^{-1}$)	0.2
Al ($\mu g.l^{-1}$)	58.08
Ba ($\mu g.l^{-1}$)	1.26
Rb ($\mu g.l^{-1}$)	1.90
Cu ($\mu g.l^{-1}$)	0.18
Fe ($\mu g.l^{-1}$)	51.79
Mn ($\mu g.l^{-1}$)	1.56
Pb ($\mu g.l^{-1}$)	0.06
U ($\mu g.l^{-1}$)	0.22
Ti ($\mu g.l^{-1}$)	11.82
Sr ($\mu g.l^{-1}$)	2.84
Zn ($\mu g.l^{-1}$)	11.74
Mo ($\mu g.l^{-1}$)	0.39

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determined in all the LC–UV chromatographic runs indicated both optimal storage conditions of the alga and mild extraction conditions.

Our LC–MS methodology also allowed identification of the membrane lipids contained in the raw extract of *Lemanea fluviatilis* (Table 2; Fig. 4c) relying on retention time on different chromatographic stationary phases (RP18 and HILIC), on full scan and tandem MS spectra obtained by positive and negative ESI ion–modes ionization. Among the structural components of chromoplast, we profiled 20 monogalactosyl diacylglycerols (MGDG), 22 digalactosyl diacylglycerols (DGDG) and 10 sulfoquinovosyl diacylglycerols (SQDG) with other cell membrane lipids were in the classes 3 diacylglyceryl N,N,N–trimethylhomoserine (DGTS) and 26 phospholipids belonging to the class of phosphaditylcholine (PC). Among galactolipids, MGDGs and DGDG bearing polyunsaturated and long fatty acyl chain (in particular EPA, the ω–3 eicosapentaenoyl chain, 20:5) were the most abundant in this class (about 67% and 52%, respectively). Curiously, concerning plasma membrane lipids, this PUFA chain is very abundant (about 85%) in PC lipid species and not present at all in any detectable DGTS species.

This hint is also in agreement with the averaged unsaturation index (UI) of each lipid class. The PUFA–richest (PC and MGDG) are also the most unsaturated lipid classes (UI = 7.6 and 6.8 respectively), followed by DGDG (4.1); the PUFA–poorest (SQDG and DGTS) are the less unsaturated lipid classes (UI = 3.3 and 2.8 respectively).

As expected, a similar trend is found for the average chain length, with PC and MGDG having the highest ACL values (38.5 and 37.9 respectively), DGDG intermediate (ACL = 35.9) whilst SGDG and DGTS have the lowest values (34.1 and 34.0).

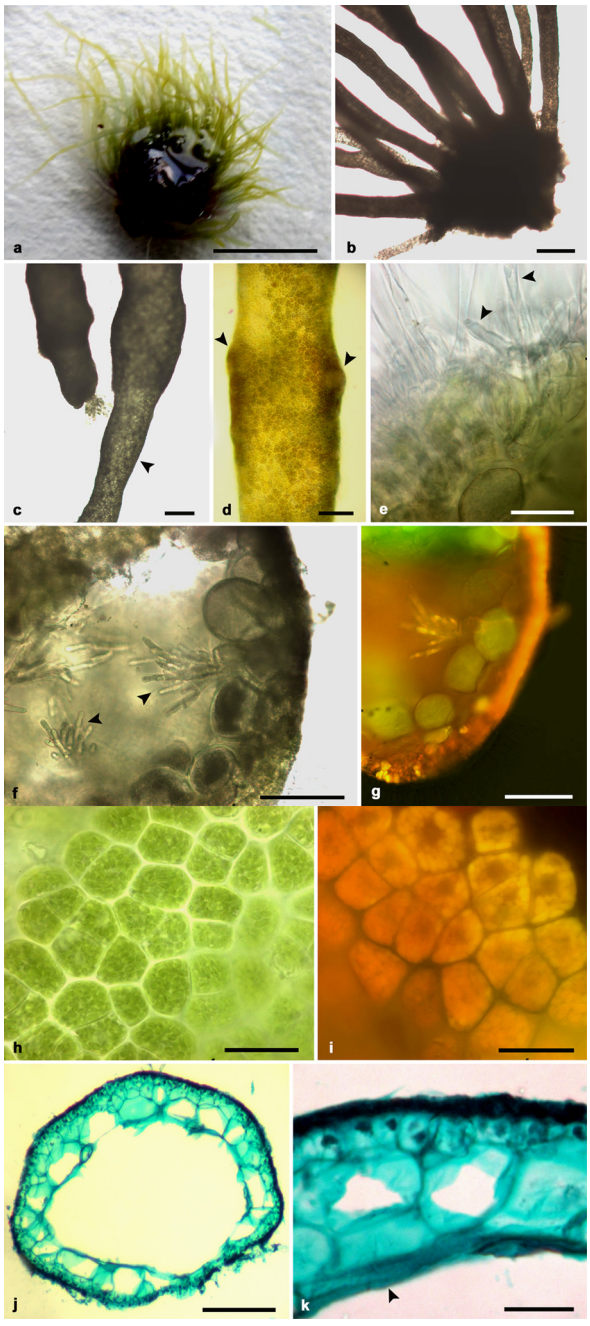

Fig. 2. Morphological characteristics for the population of *Lemanea fluviatilis*: (a) overall habit showing small–sized olive–green unbranched thalli in dense clumps; (b) basal disc from which unbranched, pseudoparenchymatous thalli arise; (c) lower part of the thallus showing a short stalk (arrowhead); (d) magnified node showing spermatangia in rusty–brown patches (arrowhead); (e) close–up view of the thallus surface showing plentiful hair cells (arrowhead). (f) cross–section of thallus showing the developing carposporophyte with filaments (arrowhead) projecting into the center of the thallus; (g) autofluorescent plastids in the outer cortical cells, and carposporophyte filaments; (h) several, parietal, disc–shaped chromoplasts; (i) autofluorescent chromoplasts; (j) light green–stained transverse section showing a thin layer of small outer cortical cells, larger inner cortical cells with an empty central region; (k) magnification of the transverse section characterized by the small outer cortical cells and the larger inner cortical ones with ray cells (arrowhead) running parallel to the cortex. Scale bars 5 mm (a), 20 µm (e, h–i, k), 200 µm (b–d), 100 µm (f–g, j).

Discussion

Most of the morphological characteristics examined would suggest that this *Lemanea* population belongs to the species *Lemanea fluviatilis*. However, the thalli collected in this study were distinctly short. The small stature of this population has been observed in other collections at the same site in different years (Cantonati personal observation). The small size may be due to the glacial mountain habitat as Cantonati et al. (2001) collected similarly small thalli from the typical high discharge/high turbidity glacial Niscli Stream (Adamello) at an elevation of 2372 m a.s.l. (46°06'50.25" N, 10°37'31.30" E). The smaller size of *L. fluviatilis* in this study might be an adaptive phenotypic mechanism to avoid the high current velocity and flow–related

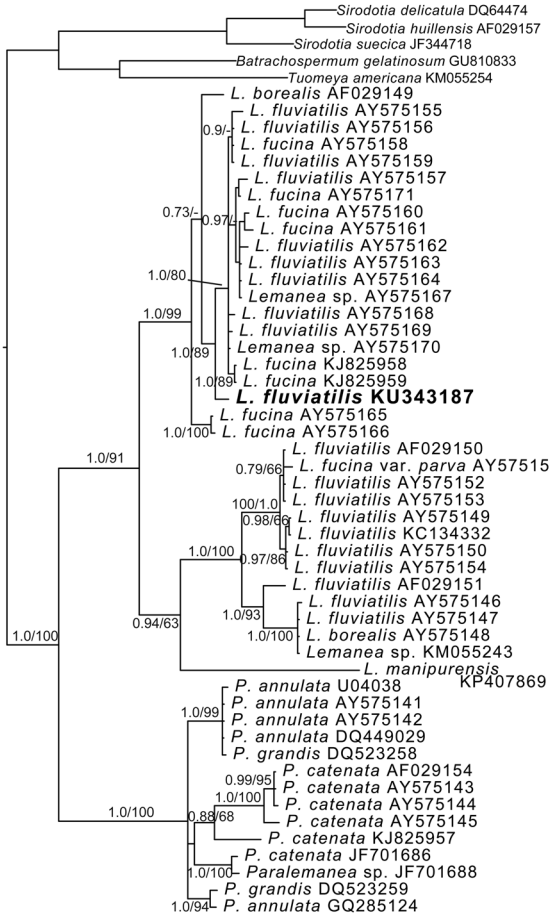

Fig. 3. Phylogenetic tree based on *rbc*L sequence data showing the relationships of *Lemanea fluviatilis* (Italy) and previously published sequences from GenBank using Bayesian Inference analysis in Mr.Bayes v.3.2. Support values are shown as BI pp/ML bootstrap. Branches without values had BI pp <0.7 and ML <60%.

stress in the montane habitat in the south–eastern Alps. Everitt & Burkholder (1991), Vis et al. (1991), Eloranta & Kwandrans (1996) and Filkin & Vis (2004) in their studies of *Lemanea* and *Paralemanea* observed these taxa at high current velocity (>1 m.s^{-1}), but with greater sized thalli. Perhaps, the smaller size is not related to current velocity, but the montane habitat (characterized, e.g., by scarce nutrient, in particular phosphorus availability). It likely seems that further studies are needed to assess how widespread the small sized thalli of *L. fluviatilis* are in high mountain populations of the Alps, and the possible adaptive significance of this feature.

The molecular data placed the *Lemanea* population from the current study as sister in a clade with sequences labelled as *Lemanea fluviatilis*, *L. fucina* and *Lemanea* sp. Unfortunately, there is no associated morphological data with the specimens from GenBank. Therefore, it is assumed that they were identified using the current circumscriptions for *L. fluviatilis* and *L. fucina*. These two taxa appear to be morphologically distinguished with *L. fucina* having many thalli branched per population in Vis & Sheath (1992) and the stalk being more tapered as well as thalli not growing as compact tufts (Eloranta et al. 2011; see Table S1). These morphological characters seem like they may be open to interpretation and could result in the species epithets being utilized differently. As well, there is the distinct possibility that these characteristics used for morphological identification may not be phylogenetically informative. Nevertheless, when future systematic studies are conducted, current *Lemanea* population will have both morphological and molecular data documented so that it can be easily included and may provide data from a more extreme montane habitat.

From the ecological standpoint, this study confirmed that *L. fluviatilis* prefers unpolluted, fast–flowing, oligotrophic, mountain streams with cold water. Accordingly, Lederer & Soukupová (2002) recorded this species in rocky mountain streams and rivers in Central Europe. Vis & Sheath (1992) suggested that cold waters with an average temperature of 13 ºC are the typical environments for members of the Lemaneaceae. They recorded populations in streams with water temperatures between 7 and 24 ºC. Kučera et al. (2008) showed that *L. fluviatilis* is a widespread species in Czech Republic, usually growing on boulders and cobbles in riffles, on weirs or in waterfalls which are partly–shaded or well–illuminated. They also confirmed that *L. fluviatilis* is not restricted to mountain streams, but occurs across an altitudinal gradient ranging from 305 to 888 m a.s.l. Accordingly, Eloranta & Kwandrans (1996) described this species from a wide range of flow velocity including strong currents (0.2–1.9 m.s^{-1}). Gutowski et al. (2004), Eloranta & Kwandrans (2007), and Ceschin et al. (2012) reported *L. fluviatilis* from oligotrophic streams. Vis & Sheath (1992) reported that *L. fluviatilis* in North America occurs over a wide pH range (5.0–8.6). Ceschin et al. (2012) determined this species in Italian watercourses with pH regimes ranging from neutral to alkaline (7.4–8.6).

One of the most notable features of our study site is the high elevation (2170 m a.s.l.). Cantonati et al. (2001) sampled *L. fluviatilis* from a stream in the same mountain range at an elevation of 2372 m a.s.l. These reports might be some of the highest altitudes for *Lemanea fluviatilis* occurrence (compare e.g. Sheath & Vis 2015, who reported elevations up to 1200 m a.s.l.).

Recent studies of Kwandrans & Eloranta (2010) and Eloranta et al. (2011) on freshwater red algae biodiversity in Italy lamented a scarcity of knowledge about the biogeographical patterns of this group in some Central European countries including Italy. Our record might contribute to a more detailed knowledge on the distribution of this species, particularly in regards to elevation and pH. Recently, combined molecular /morphological studies have provided new insights into *Lemanea/Paralemanea* biodiversity in so far poorly studied geographic areas (Ganesan et

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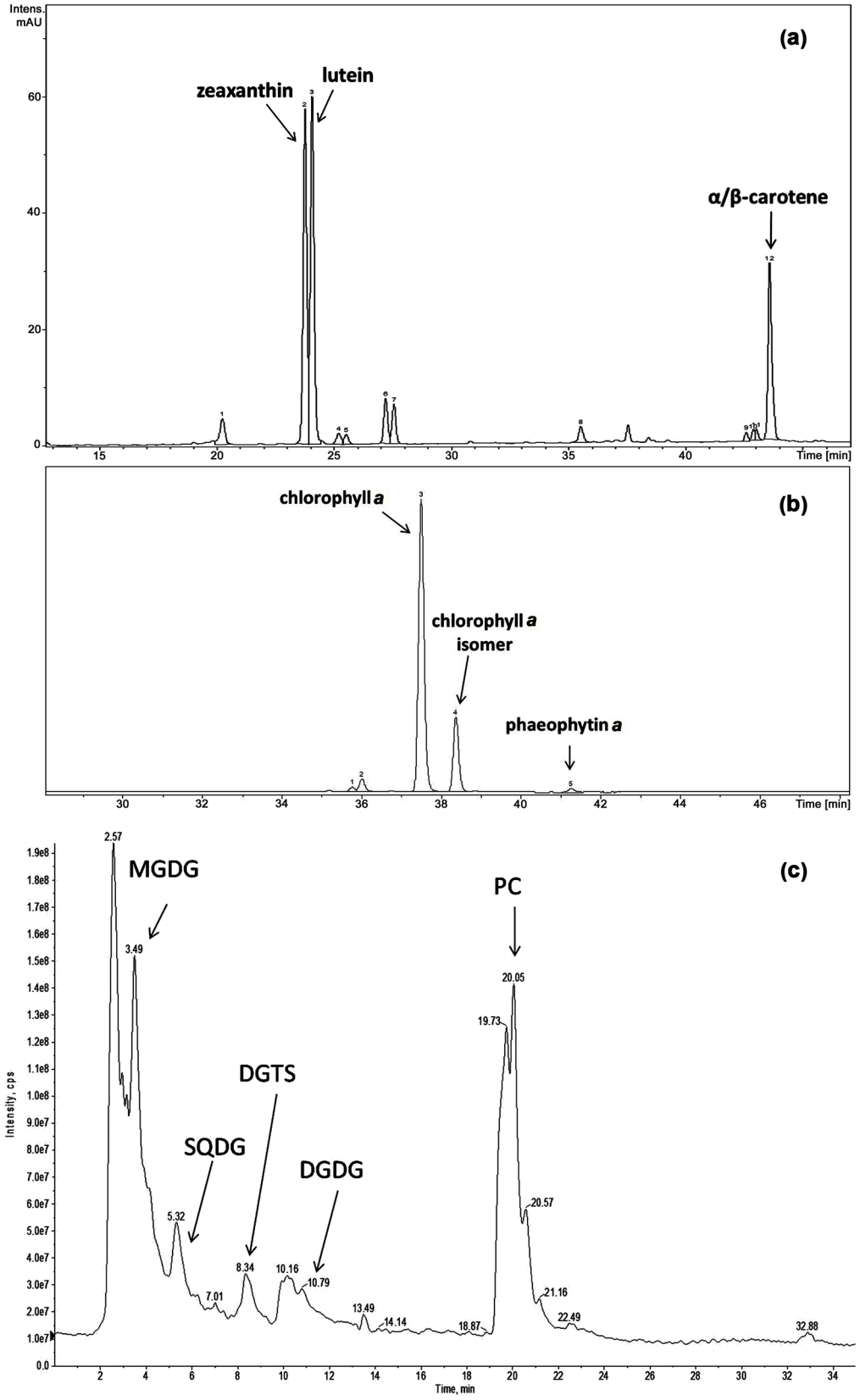

Fig. 4. Chromatograms of carotenoids, chlorophylls, and lipids for *Lemanea fluviatilis*: (a) RP8 chromatogram as detected by PDA at l 470 nm (carotenoids); (b) RP8 chromatogram as detected by PDA at l 665 nm (chlorophylls) (c) HILIC chromatogram as detected by full scan ESI(+) mass spectrometer (lipid classes). Intra–class lipid distribution was obtained in reversed–phase chromatographic conditions.

Table 2. Results of the membrane lipidomics analysis of *Lemanea fluviatilis* detailed per lipid class.

Items	Lipid classes				
	MGDG	DGDG	SQDG	PC	DGTS
species	20	22	10	26	3
UI	6.76	4.11	3.28	7.58	2.83
ACL	37.93	35.94	34.06	38.48	34
Total number of lipid molecular species				81	

MGDG: monogalactosyl diacylglycerols; DGDG: digalactosyl diacylglycerols; SQDG: sulfoquinovosyl diacylglycerols; PC: phosphaditylcholine; DGTS: diacylglyceryl *N,N,N*–trimethylhomoserine; UI: the unsaturation index of each lipid class; ACL: average chain length for each lipid class.

al. 2015).

The carotenoids profile of this alga is in fair agreement with that expected for a macrophytic type Rhodophyta (Schubert & García–Mendoza 2008). Moreover, this inventory of pigments, especially zeaxanthin and lutein, might have light–harvesting and protective functions in this harsh mountain habitat (Takaichi 2011).

Temperature and nutrient availability (specifically P) are the most influential environmental drivers affecting algal cell membrane fluidity and permeability by the presence of special algal membrane–lipids' classes, as an adaptive mechanism. DGTS are primitive lipids that are mainly determined in lower plants, algae, dinoflagellates, and mosses (Kumari et al. 2013; Riekhof et al. 2014). They are structurally similar to PC, but lack the phosphate group. In general, DGTS lipids can completely replace PC lipids in the plasma membranes, especially in organisms living in harsh environments, like those with low or very low phosphorus availability. However, the average UI and ACL determined in this *Lemanea fluviatilis* population for these two lipid classes lie at opposite extremes, being the highest (UI = 7.6 and ACL = 38.5) for PC and the lowest (UI = 2.8 and ACL = 34.0) for DGTS lipid species. Although, the *L. fluviatilis* habitat in our study has low phosphorus concentrations, nutrients in this turbulent, high–current–velocity habitat are likely to be replenished rapidly through the reduced boundary layer (MacFarlane & Raven 1985; Sheath & Hambrook 1990). Moreover, emerging evidence (GG unpublished data) seems to indicate that betaine lipids distribution mainly reflects the algal phylogenetic relationships rather than its ability to mediate changing environmental conditions.

The modifications of the unsaturation degree of the lipid acyl chains are important for maintaining adequate membrane fluidity and therefore all the metabolic processes associated to the plasma membrane, especially for organisms living in extreme environments. Accordingly, *Lemanea fluviatilis* living in lotic, very–cold streams possesses lipid species with high UI and long acyl chains. The detailed lipid analysis of this species further demonstrated that red algae are a rich source of a important ω–3 fatty acids such EPA (20:5) as already been reported in the red alga *Porphyridium cruentum* (Cohen 1990) and in several other species.

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Supplementary material

the following supplementary material is available for this article:

Table S1. Morphometrical and ecological comparative study between *L. fluviatilis* and *L. fucina* in this study and other relevant literature.

Excel file. Details of lipidomics and pigments of L. fluviatilis in this study.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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