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ORIGINAL PAPERS

The algal partnership is associated with quantitative variation of lichen specific metabolites in *Cladonia foliacea* from Central and Southern Europe

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Abstract

Lichen specific metabolites (LSMs) have interesting biological activities and quantitative variations may be present intraspecifically. For example, variations in medullary fumarprotocetraric acid (FA) and cortical usnic acid (UA) were observed in the lichen *Cladonia foliacea*, but the mechanism of variation is not well understood. The current study aimed to characterise the quantitative variation of FA and UA and to investigate the association between lichen metabolite content and ecological / biological variables. Fungal and algal trees were constructed using fungal (nrITS, RPB2) and algal (nrITS) loci, respectively. Using a chiral chromatographic method, the contents of (-)-UA were determined in 29 *C. foliacea* specimens and range from 6.88 to 34.27 mg/g dry wt. The FA contents were lower and varied from 1.44 to 9.87 mg/g dry wt. Although the fungal tree showed two well resolved clades, no significant differences of UA or FA contents were found between the two fungal clades. However, a significantly higher UA/FA ratio as well as a unique habitat were found to be associated with specimens which contained the alga *Asterochloris lobophora* than those specimens associated other *Asterochloris* algae. Taking all predictive variables into account (i.e. substrate type, elevation, collection season, photobiont identity), the multivariate data analysis indicated that photobiont identity explained most of the variance of LSM contents in *C. foliacea*. Thus future LSM biosynthetic studies should take the photobiont into consideration when dealing with intraspecific quantitative variation.

Keywords *Asterochloris* photobionts · *Cladonia convoluta* · Fumarprotocetraric acid · HPLC-PDA · UHPLC-PDA-MS · Usnic acid enantiomers

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1 Introduction

Lichens are microbial consortia where the lichen forming fungi (mycobionts) provide a framework hosting photosynthesizing partners (algae and/or cyanobacteria) and other microbes (e.g. bacteria) (Hawksworth and Grube 2020). These miniature ecosystems produce at least a thousand lichen specific metabolites (LSMs) made by mycobionts, which comprise depsides, depsidones and dibenzofurans and most of which is restricted to this group of organisms (Stocker-Wörgötter 2008). Lichens produce other secondary metabolites also found in non-lichen fungi and algae, but in this study we focus only on LSMs. LSM profiles were historically used for chemotaxonomic purposes (Culberson 1968) and their concentrations were not considered important in this respect (Hawksworth 1976). The increasing interest of LSMs nowadays relates to their antibiotic activities against various microbial pathogens, insects or vectors of pathogens (Khadri et al. 2019; Molnár and Farkas 2010; Muhoro and Farkas 2021; Xu et al. 2016). Considerable intraspecific LSM variation has been reported in either qualitative or quantitative manner (Armaleo et al. 2008 and references therein; Bokhorst et al. 2021; Egbert et al. 2022; Xu et al. 2017). Such variations have led to uncertainties with respect to species delimitation (Culberson 1986) even before molecular phylogenetics took hold. Advances in genomics have helped us to understand the qualitative differences between species and even populations (Singh 2023; Singh et al. 2021a, b), but the causes of intraspecific quantitative LSM differences are still under study. It has been suggested that water potential is a strong determinant for depside/depsidone production by mycobionts, both in vitro (Culberson and Armaleo 1992) and in natural lichens, where the combination of differing light/temperature effects on water potential in individual thalli is thought to underlie intraspecific variation of depside/depsidone concentrations (Armaleo et al. 2008).

The LSMs provide contributions for both partners enabling them to survive in various environments. Recently there has been an increasing interest as to how the various photosynthesizing partners affect the success and distribution of the lichen holobiont in nature (Dal Grande et al. 2018; Škvorová et al. 2022; Vančurová et al. 2021). For example, photobiont switching may expand the climatic niches colonised by *Stereocaulon* species (Vančurová et al. 2018). However, Škvorová et al. (2022) demonstrated on the basis of a large dataset that most *Cladonia* species are unable to increase their ecological niches by changing their photobiont partners, possibly because photobiont switching in this genus is limited to algae with similar ecological preferences. Climate and soil parameters are the main limiting factors for both partners.

The lichen *Cladonia foliacea* (Huds.) Willd. is an ideal example for investigating the mechanisms of intraspecific quantitative chemical variation of LSMs as it exhibits morphological variation in lobe shapes (Fig. 1), occurs in different habitat types (e.g. calcareous and siliceous soils) and has a wide distribution in North Africa and Europe, particularly in the Mediterranean region (Burgaz et al. 2020; Pino-Bodas et al. 2010). Our previous studies revealed quantitative differences in major LSMs between lowland and mountain populations in Hungary that was accompanied by a significant seasonal fluctuation (Veres et al. 2022b). Fumarprotocetraric acid (FA) and usnic acid (UA) are the major specialized metabolites and are produced in the medulla and cortex (Fig. 2), respectively (Veres et al. 2022b). Different localization of LSMs may imply their different biological and ecological roles. Being a cortical compound, UA primarily protects the photosynthesizing algal partner and the fungal hyphae

Fig. 1 Habitat photograph of the lichen *Cladonia foliacea*. **a** Thalli in a typical lowland steppe habitat in Hungary; **b**, **c** specimens with round lobes, **d**, **e** specimens with divided lobes. Photo E. Farkas

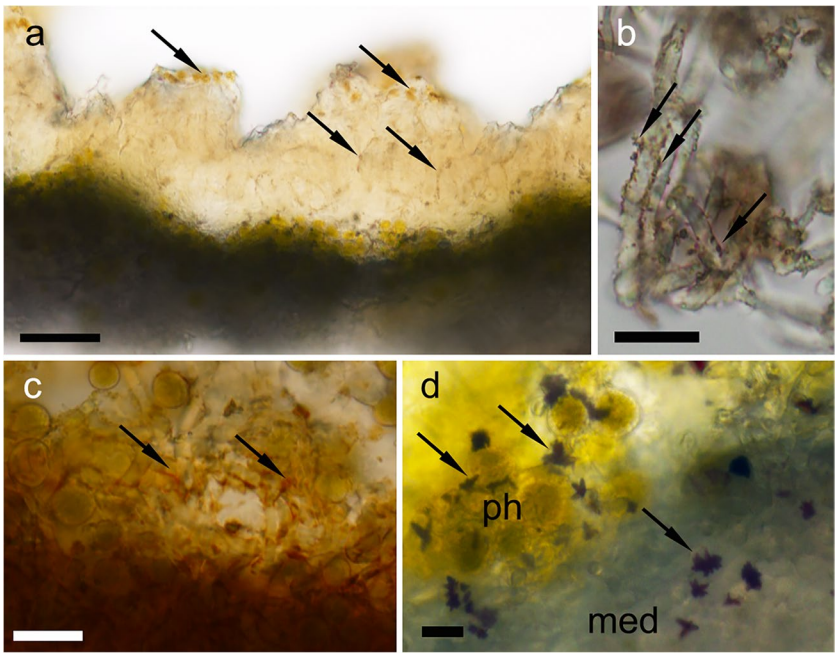

Fig. 2 Localisation of lichen secondary metabolites in *Cladonia foliacea* thallus presented in light microscopic views. **a** Cortical layer of thickened hyphae with larger (near the surface) and smaller (between the thickened hyphae) yellow UA crystals (see arrows) in water; FA crystals (see arrows) **b** on lower surface medullary hyphae stained orange, **c** on hyphae in photosynthetic layer (near algae) and **d** on hyphae in photosynthetic (ph) and medullary (med) layer stained strongly orange-red by p-phenylenediamine. Scales: a = 50 µm, b, c = 20 µm, d = 10 µm. Micrographs: E. Farkas

from solar UV irradiation (Farkas et al. 2020a, b; Veres et al. 2022a, b), UA from *C. foliacea* also exerts a phytotoxic or even a herbicidal effect on nearby bryophytes (Giordano et al. 1999). Furthermore, UA also shows antimicrobial effects (Lauterwein et al. 1995), insecticidal potential (Emmerich et al. 1993) and antioxidant activity (Anar et al. 2016; Odabasoglu et al. 2006). FA with a low pKa_1 value of 2.7 enhances tolerance to substrate acidity (Hauck et al. 2009), and has anti-herbivore effects (Nimis and Skert 2006; Tigre et al. 2015). FA also has moderate antibiotic activity against microbes and tumor cells (Bézivin et al. 2004; Yilmaz et al. 2004.

While seasonal, geographic and environmental factors are likely to drive intraspecific LSM variation (Armaleo et al. 2008; Bokhorst et al. 2021; Gauslaa et al. 2013), roles of fungal or algal partners in such variation have not been investigated. Therefore, the present study aimed to 1) characterize quantitative variation of UA and FA in the lichen *C. foliacea* in Central and Southern Europe, and 2) investigate the association between quantitative LSM variation and the following variables (season, substrate, altitude). Combined phytochemical, phylogenetic and statistical analyses were used to answer the following questions: (a) How much phytochemical quantitative variation is present in *C. foliacea*, (b) What is the mycobiont and photobiont diversity in *C. foliacea*, and (c) which variables (e.g. photobionts, mycobionts and substrates) could explained most of the variance in LSM contents?

2 Materials and methods

2.1 The investigated species

Cladonia foliacea (Huds.) Willd. (Cladoniaceae, lichenised Ascomycota) (Fig. 1) is characterized in detail in numerous publication (Burgaz and Ahti 2009; Farkas et al. 2020b; Poelt and Vězda 1977; Verseghy 1994; Wirth 1995). This lichen is relatively frequent in Hungary and widely distributed over Europe both in open, dry and sun-exposed habitats in lowland steppe and in mountain grassland communities. The thallus is dimorphic with very rare podetia and a dominant primary thallus of squamules with a stratified structure (Veres et al. 2022b). This consists of a thickened upper cortex containing the dibenzofuran UA, a photosynthetic layer containing a coccoid green alga photobiont (*Asterochloris* sp., Trebouxiaceae), a medullary layer producing the depsidone FA. The lichen has a yellowish white to white lower surface. Localisation of lichen secondary metabolites in the primary thallus is shown (Fig. 2) by microscopic views taken with a Nikon DS-Fi1c camera (with NIS-Elements BR software, Nikon Corporation, Tokyo, Japan) mounted on a Nikon Eclipse/NiU compound microscope (Nikon Corporation, Tokyo, Japan).

Based on extensive sampling of variation of the sibling species, *C. convoluta* (Lam.) Anders, was suggested to be treated as *C.'convoluta'*-like morphotype under the

species *C. foliacea* (Pino-Bodas et al. 2018). This practice was followed in this study, because both morphological and phylogenetic analyses were unable to distinguish the two taxa. The morphological variation within this species complex was interpreted as a response to environmental conditions. The morphological features of our specimens were carefully studied by using a Nikon SMZ18 stereo microscope (Nikon Corporation, Tokyo, Japan). This revealed a similarity between the former *C. convoluta* and *C. foliacea*. In the literature description (Poelt and Vězda 1977), *C.'convoluta'*-like specimens of *C. foliacea* were identified when wide rounded lobe ends were found mostly or exclusively on a specimen. In contrast *C.'foliacea'*-like specimens were characterised by lobe ends that were divided into several thinner lobes. The colours of the lower surface and of cilia (fibrils) were also recorded but found not to be as useful as lobe shapes.

2.2 Sample collection

Altogether 29 lichen samples (each comprising several thalli) of *Cladonia foliacea* were collected in Hungary, Albania and North Macedonia from typical habitats between 16 June 2013 and 9 June 2022. They were identified by Edit Farkas, László Lőkös and Nóra Varga (Supplementary Table S1 and Fig. S1). Voucher specimens were deposited in the Lichen Herbarium VBI at the Institute of Ecology and Botany of HUN-REN CER, Hungary (https://sweetgum.nybg.org/science/ih/herbarium-details/?irn=124855). Specimen information, including sampling date, elevation, location and habitat type (acidic or basic calcareous soil) were recorded during collection. Elevation ranges of ≤ 200 m a.s.l. for lowland and ≥ 600 m a. s. l. for mountain sites were established applying Gábris et al. (2018) and Whittow (1984), respectively. The sampling sites are presented in a map (Supplementary Fig. S1), constructed by the software QGIS 3.18.2 'Zürich', released in 2020.

2.3 Qualitative analysis of LSMs by HPTLC and UHPLC-PDA-MS

Collected samples were initially tested for the presence of UA and FA by HPTLC according to standard methods for analysing lichen samples described by Arup et al. (1993) and Molnár and Farkas (2011). A CAMAG horizontal chamber of 10 cm × 10 cm (DONAU LAB Kft., Budapest, Hungary), a CAMAG TLC Plate Heater III (DONAU LAB Kft., Budapest, Hungary), and 10 cm × 10 cm thin-layer chromatographic plates (Merck, Kieselgel 60 F254) were used. LSMs were tested in all the three standard solvent systems (Arup et al. 1993). However, solvent system C (toluene: acetic acid, 20: 3, v/v) was applied routinely. Compounds were identified by comparing their migration rates with UA and FA standards. Compound identity was further verified using ultrahigh performance liquid chromatography-photodiode array detection-mass spectrometry (UHPLC-PDA-MS). The Waters Arc UPLC system consisted of a quaternary solvent pump, an auto-sampler with temperature control at 10 °C, a column manager with temperature control at 25 °C, a PDA detector and a single quadrupole MS detector. One CORTECS C18 column (4.6 mm × 50 mm, 2.7 µm) was used for chromatographic separation with a flow rate of 0.5 ml min^{-1}. Solvent A was 0.1% formic acid, and solvent B was acetonitrile. The gradient elution condition was: 0–1 min, 20%B; 1–8 min, linear increase 20–100%B; 8–8.5 min, linear decrease 100–20%B; 8.5–10 min 20%B. UV spectra were recorded from 220 to 380 nm, and ions in negative ion mode were scanned from 100 to 1,000 m/z. Single ion monitoring for UA and FA was set at 343.1 and 355.1 m/z, respectively.

2.4 Quantitative analysis of fumarprotocetraric acid by HPLC-PDA

The amount of FA was measured at the Hungarian HUN-REN CER Institute of Ecology and Botany by high-performance liquid chromatography (HPLC, Alliance e2695, Waters Corporation, Milford, MA, USA) including a photodiode array detector (PDA, 2998, Waters Corporation, Milford, MA, USA). Thalli were chosen from the randomised material of each sample after cleaning and excluding thalline lobes with the obvious presence of lichenicolous fungi (cf. Asplund et al. 2018; Khadri et al. 2019). Thalli (of 1.5–2 g) were c. 4–5 cm in diameter. Portions (50 mg) of the homogenised material (20–30 g/sample) was suspended in 10 ml pure acetone and placed into an ultrasonic water bath for 20 min. The suspensions were then centrifuged at 8500 g for 20 min, and the supernatant was filtered through a Cronus Ø 25 mm PTFE syringe filter (0.22 µm). For calibration purposes, standard stock solutions (1 mg ml^{-1}) were made from reference standards for fumarprotocetraric acid (Phytolab GmbH & Co. KG, Vestenbergsgreuth, Germany) dissolved in dimethyl sulfoxide. The lichen metabolites were quantified according to a five-point (5, 10, 20, 50, 100 µg ml^{-1}) calibration. The chromatographic method based on Ji and Khan (2005) was used. For chromatographic separation, a Phenomenex Luna 5 µm C18, 150 × 4.6 mm column was used, and 10 µl of the filtered supernatant were injected. The temperature was 40 °C in the column oven and 5 °C in the sample cooler. For the baseline separation of LSMs, a gradient elution program was used. Solvent A consisted of ortho-phosphoric acid and deionised (Milli-Q ultrapure) water (0.5:99.5, v/v), and solvent B contained ortho-phosphoric acid and acetonitrile (0.5:99.5, v/v). All the chemicals used were HPLC grade. The linear gradient started with a 60% A solvent after the volume decreased to 10% within 20 min and then to 0.5% in 30 s after which the volume remained

constant for 9.5 min. The volume of solvent A was changed back to 60% within 1 min. The flow rate of solvents was 1 ml min^{-1}. The lichen metabolite FA was detected (n = 5) at 240 nm.

2.5 Determination of usnic acid enantiomers

The contents of UA enantiomers were determined at the University of Iceland (Faculty of Pharmaceutical Sciences), using a chiral chromatographic method described in details in Xu et al. (2022). Lyophilized lichen powders (less than 20 mg) were weighed, and LSMs were extracted with acetone in a sonicator. Each specimen was prepared in triplicate (n = 3). After evaporating acetone, dry residues were re-dissolved in 2 ml methanol: acetonitrile (50:50, v/v). Solutions were subjected to reversed-phase solid phase extraction, prior to chiral high performance liquid chromatography (HPLC) analysis on a Waters Arc HPLC system. Separation of enantiomers was carried out on an Amylose-1 chiral column. Concentrations of UA enantiomers in test solutions were determined using external standard curves. The detection wavelength was set at 280 nm and the flow rate was 0.5 ml/min.

2.6 DNA extraction, PCR and phylogenetic analysis

Lichen whole genomic DNA was extracted using a modified CTAB method (Cubero et al. 1999). DNA concentrations were determined using a NanoDrop. The fungal nuclear ribosomal internal transcribed spacer (nrITS) region, the second largest subunit of RNA polymerase II locus (RPB2) and algal nrITS region were amplified using the touchdown polymerase chain reaction (PCR) programs previously described (Xu et al. 2020). Amplicon sizes were assessed by 1.5% agarose gel electrophoresis. Gels were stained by SBYR safe. Amplicons were purified by ExoSAP and sent for Sanger sequencing to Macrogen Europe. The same primers were used for sequencing. Contig assembly and sequence alignment were made using the PhyDE software. Contig sequences were preliminarily identified by BLAST search against GenBank, followed by phylogenetic analyses incorporating references fungal and algal sequences. All fungal nrITS and RPB2 sequences obtained were from the fungus *Cladonia foliacea* (phylogenetic trees and reference GenBank accession numbers shown in Supplementary Fig. S2 and S3), and the algal nrITS sequences obtained were from three *Asterochloris* species, i.e. *A. lobophora*, *A. mediterranea* and *A. woessiae* (phylogenetic tree and reference GenBank accession numbers shown in Supplementary Fig. S4).

For fungal tree construction, *Cladonia cervicornis* (Ach.) Flot. and *C. pulvinata* (Sandst.) Herk & Aptroot were selected as the outgroup, since they are sister to *C. foliacea* (Pino-Bodas et al. 2010). The phylogenetic tree of each fungal locus was generated using maximum likelihood (ML) method. ML trees were inferred with the software raxmlGUI 2.0 (Edler et al. 2020) assuming the GTRGAMMA model, and bootstrap values were calculated from 500 pseudoreplicates. Fungal nrITS and RPB2 sequences were concatenated for phylogenetic inference of the fungal tree, as there are no major topological conflicts (> 75% bootstrap support) between the fungal nrITS tree and RPB2 tree. The concatenated fungal sequence alignment contains 1293 nucleotide positions (nrITS: 536; RPB2: 757). The evolutionary model for each partition was assessed using PartitionFinder v.2 (Lanfear et al. 2012) under the corrected Akaike information criterion: HKY + G model was selected for fungal nrITS, and TrNef for fungal RPB2. MrBayes v3.2 (Ronquist et al. 2012). Bayesian inference (BI) was also implemented to infer phylogenetic relationship using the selected evolutionary model for each partition. Bayesian analyses were run for ten million generations on four chains (one cold and three heated) where sampling was taken every 500 generations using Markov Chain Monte Carlo (MCMC) sampling. Two MCMC runs were carried out, and convergence between runs was checked for the average standard deviation of split frequencies (below 0.005). The first 25% trees were discarded. Phylogenetic trees were visualized using FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). For the estimation of the algal tree, *A. gaertneri* Škaloud et Peksa was selected as outgroup for the algal nrITS tree according to its sister relationship to the three annotated *Asterochloris* species (Moya et al. 2015). The evolutionary model HKY was selected for the algal nrITS sequences. Both ML and BI methods were used for the phylogenetic analyses as performed for fungal trees.

2.7 Statistical analysis

The statistical analyses for comparing concentrations were carried out with the R software version 3.6.3 (R Core Team 2020). Data normality was checked, and then T-test was applied pairwisely for comparing concentrations of samples containing the three algal photobionts.

Redundancy analysis (RDA) was carried out for analysing the effect of symbionts (species of the photobiont; categorical variable), substrate (acidic or basic; categorical variable), elevation of the collecting sites (numerical variable in metres), season of collecting date (spring or autumn; categorical variable) on the UA and FA concentration of the sampled specimens. To equalize the weight of the two examined LSMs, UA and FA concentrations were standardized to mean = 0 and standard deviation = 1. The effects of the predictors were tested using Wald tests and variation partitioning. The *p*-values were assessed by permutation tests, and they refer to individual unshared effect for each variable. The relationship between the individual LSMs and

the same dependent variables as in the RDA redundancy analysis were further tested independently by multiple linear regression (MLR). We also performed variation partitioning to explore shared effects of the explanatory variables. The redundancy analysis and its evaluation were carried out by the rda, anova.cca and varpart functions of the vegan (Oksanen et al. 2022) R package.

3 Results

3.1 Identification and quantification of LSMs

3.1.1 Compound identification

The collected samples (20 from Hungary, 1 from Slovakia, 7 from Albania and 1 from North Macedonia) were initially screened by high performance thin layer chromatography (HPTLC) and the bands corresponding to UA and FA were identified (Supplementary Fig. S5). The identification of the major LSMs, i.e. UA and FA, by ultrahigh performance liquid chromatography-photodiode array detection-mass spectrometry (UHPLC-PDA-MS) are shown in Fig. 3. These were initially identified by their retention times compared with standards: FA at 5.02 min and UA at 7.63 min. Compounds identified were further verified by their UV and MS spectra. FA shows UV maximums at 240 and 316 nm, while UA has maxima at 235 and 281 nm. MS spectrum of FA has the base peak at m/z 355 as the major fragment ion, and the deprotonated molecular ion is m/z 471 (Xu et al. 2018). UA has the deprotonated molecular ion as the base peak at m/z 343 (Xu et al. 2017). The MS total ion current chromatogram (bottom one in Fig. 3a) shows a peak at m/z 387.07, which is annotated as methyl protocetraric acid according to published MS data on French *C. convoluta*/*C. foliacea* (Bézivin et al. 2004). This compound is in minor quantity and not detected by the PDA (upper chromatogram in Fig. 3a).

3.1.2 Quantification of LSMs

The FA content was analysed in 29 specimens by HPLC–PDA and it was shown to be 1.44–9.87 mg/g dry wt, lower than that of UA (Table 1). All the 29 specimens were analysed by chiral chromatography and the presence of (-)-UA was confirmed and quantified (Table 1). The chiral chromatogram (Fig. 4) shows that only the (-)-UA was produced by the lichenised fungus *Cladonia foliacea* in all samples. The (+)-UA was not detected at the current limit of detection (ca. 18 ng/ml). In addition, the content of (-)-UA shows a substantial variation, ranging from 6.88 to 34.27 mg/g lichen dry wt, and thus the isomer could account for up to 3.4% lichen dry wt (Table 1).

3.2 Phylogenetic analysis

All 29 specimens were amplified by PCR and sequenced. In total 87 new sequences were generated from this study, and their GenBank accession numbers are listed in Supplementary Table S1 (available online): fungal nuclear ribosomal internal transcribed spacer (fungal nrITS) OQ696346-OQ696374; algal nrITS OQ985218-985,246; fungal second largest subunit of RNA polymerase II (RPB2) OQ995179-OQ995207.

3.2.1 Fungal tree

BLAST search suggested that fungal nrITS sequences are all *C. foliacea*, which is also shown in Supplementary Figure S2. Two well supported monophyletic clades are shown in the fungal tree (Fig. 5), constructed from a combined matrix containing fungal nrITS and RPB2 loci. Phylogenetically informative nucleotide variations are mainly from the RPB2 sequences. The two monophyletic clades are also found when reference *C. foliacea* RPB2 sequences were incorporated (Supplementary Fig. S3). Clade 1 in Fig. 5 of the fungal tree contains mostly the *C.'convoluta'*-like form and came from calcareous substrates and lower elevation sites. There were various exceptions (e.g. sample CLF22 – *C.'foliacea'*-like form came from an acidic substrate in high elevation; others differed either in morphology (e.g. sample CLF15) or substrate type (e.g. sample CLF18) or elevation (e.g. samples CLF21, CLF28). Clade 2 in Fig. 5 contains specimens mostly from acidic substrate type and high-elevation (Mediterranean) collections, and the specimens were either *C.'foliacea'*-like or *C.'convoluta'*-like morphology or between *C.'foliacea'*- and *C.'convoluta'*-like morphology. The UA contents as well as FA contents between two clades were compared, and no significant differences were found.

3.2.2 Algal tree

The algal nrITS phylogram (Fig. 6) shows three monophyletic clades belonging to three species of *Asterochloris* photobionts. These photobionts were identified by comparing with reference algal nrITS sequences (Supplementary Fig. S4). *A. lobophora* Škaloud et Peksa were from *C.'convoluta'*-like specimens growing on calcareous substrata under 200 m, and consisted of all lowland specimens (CLF16–17, CLF19) or low elevation (200–600 m) rocky grassland ones (CLF1–2). Specimen CLF15 should be like the specimens CLF16–18 depending on the habitat, but divided lobes were found in the majority. *A. woessiae* Škaloud et Peksa was found more often in acidic substrate-colonising specimens from higher elevations. Since the higher elevation specimens were only collected

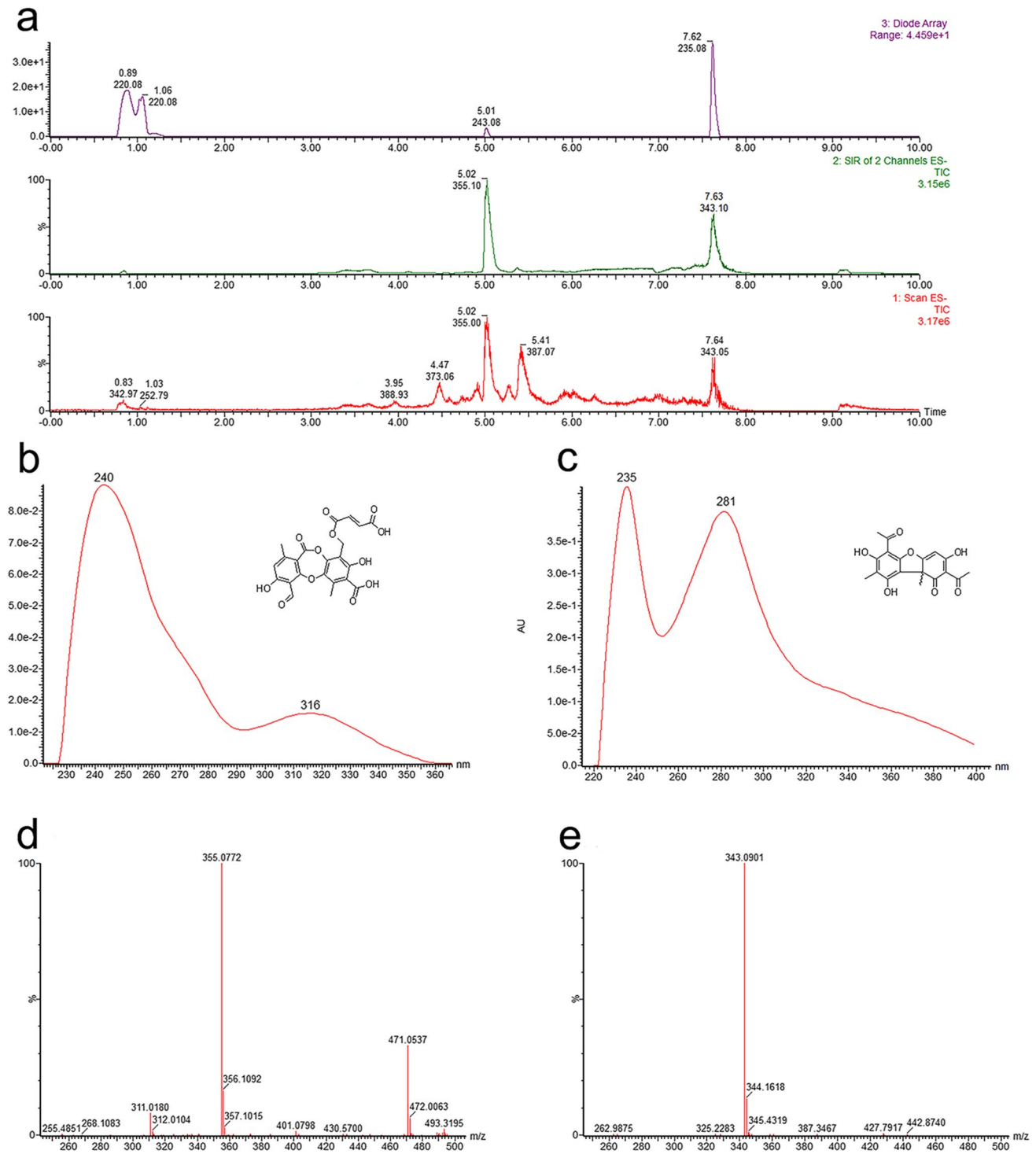

Fig. 3 Identification of lichen secondary metabolites using UHPLC-PDA-MS. **a** Chromatogram with PDA detection (top), single ion monitoring (middle) and total ion current (bottom), **b** UV spectrum and structure of fumarprotocetraric acid, **c** UV spectrum and structure of usnic acid, **d** MS spectrum for fumarprotocetraric acid, **e** MS spectrum for usnic acid

from Albania and North Macedonia, these appear to be of Mediterranean distribution. The North Macedonian specimen was however, from calcareous substrate. According to the literature (Poelt and Vězda 1977), the *C.'foliacea'*-like specimens should be more likely to occur on acidic substrate (and in rocky grasslands at higher elevation). Here both *C.'foliacea'*-like and *C.'convoluta'*-like specimens were found, although *C.'convoluta'*-like ones were more

Table 1 Mean concentrations (with standard deviation) of fumarprotocetraric acid (n = 5) and (-)-usnic acid (n = 3) in the collected samples (More details found in Supplementary Table S1)

Sample ID	Fumarprotocetraric acid* [mg/g dry wt]	(-)-usnic acid** [mg/g dry wt]
CLF1	5.36 ± 0.63	24.56 ± 1,13
CLF2	4.57 ± 0.58	34.27 ± 2,43
CLF3	5.54 ± 0.90	17.04 ± 0,41
CLF4	4.70 ± 0.41	21.58 ± 0,49
CLF5	4.09 ± 0.48	21.95 ± 1,19
CLF6	5.04 ± 0.37	10.24 ± 0,46
CLF7	5.05 ± 0.46	12.36 ± 0,53
CLF8	5.45 ± 0.27	8.05 ± 0,40
CLF9	4.34 ± 0.24	11.96 ± 0,51
CLF10	3.64 ± 0.38	11.11 ± 0,25
CLF11	4.60 ± 2.05	10.35 ± 0,37
CLF12	6.06 ± 1.62	19.51 ± 1,15
CLF13	4.41 ± 0.30	11.96 ± 0,73
CLF14	5.30 ± 0.87	15.35 ± 0,55
CLF15	3.29 ± 0.36	24.06 ± 0,70
CLF16	1.94 ± 0.14	7.37 ± 0,32
CLF17	1.71 ± 0.21	16.33 ± 0,68
CLF18	3.03 ± 0.73	6.88 ± 0,26
CLF19a	1.44 ± 0.07	16.37 ± 0,59
CLF19b	2.45 ± 0,04	30.55 ± 0,44
CLF20	5.28 ± 0.78	16.14 ± 0,23
CLF21	9.87 ± 2.27	19.87 ± 0,50
CLF22	4.60 ± 0.62	15.04 ± 0,22
CLF23	3.85 ± 0.57	17.38 ± 0,70
CLF24	4.90 ± 0.58	9.83 ± 0,19
CLF25	6.78 ± 0.59	21.27 ± 0,27
CLF26	5.98 ± 0.48	20.68 ± 0,13
CLF27	5.80 ± 0.19	13.35 ± 0,45
CLF28	7.02 ± 0.29	11.59 ± 0,22

* Mean concentration measured by HPLC–PDA. ** Mean concentration measured by chiral HPLC

frequent. All noted morphological and habitat (substrate type, elevation) features in specimens containing *A. mediterranea* E. Barreno et al. showed a high diversity. UA and FA contents when compared to specimens containing other algal partners which shows the importance of algae in quantitative variations (Fig. 7). It is interesting that specimens associated with the alga *A. lobophora* (AL group in boxplots of Fig. 7) tend to have higher UA contents, but the difference was not significant. Similarly the comparison of total metabolite contents (UA + FA) between different photobiont groups (Supplementary Fig. S6) resulted in no significant ($p < 0.05$) differences. On the other hand, FA content in the *A. lobophora* (AL) group was significantly lower than that in the *A. mediterranea* (AM) group ($p < 0.01$) and in the *A. woessiae* (AW) group ($p < 0.05$). Here, an „inverse" trend in the quantities of UA vs. FA seems discernible, despite the lower significance among the UA differences.

To better define any differences in LSM pattern to photobiont types, a scatter plot was constructed by plotting the contents of UA against the contents of FA (Fig. 8a). Lichens containing the *A. lobophora* photobiont (AL) tend to separate from lichens containing *A. mediterranea* (AM) and *A. woessiae* (AW). The AL group seems to have relatively lower FA contents but relatively higher UA contents (Fig. 8a). In fact, by combining the Fig. 7 data as UA/FA ratios according to photobiont groups (Fig. 8b), significantly ($p < 0.01$) higher UA/FA ratio is observed in the lichens containing *A. lobophora* as the major photobiont – the AL group.

3.2.3 Model evaluation

The evaluation of RDA and MLR is summarized in Table 2. According to the RDA, the algal symbiont, the substrate, the elevation, and the collecting date altogether explained 42.8% ($F = 3.447$, $p = 0.001$) of the total variance in the two LSM concentrations. Within these models, the algal symbiont had the strongest effect ($F = 4.439$, $p = 0.005$), followed by the substrate type ($F = 4.102$, $p = 0.018$) and the date ($F = 3.272$, $p = 0.036$), while the effect of altitude was not significant ($F = 1.004$, $p = 0.342$). In addition, variation partitioning revealed that the altitude and date had no interpretable unshared effect; their explained variations are due to shared effect with the algal symbiont. In contrast, the largest parts of the effect of the algal symbiont and the substrate are unshared (Fig. 9). The MLRs revealed that the concentration of FA is influenced by the same variables in similar magnitudes: the algal symbiont with the strongest effect ($F = 7.519$, $p = 0.003$), followed by substrate ($F = 6.260$, $p = 0.020$), while altitude ($F = 0.778$, $p = 0.387$) and date ($F = 1.649$, $p = 0.212$) with negligible effects. In total, this model explained 51.0% of the total variation of FA ($F = 4.796$, $p = 0.004$). In contrast, the same set of variables had much weaker effect on the UA concentration. None of the variables had significant individual effect (see Table 2), and the total model explained only 34.6% variation ($F = 2.437$, $p = 0.065$).

4 Discussion

Both UA and FA contents in *Cladonia foliacea* were comparable to those previously measured (Farkas et al. 2020a, b; Veres et al. 2022a, b. This is the first study using chiral chromatography to confirm the production of (-)-UA in *C. foliacea* collected in Hungary and nearby countries. Our wider sampling provided more support for the enantiomer

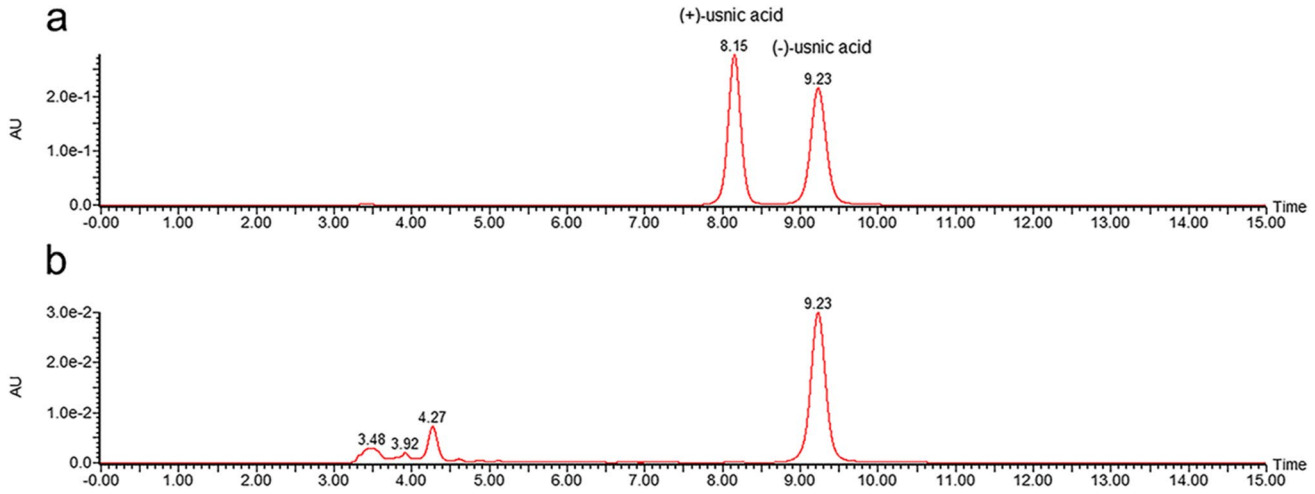

Fig. 4 Chiral separation of the usnic acid enantiomers. **a** The chiral HPLC chromatogram showing separation of the usnic acid enantiomer mixture standard: (+)-usnic acid elutes at 8.15 min and (-)-usnic acid elutes at 9.23 min. **b** Sample chromatogram showing that only (-)-usnic acid is present in *Cladonia foliacea*

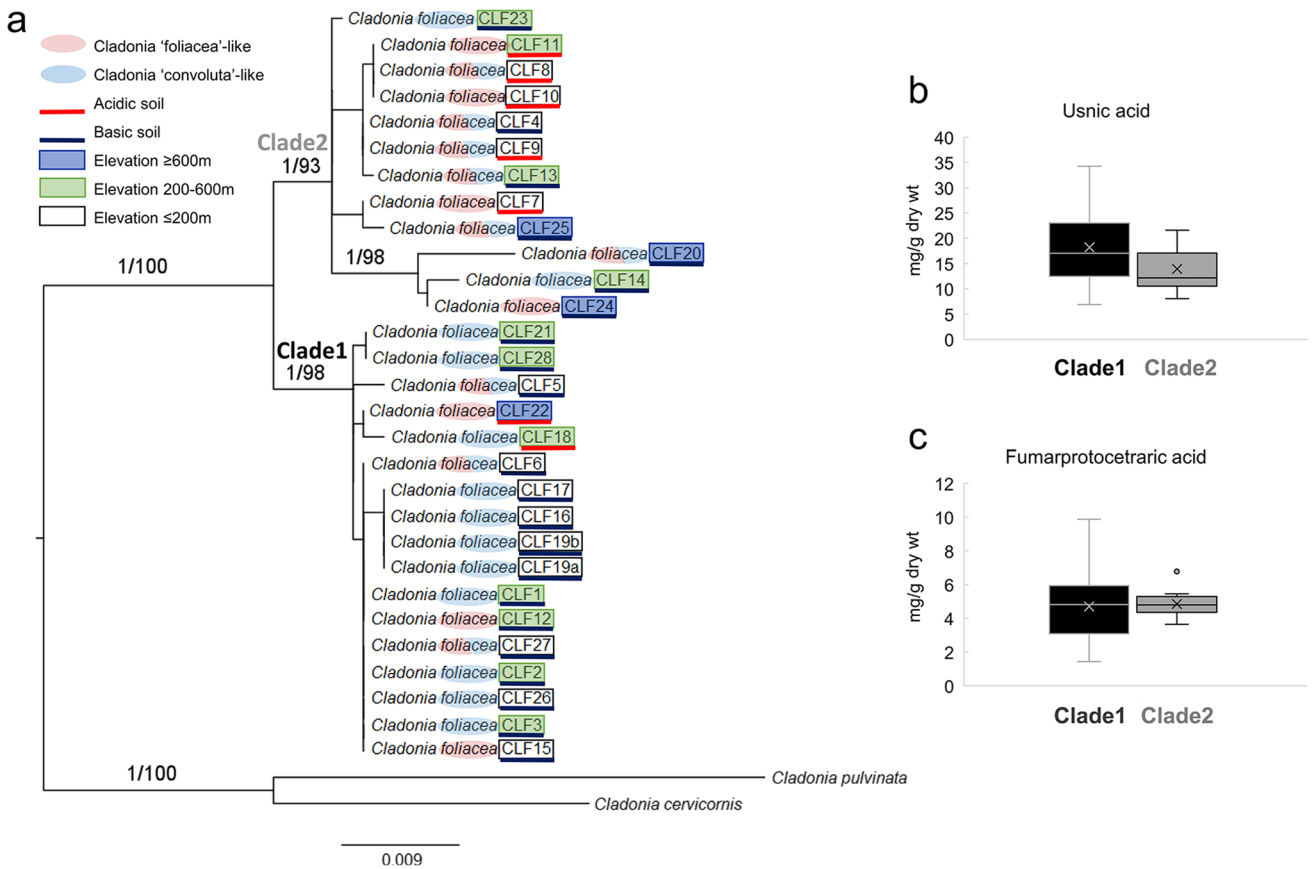

Fig. 5 **a** The Maximum Likelihood fungal tree of *Cladonia foliacea* using a concatenated matrix of fungal nrITS and RPB2. Specimen ID (CLF1-28) is provided (Supplementary Table S1). Posterior probabilities (PP) from Bayesian inference and bootstrap values (BS) from Maximum Likelihood method are shown at each node as PP/BS. **b** Concentration of UA. **c** Concentration of FA. In the boxplots, the vertical lines span the minimum and maximum values, and the boxes include from 25 to 75% of the data, with the horizontal line representing the median. Compound concentrations between clades are not significantly different ($p \geq 0.05$)

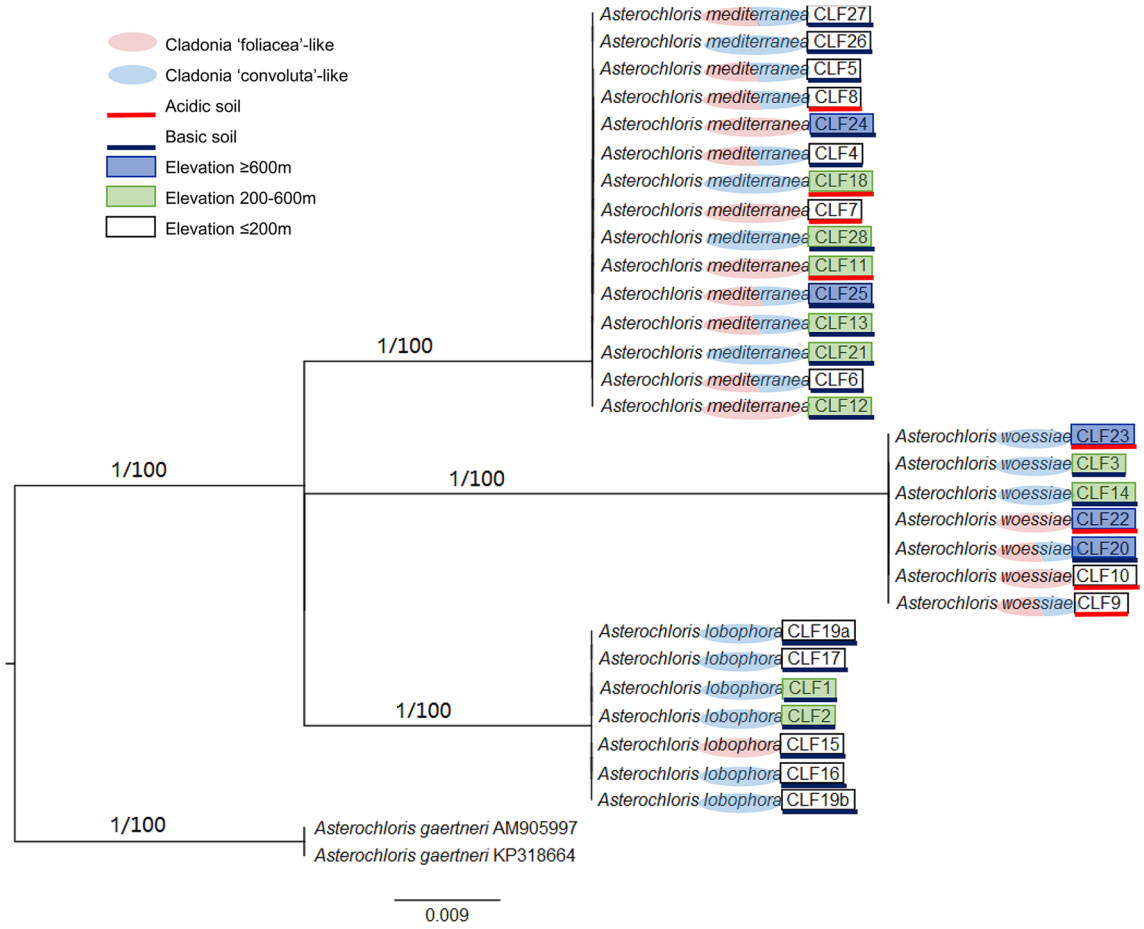

Fig. 6 Algal nrITS gene tree of *Cladonia foliacea*. Specimen ID (CLF1-28) is provided (Supplementary Table S1). Posterior probabilities (PP) from Bayesian analysis and bootstrap values (BS) from Maximum Likelihood method are shown at each node as PP/BS

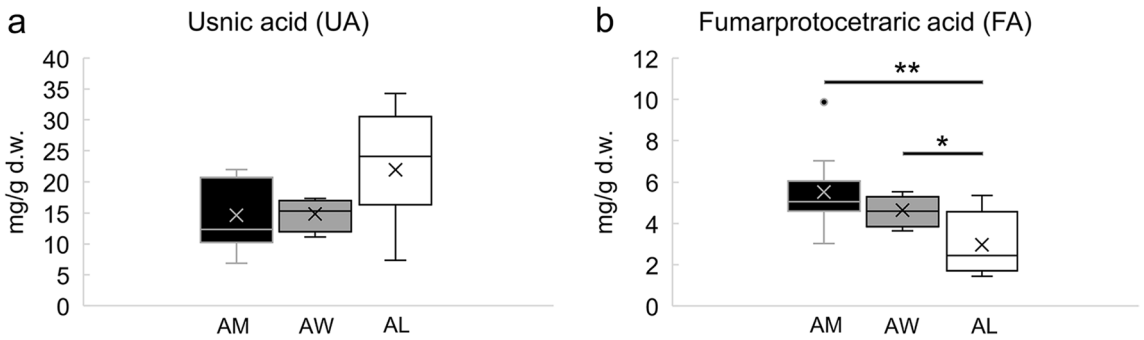

Fig. 7 Lichen secondary metabolite contents in relation to algal partners associated with *Cladonia foliacea*. **a** Concentration of UA according to photobionts. **b** Concentration of FA according to photobionts. In the boxplots, the lines represent the minimum and maximum values; the boxes include from 25 to 75% of the data, with the horizontal line representing the median. Levels of statistical significance were marked with asterisks, where * and ** indicate *p*-values under 0.05 and 0.01, respectively. Abbreviations: AL − *Asterochloris lobophora*, AM − *Asterochloris mediterranea*, AW − *Asterochloris woessiae*, FA − fumarprotocetraric acid, UA − usnic acid

production pattern in the lichen, and also provided direct evidence that (+)-UA is not present in detectable amounts. Previously isolated (-)-UA from Italian (Cavalloro et al. 2021) and French samples (Bézivin et al. 2004), supports that *C. foliacea* is conservative in only producing the (-)-UA isomer. The quantitative variation in these lichen metabolites

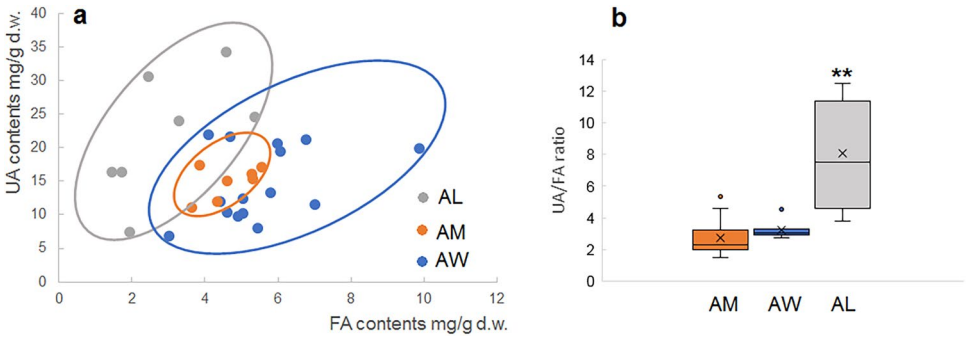

Fig. 8 Lichen secondary metabolite contents in relation to algal partners associated with *Cladonia foliacea*. **a** A scatter plot showing patterns according to photobionts. **b** A bar plot showing significantly higher UA/FA content ratio in the specimens containing the AL photobiont. Level of statistical significance were marked with asterisks, where ** indicate *p*-value under 0.01. Abbreviations: AL − *Asterochloris lobophora*, AM − *Asterochloris mediterranea*, AW − *Asterochloris woessiae*, FA − fumarprotocetraric acid, UA − usnic acid

is large (5–6-times difference between the lowest and the highest) in both UA (6.88 to 34.27 mg/g dry wt) and FA (1.44 to 9.87 mg/g dry wt) content among the various tested lichen specimens. There was also up to two-fold differences between the specimens collected in the same or very near sites (e.g. samples CLF1–3, CLF4–6; CLF16–17, CLF19a–19b or CLF26–27).

Previous studies (Veres et al. 2022b) showed that FA concentration is higher ($p < 0.0001$) in the specimens collected in mountain areas than those in the lowland, whereas the UA concentration was significantly higher ($p < 0.0001$) in lowland samples than in mountain samples. The current study did not support these findings (Fig. 10). The difference may be explained by different sampling strategies. Sampling in our previous study (Veres et al. 2022b) was done regularly every six months at the same time (of year within 3 years in Hungary. In the current study, samples were collected at various months and seasons over some 10 years and across a broader geographic range. The seasonal variation is the result of alternating periods of decomposition and light-induced production phases as a result of variation in humidity as was shown earlier (Veres et al. 2022a). In that study a significant ($p < 0.0001$) seasonal change in UA concentration was found in *C. foliacea*, which was lower in summer and spring, and higher in winter and autumn. Furthermore, UA did not differ significantly between thalli collected in sunny and shaded sites. The conclusion is that UA and FA contents may be affected by temporal and environmental factors.

Despite temporal, environmental and geographic effects on the fluctuation of UA and FA contents, in this study we still found significant differences of FA contents and UA/FA ratios between specimen groups differing in algal partners. *Asterochloris mediterranea, A. lobophora* and *A. woessiae* have been reported to be associated with the mycobiont of *C. foliacea* (Moya et al. 2015, Pino-Bodas and Stenroos 2021, Škvorová et al. 2022). Previous studies have shown that climatic factors (e.g. sun exposure, rain precipitation, etc.) and soil chemistry regulate photobiont switching in the genus *Cladonia* (Peksa and Škaloud 2011, Pino-Bodas and Stenroos 2021; Škvorová et al. 2022). Specimens containing the photobiont *A. lobophora* stand out by having a significantly higher UA/FA ratio than the other photobiont groups (Fig. 8/b). Interestingly, a comprehensive study (Škvorová et al. 2022) linking symbiotic associations to climatic factors shows that mycobionts associated with *A. lobophora* represent a unique symbiosis, different from the symbiotic form for association with *A. mediterranea* or *A. woessiae*. The association with *A. lobophora* tends to prefer lower isothermality, lower evapotranspiration and higher precipitation of warmest quarter during the year (Škvorová et al. 2022) and appears to be restricted to calcareous substrates in low

Table 2 Evaluation statistics for models of relationship between explanatory variables (algal symbiont, substrate, altitude, collection date) and response variables (UA and FA contents)

Redundancy analysis on FA and UA concentrations			
explanatory variable	*df*	*F*	*p*
algal symbiont	2	4,4287	0,00199
substrate	1	4,016	0,02283
altitude	1	0,5835	0,5652
date	1	0,7226	0,49,443
Multiple linear regression on FA			
explanatory variable	*df*	*F*	*p*
algal symbiont	2	7,5192	0,003071
substrate	1	6,2597	0,019908
altitude	1	0,7783	0,386,803
date	1	1,6486	0,211,929
Multiple linear regression on UA			
explanatory variable	*df*	*F*	*p*
algal symbiont	2	2,1141	0,1436
substrate	1	2,3357	0,1401
altitude	1	0,4376	0,5148
date	1	0,0291	0,8661

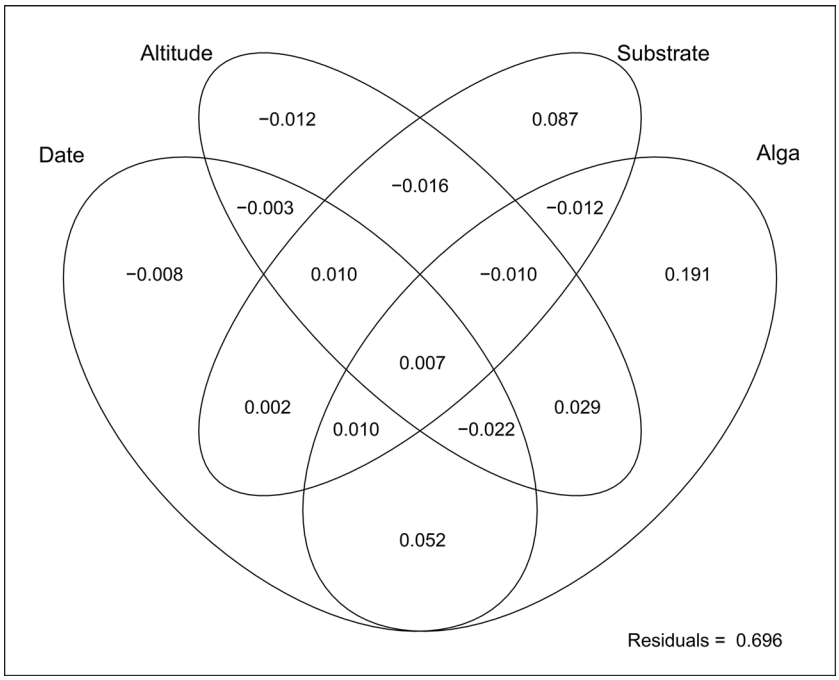

Fig. 9 Variation partitioning of explanatory variables (algal symbiont, substrate, altitude, collection date). Adjusted R-squared (R^2) values below 0 are not interpreted

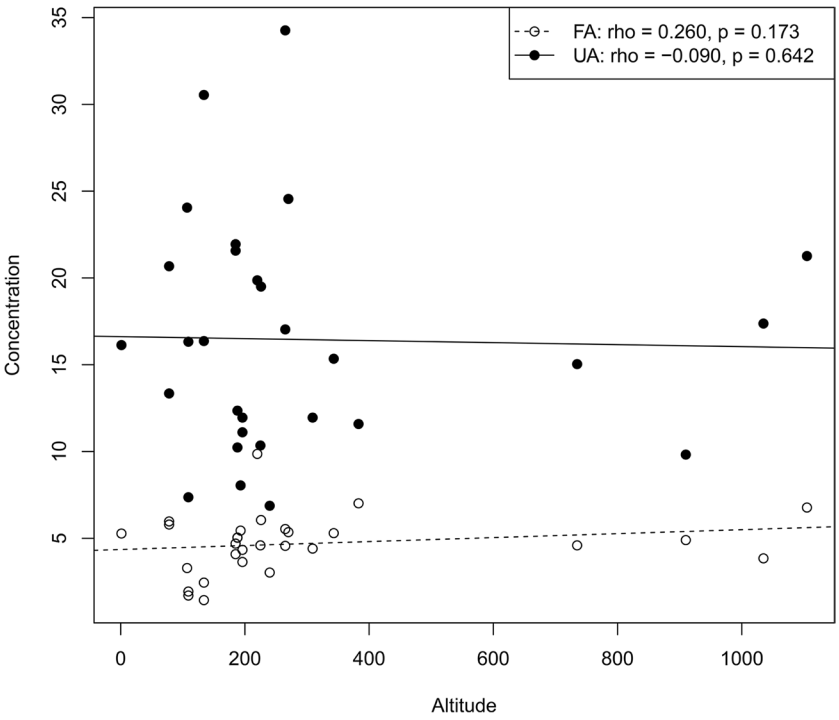

Fig. 10 Linear regressions (UA – top; FA – bottom) presenting lichen secondary metabolite contents (mg /g dry wt) in relation to altitude (m) of collecting sites of *Cladonia foliacea* samples. Spearman's rank correlation coefficients (ρ) are 0.260 and 0.090; *p*-values 0.173 and 0.642 for FA and UA respectively. Abbreviations: FA − fumarprotocetraric acid (open circles), UA − usnic acid (dots)

elevation less than 280 m in Central European samples (Supplementary Table S1).

Taking all environmental and biological variables into account, the RDA and MLR models indicated the impact of photobiont species on the quantitative variation of LSMs, particularly on the production of FA. However, the tested variables could not explain the significant variation in UA concentration.

Similar quantitative LSM variations (with no significant UA variation, but significant in FA variation) have been reported in *Usnea antarctica* (Bokhorst et al 2021). Low FA contents are associated with high nitrogen availability in Antarctic. This is also true in boreal forest lichens where high nitrogen availability may lower the LSM contents (Solhaug and Gauslaa 2012). It will be interesting to test the nitrogen contents in *C. foliacea* thalli in future studies.

According to former studies (Engelen et al. 2010; Osyczka et al. 2021; Romeike et al. 2002; Vančurová et al. 2021) lichens facing to stress caused by their environment (*cf.* light stress caused by a considerable UV and solar radiation in open vegetation, like lowland steppe or rocky grassland which are the typical habitats of the here studied *C. foliacea*) have lower selectivity in their photobiont partner. It explains why *C. foliacea* has various photobionts over the investigated areas across Europe where the environmental tolerance of the partners are expected to be matching (Casano et al. 2011; Peksa et al. 2022). The mechanisms for tolerating desiccation-rehydration stress evolved differently in two *Trebouxia* species according to habitat humidity (Catalá et al. 2016; Centeno et al. 2016; Hell et al. 2019, 2021). A species-specific induction of the antioxidant system supposedly may exist in various *Asterocholoris* species though so far only the adaptation of *A. irregularis* containing *Cladonia borealis* in Antarctica has been studied by measuring ecophysiological responses to various microclimatic conditions (Cho et al. 2020). As shown under controlled experimental conditions by Culberson and Armaleo (1992) the regulation of light influenced the changes in temperature and water relations, and this latter has the most important role in the induction of LSM production by the fungal partner even without the photobiont. Based on this former knowledge Armaleo et al. (2008) carried out LSM measurements in the corticolous *Parmotrema hypotropum* (Nyl.) Hale collected from controlled natural environment (Durham, North Carolina, USA), where radiation was restricted to be the only variable. They could confirm that changing light conditions directly determined changes in temperature and humidity but with opposite trends: the growing temperature followed the increase of light and the tendencies were different in case of humidity. Though no microclimatic parameters of collecting sites were measured in our investigations, it can be assumed that these correlations exist in nature in general and are valid also at the investigated natural habitats of *C. foliacea*. The opposite trends in the change of environmental conditions (i.e., temperature and humidity) was coupled with opposite trends in the production between cortical and medullary LSMs (Armaleo 1995; Armaleo et al. 2008; Culberson and Armaleo 1992; Hamada 1981, 1996; Hamada and Miyagawa 1995; Hamada et al 1996). Similar trends can be observed comparing the production of the cortical UA and medullary FA in specimens of *C. foliacea* containing different *Asterochloris* species (Figs. 7 and 8) in the current study. Comparing the sites where these lichen specimens were collected, it can be concluded that the *A. lobophora* containing specimens favour calcareous lowland sites where due to the high radiation, the higher temperature and more arid climate induce an increased production of UA in the cortex and opposite trends are prevailing in the production of FA in the medulla. Thus the various associated algal species (based on the RDA and LMR models) explaining largely the quantitative variation of LSMs produced by the fungal partner serve as a kind of indicators concerning to the microclimatic parameters (esp. humidity), and they are practically uniting and representing the otherwise not measured variables.

Furthermore, the less obvious variations in LSM concentration values can also be explained by the differences of the here investigated *C. foliacea* thalli originating from various environmental conditions (i.e., light, temperature, moisture) from the *P. hypotropum* thalli naturally growing among very similar conditions in the North Carolinian study (Armaleo et al. 2008). Additionally, the morphology of *C. foliacea* thalli also resulted in a varied surface with its often folding nature showing upper and lower surface of lobes alternating towards light.

Recent advances in fungal genomics and molecular biology have considerably broadened our knowledge about the biosynthesis of LSMs, which are mostly catalysed by polyketide synthase and specific oxidative enzymes, e.g. cytochrome P450 (Armaleo et al. 2011; Singh et al. 2021a, Singh 2023). Considerable intraspecific variation of biosynthetic genes has also been reported at the population level (Singh et al. 2021b). These studies explain qualitative, but not quantitative LSM variations. The correlation we found between photobiont species and LSM quantity in *C. foliacea* could have several explanations. It has been hypothesized that algae may produce signalling substances mediating the transformation between protocetraric acid and FA (Diaz et al. 2016) or inhibiting the oxidation from depside to depsidone (Armaleo 1995). Algae may broadly influence LSM contents by controlling the supply of photosynthesized carbohydrates to lichen-forming fungi, and algal species may differ in their carbohydrate production capacity. A study in which cultured *C. rangiferina* was fed with different carbohydrates, showed that only ribitol, a common transfer-polyol in lichens (Richardson et al. 1968) induced the production of FA from the fungus (Elshobary et al. 2016). However, changing algal carbohydrate fluxes alone cannot explain why cortical LSMs may increase while medullar LSMs decrease in the same lichen in response to the same stimulus (Armaleo et al. 2008, and references therein). Our data indicate that *A. lobophora,* in contrast to *A. mediterranea* and *A. woessiae*, prefers lower elevations, i.e. drier and warmer locations (Fig. 6). The lichens with *A. lobophora* contain the highest amounts of cortical UA and the lowest amounts of medullar FA (Figs. 7 and 8). Combining these findings with the data in Armaleo et al. (2008) which show a similar behaviour of cortical atranorin *vs.* medullary norstictic acid in response to light, temperature and moisture, we hypothesize that the correlation observed between algal species and lichen LSMs is due to the fact that the alga tracks the same light, moisture and temperature conditions that influence the production of an adaptive ratio of cortical *vs.* medullary LSMs.

In addition to the present results in LSM production, analysis of data for the circumscription of *C. foliacea* was performed. The previously described "morph *convoluta*" (Pino-Bodas et al. 2018), i.e. *C. convoluta* morphotype, has a broad geographic distribution in Europe that covers our sampling area. Specimens representing this morphotype occur with *C. foliacea* specimens (Supplementary Fig. S2 and S3), instead of forming their own monophyletic clade. Our phylogenetic analyses using either fungal nrITS or RPB2 loci did not support the monophyly of *C. convoluta* or the *C. convoluta* morphotype. The RPB2 gene tree shows two monophyletic clades, while each clade contains mixed morphotypes and habitat types. We have incorporated published sequences from *C. convoluta* and *C. foliacea* into our RPB2 data matrix, and the two monophyletic clades still exist with high bootstrap support values (Supplementary Fig. S3). In addition, we also found intermediate morphological forms between *C. convoluta* and *C. foliacea* morphotypes, that varied with habitat types, which has also been reported before (Pino-Bodas et al. 2010; Burgaz and Escudero 1993). All lines of evidence suggest the presence of incomplete lineage sorting (Pino-Bodas et al. 2010). This species delimitation challenge may be resolved by using advanced methods, such as phylogenomics.

It was shown that lichens of the same habitat share a group of photobiont specific to their environment (Peksa et al. 2022). It is not rare that a lichen is associated with different photobionts and thus we found three species of the four taxa (OTUs) known from *Cladonia foliacea* worldwide (Pino-Bodas and Stenroos 2021). The increased number of photobionts support adaptation to various environmental conditions and it explains why this species is a relatively widely distributed species. Current study added interesting knowledge regarding the ecological requirements (symbiotic feature / partner selection and habitat preference) of three *Asterochloris* species (*A. lobophora, A. mediterranea* and *A. woessiae*). Selection of diverse photobionts allows the mycobiont to adapt to different habitats of the studied sites (Vančurova et al. 2018). From a global study, the alga *A. lobophora* differs from *A. mediterranea* and *A. woessia* in its habitat, being more common in anthropogenic habitats, especially soils rather than rocks (Vančurova et al. 2018). The present study shows that the lichens associated with *A. lobophora* prefer relatively low altitude and basic soil substrates, and *A. mediterranea* has a much wider geographical distribution. *A. mediterranea* is previously reported as a frequent photobiont associating with different lichen-forming fungi from the Canary Islands, Madeira, Sicily, and the Aeolian Islands (Vančurová et al. 2021), and it was also detected in specimens from Central Europe (Škvorová et al. 2022). The presence of this species also predicts an adaptation to habitats with considerable water deficit for extended periods (Pino-Bodas and Stenroos 2021).

5 Conclusions

This study combined phytochemical, phylogenetic and statistical analyses, in order to investigate the association between environmental / biological variables and LSM contents in the lichen *Cladonia foliacea*. The results showed that among these variables, the type of photobiont has the most significant effect on quantitative LSM variations, particularly for FA and probably for UA in *C. foliacea*, in addition to previously recognized environmental factors. We suggest that the correlation between algal type and LSM is due to the fact that the alga tends to track the environmental factors that directly affect LSM production by the mycobiont. This insight suggests taking also the alga partner into consideration for future studies investigating quantitative LSM variation, instead of solely focusing on the fungal partner.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13199-024-00982-8.

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Author contributions Conceptualization: Edit Farkas, Arthur Macharia Muhoro and Maonian Xu; Methodology: Edit Farkas, Krisztina Szabó, Attila Lengyel and Maonian Xu; Formal analysis: Edit Farkas, Maonian Xu, Arthur Macharia Muhoro and Attila Lengyel; Funding, resources: Edit Farkas, Elín Soffia Ólafsdóttir and Starri Heiðmarsson; Data curation: Edit Farkas, Krisztina Szabó and Maonian Xu; Writing—original draft preparation: Edit Farkas and Maonian Xu; Writing—review and editing: Edit Farkas, Maonian Xu, Arthur Macharia Muhoro, Krisztina Szabó, Attila Lengyel, Elín Soffia Ólafsdóttir, Elvar Örn Viktorsson and Starri Heiðmarsson; Visualization: Edit Farkas and Maonian Xu; Supervision: Edit Farkas, Elín Soffia Ólafsdóttir, and Starri Heiðmarsson. All authors have read and agreed to the published version of the manuscript.

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Data availability The following public databases were used: IndexFungorum (http:// www.indexfungorum.org/, accessed on 20 March 2023); Index Herbariorum (http://sweetgum.nybg.org/science/ih/, accessed on 20 March 2023); MycoBank (https://www.mycobank.org/, accessed on 20 March 2023); Recent literature on lichens (http://nhm2.uio.no/botanisk/lav/ RLL/RLL.HTM, accessed on 10 March 2023). The data presented in this study are available in the Lichen Herbarium VBI (Vácrátót, Hungary) [see Index Herbarium at above link] and

in the GenBank, if otherwise not, then data are available on request from the corresponding author [Edit Farkas] and the second author [Maonian Xu].

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Compliance with Ethical Standards Experimental research and field studies on the collected lichens in nature comply with relevant institutional, national, and international guidelines and legislation. No threatened species according to IUCN (IUCN Policy Statement on Research Involving Species at Risk of Extinction – https://portals.iucn.org/library/efiles/documents/PP-003-En.pdf, accessed on 14 August 2023) were involved in the current research.

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