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Abstract

The lichen *Cetraria islandica* or Iceland Moss is commonly consumed as tea, food ingredients (e.g. in soup or bread) and herbal medicines. *C. islandica,* which has two chemotypes, can be difficult to distinguish from the sister species *Cetraria ericetorum*. They are collectively referred to as the *Cetraria islandica* species complex. This study aimed to use an UPLC-QToF-MS chemical profiling together with DNA barcoding to distinguish species and chemotypes of the *C. islandica* species complex. Our results show that the two chemotypes of *C. islandica* are clearly distinguishable from each other and from *C. ericetorum* by the chemometric approach. The RPB2 barcode was able to differentiate *C. islandica* from *C. ericetorum* with a barcode gap, but the widely used nrITS barcode failed. Neither of them could discriminate chemotypes of *C. islandica*. In conclusion, this integrative approach involving chemical profiling and DNA barcoding could be applied for authentication of Iceland Moss materials.

Keywords: *Cetraria islandica*, *Cetraria ericetorum*, DNA barcoding, chemical profiling, authentication

1. Introduction

Taxonomically, *Cetraria islandica* or Iceland Moss is not a moss species but a lichen taxon and the classification is based on the symbiotic fungal partner (Parmeliaceae, ascomycete) (Ingolfsdóttir, 2000). This lichen is consumed in Iceland as tea, food ingredients (e.g. in milk soups and bread) and herbal medicines (Xu et al., 2016). Considerable morphological and chemical variations have been found among Icelandic populations of *C. islandica* and two chemotypes have been reported (Kristinsson, 1969). They consist of the fumarprotocetraric acid (FA)-producing and FA-deficient races, where the latter chemotype has exclusively been found in Iceland (Kristinsson, 1969). Traditional use seems to favor the FA-deficient chemotype of *C. islandica*, which is believed to be less bitter (Kristinsson, 1968). Furthermore, the species boundaries between *C. islandica* and its sibling species *Cetraria ericetorum* are still ambiguous: *C. ericetorum* has similar morphology to certain morphotypes of *C. islandica* and it is reported to be FA-deficient. Together *C. islandica* and *C. ericetorum* are collectively called the *Cetraria islandica* species complex (Kristinsson, 1969; Thell, Stenroos, & Myllys, 2000). Their chemical profiles, particularly of the *C. islandica* FA-deficient chemotype and *C. ericetorum*, have not been thoroughly investigated for food safety, and an accurate identification method for these lichen materials is needed.

Chemical profiling or fingerprinting, in particular when using an untargeted approach, can directly detect chemical hazards and contaminants in food or herbal materials, with the limitation that their species sources cannot be determined (de Boer, Ichim, & Newmaster, 2015). Chemometric analysis using complex metabolite datasets has shown great potential in the inspection of food adulteration as well as in the characterization of markers for adulteration detection (Cubero-Leon, Peñalver, & Maquet, 2014).

Untargeted chemical profiling is especially useful in the distinction of closely-related plant species, where certain genetic markers may not be informative (Messina, Callahan, Walsh, Hoebee, & Green, 2014). That approach has been successfully applied to the lichen *Ramalina siliquosa* complex using liquid chromatography-mass spectrometry (LC-MS) (Parrot, Jan, Baert, Guyot, & Tomasi, 2013).

Recently, DNA barcoding has emerged as an effective tool in the identification of plant and animal materials using defined species-specific DNA markers. It has found wide application in the authentication and traceability of food materials (Galimberti et al., 2013). This approach has been extended to the authentication of multiple ingredients samples using a more advanced DNA metabarcoding approach, which involves next generation sequencing (Staats et al., 2016). Practically, DNA barcoding has been applied for authentication of fungi-based dietary products (Raja, Baker, Little, & Oberlies, 2017). Furthermore, identification of lichenized fungi using DNA barcoding has been successfully performed using the fungal nuclear ribosomal internal transcribed spacer region (nrITS) (Kelly et al., 2011), which has been proposed as the universal DNA barcode for fungi (Schoch et al., 2012).

Two DNA barcodes, nrITS and RPB2 (the second largest subunit of ribosomal polymerase II) were selected for this study on the *C. islandica* lichen materials. Although the widely used nrITS region is known to provide a sufficient amount of variation to distinguish between most fungal species and is represented by many reference sequences in public databases, some drawbacks for DNA barcoding and especially DNA metabarcoding have been reported (Větrovský, Kolařík, Žifčáková, Zelenka, & Baldrian, 2016). While nrITS can identify species, its multi-copy nature of the ITS region may render problems with relative quantification of species in mixed samples. In other cases, intra-individual polymorphism including multiple functional genes, putative pseudo genes or recombinants hamper identifications (Mark, Cornejo, Keller, & Flück, 2016). The single-copy RPB2 gene has been proposed as an alternative to the nrITS region, which could overcome some of these challenges (Větrovský et al., 2016). The performance of RPB2 will be compared to that of nrITS in our study.

The overall aim of this study was to explore the usefulness of an authentication approach for *Cetraria islandica* species complex using UPLC-QToF-MS chemical profiling and DNA barcoding. Specific objectives were: Firstly, to distinguish chemotypes of the species complex by comparing their UPLC-QToF-MS chemical profiles using chemometric data analysis, and secondly to compare the discriminatory power of RPB2 and nrITS barcodes for the taxa of the *C. islandica* species complex.

2. Materials and Methods

2.1 Sampling and chemotype identification

Cetraria islandica (L.) Ach. (English: Iceland Moss; Icelandic: fjallagrös) and *Cetraria ericetorum* Opiz specimens were collected in Iceland. Authentic specimens of *C. islandica* are provide by lichenologists from the Icelandic Institute of Natural History, Akureyri, Iceland (AMNH). Collected voucher specimens are deposited at the AMNH herbarium. Intraspecific morphological variation of Iceland Moss (*C. islandica*) as well as the interspecific similarity between the two species (*C. islandica* and *C. ericetorum*) can be seen in Figure 1. Additional *C. ericetorum* type specimens were kindly provided by Dr. Stefan Ekman, Museum of Evolution, Uppsala University, Uppsala, Sweden. The chemotypes of *C. islandica* and *C. ericetorum* were tested using a conventional spot testing method (Kristinsson, 1969). Briefly, a small fragment was cut from a thallus with a blade, and drops of *p*-phenylendiamine (PD) solution (ca. 2% in ethanol) were added to lichen medulla on a white paper with a glass capillary tube. Then the medullary color reactions were visualized under a stereoscope. Specimens showing a red medullary color after spot testing were assigned as PD+, while the ones without color change as PD-. PD spot testing was carried out on fragments from three different parts of the thallus, to make sure the chemotype. Fragments were discarded immediately after testing. Voucher information and gene accession numbers are provided in Online Resource (see Table S1). In total, 30 specimens of PD+ *C. islandica*, 15 specimens of PD- *C. islandica* and 18 specimens of uniformly PD- *C. ericetorum* were identified.

2.2 Chemical profiling

2.2.1 LC-MS analysis

Air-dried lichen thallus (ca. 20 mg) was weighed and ground into powders under liquid nitrogen. Powdered lichen materials were macerated with acetone under shaking in ambient temperature for 2 h. The extraction was repeated twice. Extracts were combined and evaporated with nitrogen gas flow. Dried residues were then solubilized in HPLC-grade acetonitrile (ACN), diluted into 0.1 mg/mL and filtered (pore size 0.45 µm; GE healthcare, UK) before analyses by Waters ACUITY UPLC^{TM} (Waters Corporation, Milford, MA, USA) coupled to Waters Q-ToF SYNAPT G1 mass spectrometer (Waters MS Technologies, Manchester, UK).

The UPLC system was equipped with a binary solvent delivery system and autosampler. Chromatographic separation of lichen compounds was conducted on an ACQUITY UPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm; Waters corp., Milford, MA, USA). The column oven was kept at 40ºC and the autosampler was maintained at 6.0ºC. The mobile phase consisted of solvent A: H_2O with 0.1% formic acid in water and solvent B: 0.1% formic acid in ACN. Gradient elution was used at a flow rate of 0.40 mL/min as follows: 30% B, 0-1 min; linear gradient from 30% B/70% A to 70% B/30% A, 1-3 min; linear gradient from 70% B/30% A to 100% B, 3-9 min; holding at 100% B, 9-13 min; linear gradient from 100% B to 30% B/70% A, 13-14 min; holding at 30% B/70% A, 14-15 min. Pooled samples were used as quality control. The injection volume was 5 µL.

The Synapt G1 QToF-MS mass spectrometer was operated in negative electrospray ionization mode (capillary voltage 3.0 kV, source temperature 120ºC, desolvation temperature 400ºC, cone gas flow 50 L/h, desolvation nitrogen gas flow 800 L/h). Ions with mass range 50 to 1600 m/z (mass to charge ratio) were scanned. All samples were analyzed in triplicates. Details of UPLC-QToF-MS analysis are as previously described (Xu et al., 2017). The UPLC-QToF-MS system and data acquisition were controlled by the MassLynx v4.1 software (Waters Corp., Milford. USA).

2.2.2 Chemometric data analysis

MS spectra were aligned and normalized using MakerLynx v4.1. Collection parameters were set as 50 counts, mass window 0.05 Da and retention time window 0.2 min. Replicate percentage value was set at 50%. Normalized data were introduced into SIMCA v14.1 software (Sartorius Stedim Data Analytics, Umeå, Sweden) for principal component analysis (PCA). PCA could provide a holistic overview of the grouping of lichen specimens, which was based on chemical data from organic extracts. Compounds were identified by comparing their MS/MS spectra and fragmentation patterns with those from isolated pure compounds, previously published data and public databases (Metlin and ChemSpider).

2.3 Molecular analysis

2.3.1 DNA extraction, PCR and sequencing

Air-dried lichen residues after acetone maceration were used for total DNA extraction following the CTAB protocol (Cubero, Crespo, Fatehi, & Bridge, 1999). Lichen DNA extracts were stored in TE buffer (pH 8.0) at -20°C until use. Polymerase chain reactions (PCRs) were performed to amplify the fungal nuclear ribosomal internal transcribed spacer (nr ITS) and the second largest subunit of RNA polymerase II (RPB2). Each reaction (25 μL) contained 1×standard Taq reaction buffer, 200 μM dNTPs, 0.2 μM forward and reverse primer, 1.25 units of Taq DNA polymerase (New England Biolabs), 1 μL DNA template, and PCR-grade water. The fungi-specific primers used for the amplification of nr ITS region were: ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCC CCGCTTATTGATATGC-3') (White, Bruns, Lee, & Taylor, 1990), while the primers for the RPB2 regions were: RPB2-6F (5'-TGGGGKWTGGTYTGYCCTGC-3') (Liu, Whelen, & Hall, 1999) and fRPB2-7cR (5'-CCCATRGCTTGYTTRCCCAT-3') (Liu et al., 1999). PCR amplification was carried out in a Perkin-Elmer Gene Amp PCR system 9700 thermal cycler. The PCR cycling conditions for nrITS were: initial denaturation at 94°C for 3 min, 34 cycles of 94°C for 40 s, 54°C for 40 s, 68°C for 1 min, then final extension at 68°C for 5 min before cooling down to 4°C. A touchdown PCR program was used for RPB2 region: 94°C for 4 min, followed by 6 cycles of 94°C for 1min, 55-50°C (decrease 1°C per cycle) for 1min and 68°C for 1min, then 32 cycles of 94°C for 1min, 50°C for 1min and 68°C for 1min, and final extension at 68°C for 7min, before cooling down at 4ºC. Amplicons were visualized in 1.3% agarose gel (gel picture refers to Online Resource Figure S1), purified using ExoSAP (Fermentas) and sent for Sanger sequencing by Marogen Inc. using the same set of primers as used in PCRs.

2.3.2 DNA barcoding analysis

The discriminatory power of the nrITS and RPB2 barcodes was assessed according to the monophyly criterion and the DNA barcoding gap concept (Meyer & Paulay, 2005). The sequences representing each barcode were aligned using MAFFT v7.215 (Katoh & Standley, 2013) with default parameters and trimmed if necessary. Phylogenetic trees using the Genbank sequences of *C. sepincola* (accession number KC990137.1 for nrITS) as outgroups were built with RAxML v. 8.0.26 (Stamatakis, 2014) with 100 rapid bootstrap replicates under the GTRGAMMA model. The DNA barcode gap analysis was conducted on *C. islandica* and its sister species *C. ericetorum* using the R package SPIDER (Brown et al., 2012), using the best-fitting substituion models to measure pair-wise distances. Best-fitting substitution models for each region (TIM2+G for nrITS; TIM2+I for RPB2) were chosen by using the Aikaike Information Criterion (AIC) in jModelTest 2 (Darriba, Taboada, Doallo, & Posada, 2012). Because the TIM2+G and TIM2+I models were not available in the R package APE (Paradis, Claude, & Strimmer, 2004), which was used to calculate pairwise distances, the next best-fitting model for both alignments, TrN+G, was used.

The number of false positive and false negative identifications along a DNA divergence threshold were calculated and plotted using the R package SPIDER (Brown et al., 2012). Additionally, the minimum interspecific and maximum intraspecific divergence for each sequence was calculated and plotted.

3. Results and Discussion

3.1 Chemical profiling and chemometric data analysis

Chemical profiling of *C. islandica* organic extracts has been carried out before using high performance liquid chromatography-ultraviolet detection (HPLC-UV) (Fernández-Moriano, Divakar, Crespo, & Gómez-Serranillos, 2015; Gudjónsdóttir & Ingólfsdóttir, 1997). Those previously used methods lack separation efficiency and sensitivity, and thus may underestimate the chemical diversity of *C. islandica*, e.g. (+)-roccellaric acid **6** was found in *C. islandica* using a fluorous tag-catch and release approach (Horhant, Lamer, Boustie, Uriac, & Gouault, 2007), but was previously overlooked due to poor chromatographic separation. Additionally, HPLC-UV (Fernández-Moriano et al., 2015) analysis using high UV wavelengths may overlook the content of those aliphatic lichen acids (i.e. compounds **5-8**), which are poor UV absorbants. The current UPLC method achieved the separation of compound **6** from its analogues **7** and **8**. A list of detected compounds is provided in Table S2, and the structures of major lichen secondary metabolites from the *C. islandica* species complex are illustrated in Figure 2.

Compounds **1-8** were identified in our study by comparing their molecular masses, fragmentation pathway and chromatographic properties with reference data as well as authentic standards isolated in previous studies (Bessadóttir et al., 2014; Gudjónsdóttir & Ingólfsdóttir, 1997). MS^2 spectra of each compound and their fragmentation patterns are provided in Online Resource (see Figures S2-S4). MS chromatograms (Figure 3) show that (+)-protolichesterinic acid **7** and its derivatives (i.e. **6** and **8**) are the dominant compounds in organic extracts detected in negative ion mode, followed by minor components, such as protocetraric acid **1** and fumarprotocetraric acid

3. The stereochemical diversity of (+)-protolichesterinic acid **7** seems to be largely underestimated before, since two compounds (i.e. **7A** and **7B**) were detected with the same molecular formula, molecular ions and fragmentation patterns (Figure S4) with compound **7** (Table S2 and Figure 3). In *C. ericetorum,* an additional unknown compound **6A** in the peak eluting out at 5.77 min (Figure 3c) was detected having the same mass to charge ratio as well as fragmentation pattern as (+)-roccellaric acid **6**, which suggests that **6A** could be a stereoisomer of compound **6** (Figure S4). Up to now, only one stereochemical form of roccellaric acid has been reported in nature, namely (+)-roccellaric acid **6** in *C.islandica* (Horhant et al., 2007). Three additional stereochemical forms have been synthesized by Mulzer et al. (Mulzer, Salimi, & Hartl, 1993). Minor compounds **1** and **3** eluted quite early (t_R = 2.49 and 2.96 min, respectively) under the chromatographic conditions used, reflecting that they are more water-soluble than compounds **5**-**8**. Fumarprotocetraric acid **3** (50 mg) is reported to be moderately soluble in 30 mL phosphate buffer at pH 7.4 (Syers, 1969), while the solubility of compound **3** is low (1 mg/L) in 90% acetonitrile with 1% phosphoric acid (Gudjónsdóttir & Ingólfsdóttir, 1997). Thus, polarity and pH of the extraction solvent can be expected to have considerable influence on the extraction efficiency of these lichen acids (i.e. compounds **1**-**4**).

As a conventional diagnostic tool, the PD spot test was used to check the chemotype and the presence of compound **3** in *C. islandica* (Kristinsson, 1969). From LC-MS chromatograms shown in Figure 3, the red color reaction by PD spot testing was found to correlate with the presence of compouds **1** and **3**, while these compounds were absent in the PD- *C. islandica* chemotype and *C. ericetorum*. The presence of the aliphatic lichen acids (i.e. compounds **5**-**8**) did not result in a red color reaction. The co-occurence of compounds **1** and **3** in organic extracts of *C. islandica* has been found in literature (Fernández-Moriano et al., 2015).

The two-component PCA score plot (Figure 4) of the UPLC-QToF-MS data provides the visualization of how different chemical groups relate to each other. Three chemical groups were formed based on their chemical profiles, representing the PD- (CI PD-) and PD+ chemotypes (CI PD+) of *C. islandica* and *C. ericetorum* (CE). The first component explains 43.6% chemical variations, mainly interspecific differences between CE and CI. The secondary component accounts for 10.4% variations, mainly intraspecific differences between CI PD+ and CI PD-. PCA is a useful tool in summarizing metabolite data and revealing groupings of food ingredients from different biological origins (Azilawati, Hashim, Jamilah, & Amin, 2015; Cubero-Leon et al., 2014). From the Figure 4, the lichen *C. islandica* shows high intraspecific chemical variations in Iceland with two chemotypes recognized as reported before (Kristinsson, 1969), while Icelandic *C. ericetorum* shows relatively less variation, even when compared with non-Icelandic *C. ericetorum* specimens. This could partly be explained by the limited distribution of CE, resulting in less variation. CE has a restricted geographic distribution in north and east Iceland, whereas CI has a rather wide distribution around Iceland (Thell & Moberg, 2011).

3.2 DNA barcoding

Sizes of PCR products were ca. ~600-900 bp for fungal nrITS and ca. ~900 bp for fungal RPB2 (Online Resource Figure S1). The variation of the fungal nrITS PCR products was due to the presence of a group I intron sequence in the longer amplicons, and absence in the short ones. In total 97 new sequence were obtained, including 48 for RPB2 and 49 for fungal nrITS. PCRs of a few old herbarium reference specimens were not successful (Online Resource Table S1).

The phylogenetic tree based on the ITS region (Figure 5a) shows that *C. ericetorum* is paraphyletic and nested within the *C. islandica* clade, whereas both *C. ericetorum* and *C. islandica* are monophyletic in the RPB2 tree (Figure 5b). Therefore the phylogenetic analyses support RPB2 as a barcode with power to discriminate between the two species of *Cetraria*, but shows that the nrITS barcode does not discriminate the species under study. The pairwise distance analysis supports this interpretation, revealing that RPB2 is able to distinguish the two taxa, whereas the nrITS region always yields false positive or negative identifications (Figure 6). There are other cases where the nrITS region fails to discriminate lichenized fungi (Kelly et al., 2011; Pino-Bodas, Martín, Burgaz, & Lumbsch, 2013), though the nrITS region revealed interspecific barcoding gaps among most species of genera *Melanelia* and *Montanelia* (Leavitt et al., 2014; Pino-Bodas et al., 2013). The failure of the nrITS region in this case might be attributed to intragenomic polymorphism in the ITS region, a phenomenon described in other lichen-forming fungi (Kelly et al., 2011; Mark et al., 2016). It has also been reported that the nrITS region alone is not suitable to estimate the phylogenetic relationships within the *C. islandica* group (Thell et al., 2000).

The limited application of RPB2 as a barcoding region has been explained by difficult PCR amplification and sequencing (Schoch et al., 2012). Specimens stored for over 3 years have shown problems in PCR amplification (Kelly et al., 2011). We have, however, not encountered a PCR amplification problem for either locus even with specimens which are 15 years old. Successful PCR amplification of the RPB2 region using even older specimens (*Cladonia* sp.) of about 30 years has also been recorded (Pino-Bodas et al., 2013). Age-dependent problems with PCR amplification may be taxon-specific, as well as influenced by the DNA extraction method in use. We noted that the sequence alignment of RPB2 is much simpler. In contrast to the hypervariability of the nrITS region, RPB2 is also recommended as an alternative marker for phylogenetic analysis (Větrovský et al., 2016). We therefore reject the nrITS region and propose the RPB2 region as an efficient DNA barcode for testing medicinal products containing Iceland Moss, at least in terms of discriminating between *C. ericetorum* and *C. islandica*.

Although the RPB2 region is effective for discriminating between species, chemotypes of *C. islandica* are not discriminated (Figure 5). In Figure 5b, *C. islandica* specimens from Iceland show two strongly supported clades, I and II. All of the *C. islandica* PD- chemotype specimens fall into clade I, but are interspersed with PD+ chemotypes, while clade II contains exclusively PD+ *C. islandica* specimens. Some lichen chemotypes have been shown to be monophyletic (Fehrer, Slavíková-Bayerová, & Orange, 2008), but they can also be not (Lutsak, Fernández-Mendoza, Nadyeina, Şenkardeşler, & Printzen, 2017).

Domestically, *Cetraria islandica* is sold as whole lichen-thalli food ingredients or tea in Iceland. Accurate identification is generally not difficult for taxonomic experts, but it may prove intractable to identify powdered lichen materials, which lack morphological or sometimes chemical characters. DNA barcoding as outlined here could substantially facilitate identification by comparing new sequence data with reference data generated from expertly identified voucher specimens.

The current study focused on the authentication of natural lichen materials without downstream processing. In case of highly processed herbal materials where DNA may undergo considerable degradation, an alternative method could be double gene targeting PCR, which amplifies selected shorter regions (e.g. 70-150 bp) (Hossain et al., 2016, 2017).

3.3 The advantage of the integrative approach for authentication of Iceland Moss

Lichen material authentication generally operates at the species level and as shown in this study DNA barcoding using RPB2, is an efficient method for species identification in the *C. islandica* species complex. The advantage of DNA barcoding is in identification of raw plant materials, sources of contaminants and species composition (de Boer et al., 2015), which is beyond the scope of chemical analysis. Generally, chemical profiling of lichen secondary metabolites do not have the independent role in identification/authentication of lichen species (Lumbsch, 1998). First, the utility of metabolite data in lichen identification varies among lichen taxa/populations. Our results (Table S2 and Figure 4) have demonstrated the utility of chemical profiling in discriminating species (*C. islandica* and *C. ericetorum*) and chemotypes (PD+ and PD-) in the *Cetraria islandica* species complex. However, chemical profiling may have limited utility in species discrimination where remarkable chemical variations (e.g. different in major lichen compounds) are present, such as the lichen *Ramalina siliquosa* (Lumbsch, 1998; Parrot et al., 2013). Such a huge variation may pose a challenge in lichen identification: how much chemical variation is allowed to define a species? To address this problem, it has been suggested that chemical characters be correlated with other characters, preferentially genetic sequence data (Lumbsch, 1998). The correlation between phylogenetic relationship and chemotyping (i.e. PD+ and PD-) was investigated in our study (Figure 5b).

Although chemical profiling does not have an independent role in lichen species identification/Iceland Moss authentication, it is indispensable for the quality control of marker or health-beneficial components. It can provide both qualitative and quantitative information on phytochemical composition during extraction and downstream processing. Coupled to chemometric tools, chemical profiling could also be used in differentiation of intraspecific chemical variants, which is superior to DNA barcoding.

In conclusion, this study highlights the integrative use of chemical profiling and DNA barcoding for the authentication of Iceland Moss. The members of *Cetraria islandica* species complex were easily characterized using chemometric tools. Furthermore, DNA barcodes were compared and the locus RPB2 proved to be superior to nrITS in distinguishing species of *C. islandica* species complex. Our study shows how chemical profiling and DNA barcoding can be used to differentiate chemical variants and species in the complex, and suggests the use of this integrated approach for accurate characterization of this closely related taxa as well as other plant materials used for human consumption.

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Conflicts of interest: none

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Figure captions

Figure 1. Morphological variation and similarity of Iceland Moss (*Cetraria islandica*) chemotypes and its sibling species *Cetraria ericetorum. C. islandica* shows considerable morphological variation, from wide (**a** and **c**) to narrow thallus (**b** and **d**). Two chemotypes were identified in *C. islandica* specimens using *p*-phenylendiamine (PD) spot testing/staining, including PD+ (red medullary color after staining; **a** and **b**) and PD- (no red medullary color after staining; **c** and **d**). The lichen *C. ericetorum* (**e**) is uniformly PD- and has narrow thallus. Scale: 1 cm.

Figure 2. Chemical structures of major lichen secondary metabolites detected in the *Cetraria islandica* species complex. Compounds include protocetraric acid **1**, succinprotocetraric acid **2**, fumarprotocetraric acid **3**, virensic acid **4**, nephrosterinic acid **5**, (+)-roccellaric acid **6**, (+)-protolichesterinic acid **7** and (+)-lichesterinic acid **8**. Minor compounds refer to Table S2.

Figure 3. MS chromatograms of the PD+ (**a**) and PD- (**b**) chemotypes of *Cetraria islandica* and PD- *C. ericetorum* (**c**) and thallus color reaction by PD staining of PD+ (**d**) and PD- (**e**) chemotypes of *Cetraria islandica* and PD- *C. ericetorum* (**f**).

Major secondary metabolites are labelled corresponding to structures **1-8** in Figure 2. Compounds **7A** and **7B** were tentatively identified as stereoisomers of (+)-protolichesterinic acid **7**, and **6A** a stereoisomer of (+)-roccellaric acid **6**. Scale = 0.5 mm.

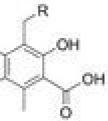
Figure 4. PCA plot giving an overview of metabolite data and indicative grouping of species and chemotypes in the *Cetraria islandica* species complex. Three chemical groups include PD-chemotype (CI PD-), *C. islandica* PD+ chemotype (CI PD+) and *C. ericetorum* (CE). Authentic herbarium specimens were marked as dark green (CI PD+), dark red (CI PD-) and grey (CE).

Figure 5. Maximum likelihood (ML) trees of the *Cetraria islandica* species complex reconstructed using barcode markers. (**a**) ML tree reconstructed using the nrITS barcode, with *C. ericetorum* specimens marked in red; (**b**) ML tree using the RPB2 marker, where tree well-supported clades were identified: I, II and III. The PD+ chemotype is labelled with a red dot after each specimen. Bootstrap values > 70 are shown above branches in both trees.

Figure 6. Barcoding gap analysis of *Cetraria islandica* species complex for each marker. (**a**) Number of false positive and

false negative identifications along a threshold from 0.1% – 4% DNA divergence; (**b**) Evaluation of inter- vs. intraspecific divergence. The distances for each gene were calculated according to the best model of evolution. Samples that are in the top-left half of the plot have a greater minimum interspecific than maximum intraspecific divergence and exhibit a barcode gap.

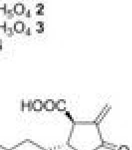

b
e
d



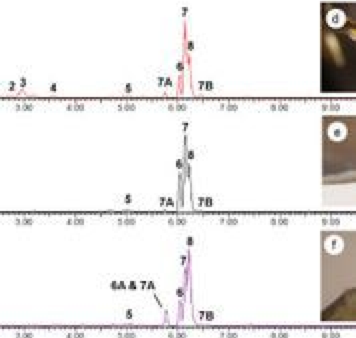
1
H_5O_4 2
H_3O_4 3

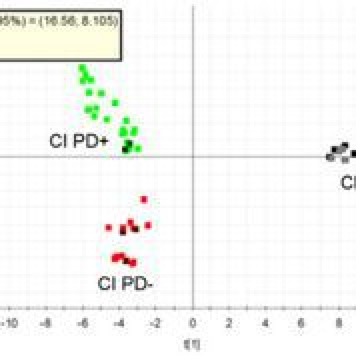
6

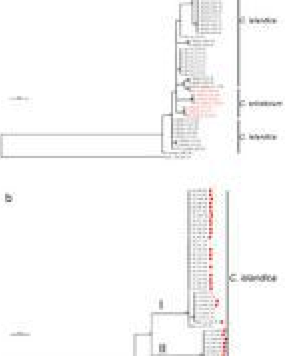
7

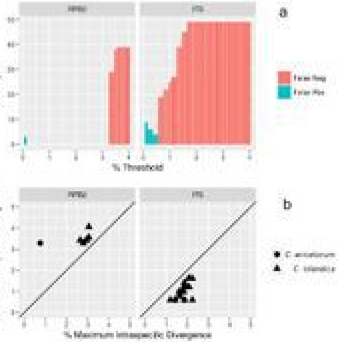


8


7
8
6
2
3
4
5
7A
7B
3.00
4.00
5.00
6.00
7.00
8.00
9.00
d
7
8
6
5
7A
7B
3.00
4.00
5.00
6.00
7.00
8.00
9.00
e
8
7
6
6A & 7A
5
7B
3.00
4.00
5.00
6.00
7.00
8.00
9.00
f


95%) = (16.56; 8.105)
CI PD+
CI
CI PD-
-10
-8
-6
-4
-2
0
2
4
6
8
t[1]


C. islandica
C. ericetorum
C. islandica
b
C. islandica
I
II


a
RPB2
ITS
Samples
0
10
20
30
40
50
0
1
2
3
4
% Threshold
False Neg
False Pos
b
% Minimum Interspecific Divergence
% Maximum Intraspecific Divergence
0
1
2
3
4
5

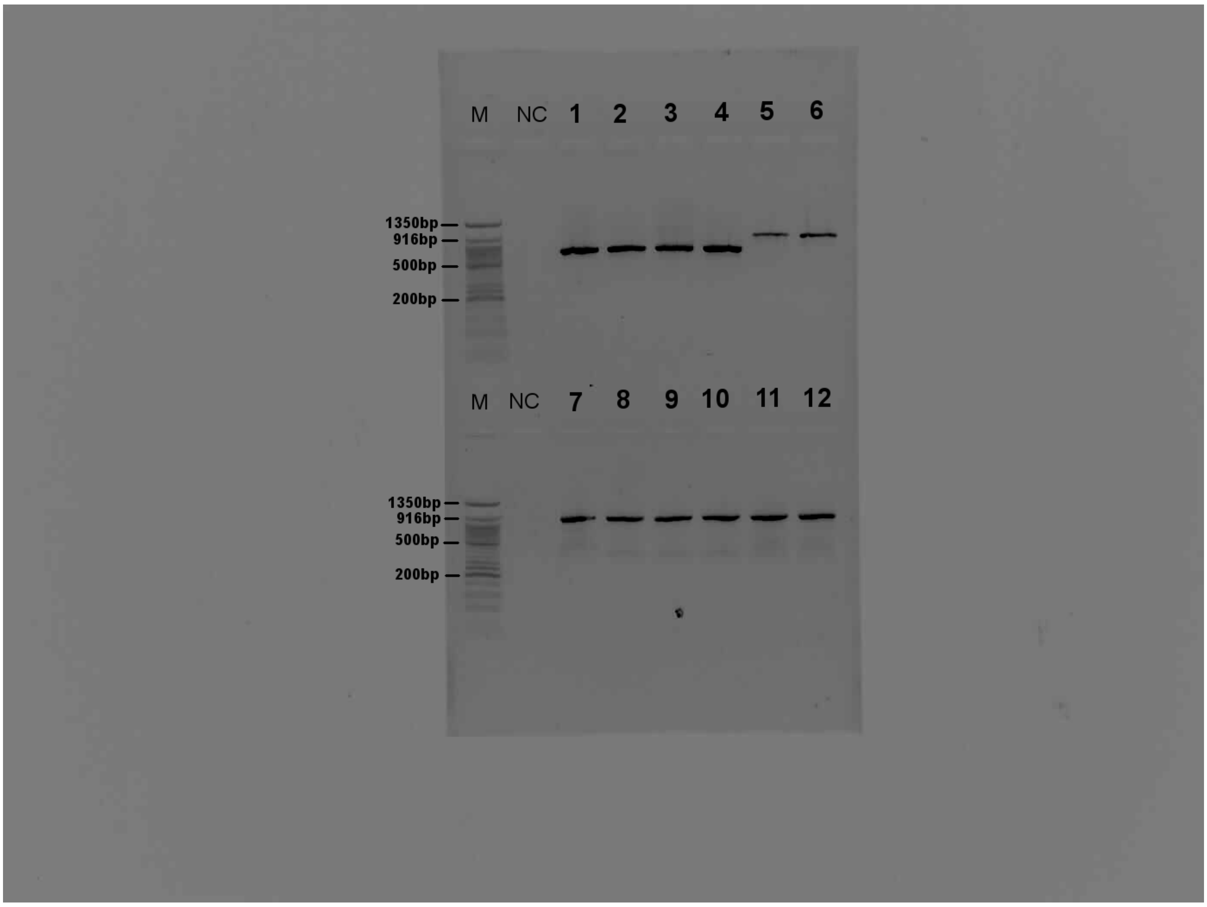


Figure S1. Agarose gel electrophoresis of PCR products from fungal nrITS and RPB2 loci. Lane 1-6: PCR products of fungal nrITS locus, ranging from 600 (intron-absent) to 900 bp (intron-present). Lane 7-12: PCR products of fungal RPB2 locus of ca. 900 bp. M: ladder. NC: negative control.

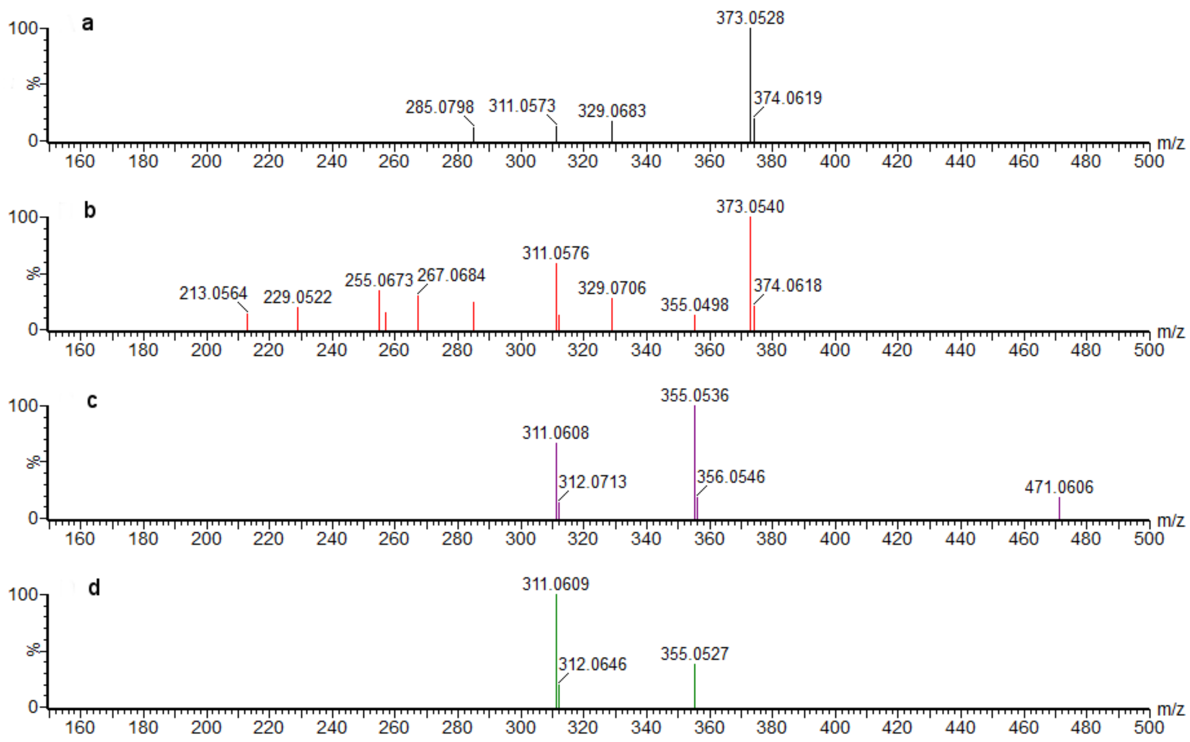


Figure S2. MS spectra of major depsidones in the PD+ *Cetraria islandica* chemotype. MS (**a**) and MS^2 (**b**) spectra for protocetraric acid **1**; MS (**c**) and MS^2 (**d**) spectra for fumarprotocetraric acid **3**.

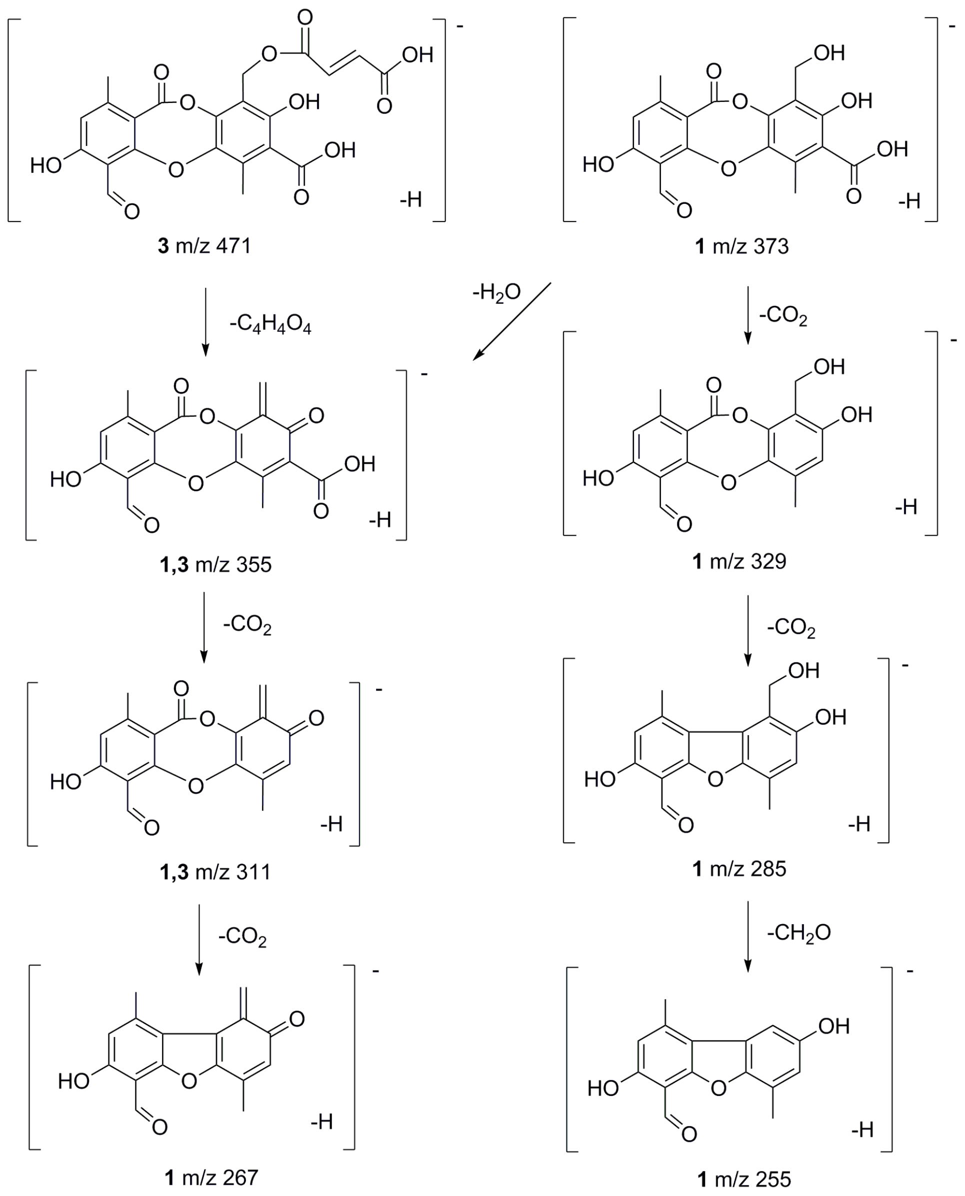


Figure S3. MS fragmentation patterns for major depsidones (protocetraric acid **1**; fumarprotocetraric acid **3**) in the PD+ *Cetraria islandica* chemotype.

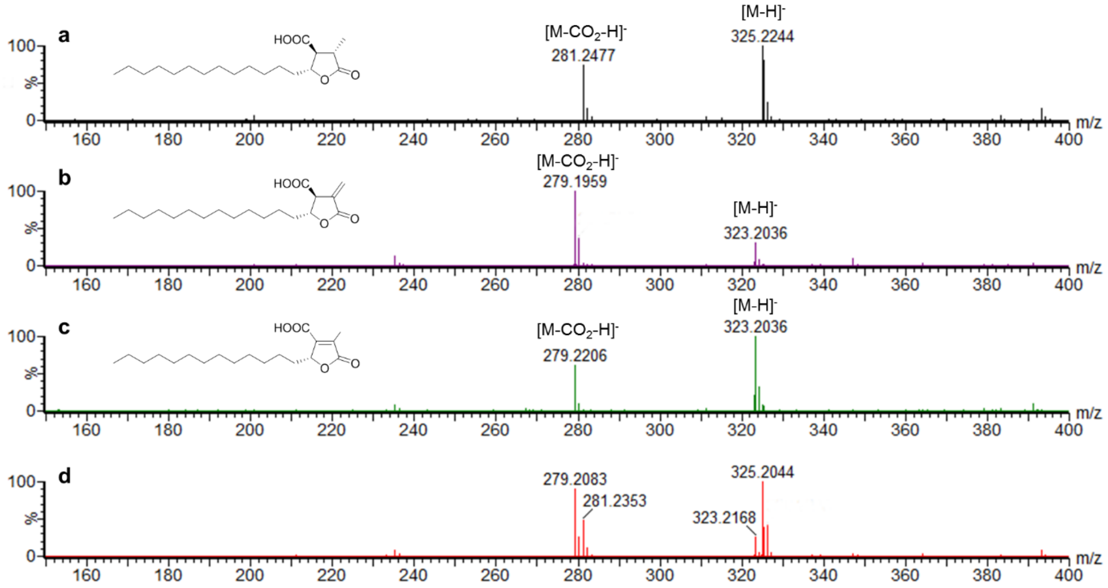


Figure S4. MS spectra for major paraconic acids in *Cetraria islandica* species complex. (**a**) MS spectrum of (+)-roccellaric acid **6**; (**b**) MS spectrum of (+)-protolichesterinic acid **7**; (**c**) MS spectrum of (+)-lichesterinic acid **8**. MS fragment ions of compounds **7** and **8** differed in the ratio of molecular ion [M-H]$^-$ to the decarboxylated molecular ion [M-CO$_2$-H]$^-$. The higher stability of lichesterinic acid molecular ion could be explained by hyperconjugation, where the electrons in the C-C bond between lactone ring and carboxylic group interacts with the unhybridized *p*-orbital in the adjacent ethylenic carbon; (**d**) MS spectrum of the peak eluting out at t$_R$ 5.87 min containing two compounds **6A** and **7A** in Fig 3. They are tentatively identified as a stereoisomer (compound **6A**; m/z 325.2 and 281.2) of **6** and a stereoisomer (compound **7A**; m/z 323.2 and 279.2) of **7**, respectively.

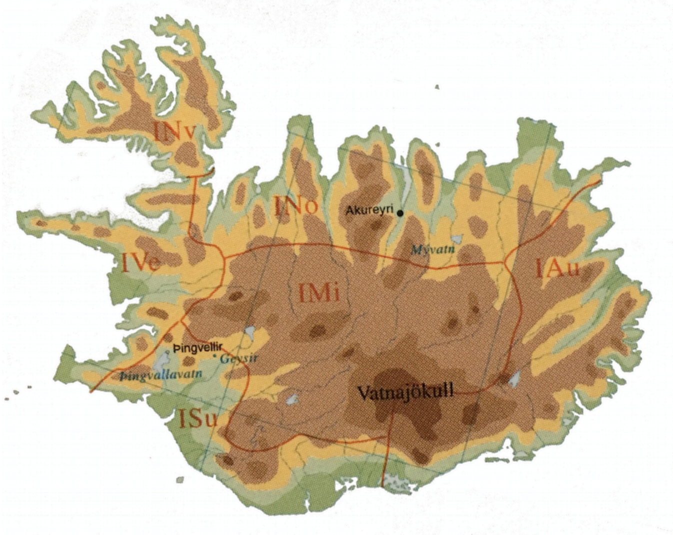
Table S1. Voucher specimens of *Cetraria islandica* species complex used in the current study, including country, collection date, voucher number, spot test results/chemotype, DNA isolate number and GenBank accession numbers.

Country[a]	Collection date	Specimen voucher[b]	Spot test[c]	Collector	DNA Isolate	GenBank accession number	
						RPB2	nrITS
Cetraria islandica							
Iceland: IVe	21-Aug-2012	**LA31863**	PD+	Starri Heidmarsson	CI1	KY768945	KY764967
Iceland: IAu	16-Aug-2012	**LA31864**	PD+	Starri Heidmarsson	CI4	KY768946	KY764968
Iceland: INo	21-Aug-2012	LA31865	PD+	Starri Heidmarsson	CI6	KY768947	KY764969
Iceland: IVe	23-Aug-2012	LA31866	PD+	Starri Heidmarsson	CI7	KY768948	KY764970
Iceland: INo	8-Jul-2013	LA31867	PD-	Starri Heidmarsson	CI11	KY768949	KY764971
Iceland: IVe	23-Jul-2013	LA31868	PD+	Starri Heidmarsson	CI12	KY768950	KY764972
Iceland: IVe	25-Jul-2013	LA31869	PD+	Starri Heidmarsson	CI13	KY768951	KY764973
Iceland: IVe	25-Jul-2013	LA31870	PD+	Starri Heidmarsson	CI14	KY768952	KY764974
Iceland: INo	16-Aug-2012	LA31871	PD+	Starri Heidmarsson	CI15	KY768953	KY764975
Iceland: ISu	12-Jul-2013	LA31872	PD+	Starri Heidmarsson	CI16	KY768954	KY764976
Iceland: IVe	11-Jul-2013	LA31873	PD+	Starri Heidmarsson	CI17	KY768955	KY764977
Iceland: IVe	11-Jul-2013	LA31874	PD+	Starri Heidmarsson	CI18	KY768956	KY764978
Iceland: IVe	23-Jul-2013	LA31875	PD+	Starri Heidmarsson	CI19	KY768957	KY764979
Iceland: IVe	9-Aug-2013	LA31876	PD+	Starri Heidmarsson	CI25	KY768958	KY764980
Iceland: IVe	23-Jul-2013	LA31877	PD+	Starri Heidmarsson	CI26	KY768959	KY764981
Iceland: IVe	8-Jul-2013	LA31878	PD+	Starri Heidmarsson	CI27	KY768960	KY764982
Iceland: IVe	8-Jul-2013	LA31879	PD+	Starri Heidmarsson	CI28	KY768961	KY764983
Iceland: IVe	11-Jul-2013	LA31880	PD+	Starri Heidmarsson	CI29	KY768962	KY764984
Iceland: IVe	8-Jul-2013	LA31881	PD+	Starri Heidmarsson	CI30	KY768963	KY764985
Iceland: IVe	21-Aug-2013	LA31882	PD+	Starri Heidmarsson	CI31	KY768964	KY764986
Iceland: IVe	8-Jul-2013	LA31883	PD+	Starri Heidmarsson	CI32	KY768965	KY764987
Iceland: INo	24-Jul-2012	LA31884	PD-	Starri Heidmarsson	CI57a	KY768966	KY764988
Iceland: INo	24-Jul-2012	LA31885	PD-	Starri Heidmarsson	CI57b	KY768967	KY764989
Iceland: INv	30-Aug-2013	LA31886	PD+	Starri Heidmarsson	CI58	KY768968	KY764990
Iceland: IAu	15-Aug-2012	LA31887	PD+	Starri Heidmarsson	CI59	KY768969	KY764991

Iceland: INv	22-Aug-2013	LA31888	PD-	Starri Heidmarsson	CI60	KY768970	KY764992
Iceland: INv	26-Aug-2013	LA31889	PD-	Starri Heidmarsson	CI61	KY768971	KY764993
Iceland: INo	14-Aug-2012	LA31890	PD+	Starri Heidmarsson	CI62	KY768972	KY764994
Iceland: IVe	15-Aug-2012	LA31928	PD+	Starri Heidmarsson	CI63	KY768973	KY764995
Iceland: INo	21-Aug-2012	LA31891	PD+	Starri Heidmarsson	CI64	KY768974	KY764996
Iceland: INo	28-Jun-2012	LA31929	PD-	Starri Heidmarsson	CI65	KY768975	KY764997
Iceland: IVe	12-Jul-2013	LA31892	PD+	Starri Heidmarsson	CI66	KY768976	KY764998
Iceland: INv	22-Aug-2013	LA31893	PD-	Starri Heidmarsson	CI67	KY768977	KY764999
Iceland: INo	24-Jun-2012	LA31894	PD+	Starri Heidmarsson	CI68	KY768978	KY765000
Iceland: INo	2012	LA31895	PD-	Starri Heidmarsson	CI69	KY768979	KY765001
Iceland: INo	2012	LA31896	PD-	Starri Heidmarsson	CI70	KY768980	KY765002
Iceland: INo	8-Aug-2012	LA31897	PD+	Starri Heidmarsson	CI77	KY768981	KY765003
Iceland: INo	28-Jun-2012	LA31898	PD+	Starri Heidmarsson	CI78a	KY768982	KY765004
Iceland: INo	28-Jun-2012	LA31899	PD+	Starri Heidmarsson	CI78b	KY768983	KY765005
Iceland: INo	23-Aug-2012	LA31927	PD-	Starri Heidmarsson	CI87a	KY768984	KY765006
Iceland: INo	11-Jul-2002	**LA30017**	PD-	Hordur Kristinsson	CI115	KY768985	KY765007
Iceland: INo	14-Aug-2012	LA31900	PD-	Starri Heidmarsson	CI117a	KY768986	KY765008
Iceland: INo	10-Aug-2006	**LA31128**	PD-	Hordur Kristinsson	CI113	-	-
Iceland: INo	10-Jul-1998	**LA17549**	PD-	Hordur Kristinsson	CI36	-	-
Iceland: INo	5-Jul-1998	**LA17221**	PD-	Hordur Kristinsson	CI37	-	-
Cetraria ericetorum							
Iceland:IAu	10-Aug-1997	LA18976	PD-	Hordur Kristinsson	CE1	-	KY765009
Finland: Sodankylä	21-Aug-2003	**NO2530**	PD-	Beata Krewicka	CE6	KY768987	KY765010
Sweden: Uppsala	20-May-2002	**NO23002**	PD-	Leif Tibell	CE8	KY768988	KY765011
Sweden: Uppsala	18-Oct-2015	**NO5626**	PD-	Stefan Ekman	CE11	KY768989	KY765012
Iceland: IAu	13-Jul-2014	LA20746	PD-	Hordur Kristinsson	CE13	KY768990	KY765013
Iceland: INo	29-Aug-2016	LA31901	PD-	Hordur Kristinsson	CE15	KY768991	KY765014
Iceland: INo	1-Sep-2010	LA31538	PD-	Hordur Kristinsson	CE16	KY768992	KY765015
Iceland: IAu	9-Aug-1997	LA27354	PD-	Hordur Kristinsson	CE2	-	-

Sweden: Gävleborg	15-Jun-1997	**NO501**	PD-	Ake Agren	CE3	-	-
Russian: Komi	6-Jul-2000	**L135019**	PD-	-	CE4	-	-
Canada: Quebec	2-Jul-1999	**NO5021**	PD-	Jan-Eric Mattsson	CE5	-	-
Poland: Silesia	19-Jul-1998	**KO2101**	PD-	-	CE7	-	-
Russia: Komi	2-Jul-1997	**NO7971**	PD-	Björn Larsson	CE9	-	-
Iceland: INo	31-Jul-1996	**NO720**	PD-	Starri Heidmarsson	CE10	-	-
Iceland: INo	18-Aug-1998	LA20809	PD-	Hordur Kristinsson	CE12	-	-
Iceland: INo	7-Jun-1998	LA18310	PD-	Hordur Kristinsson	CE14	-	-
Iceland: INo	19-Aug-1998	LA29284	PD-	Hordur Kristinsson	CE17	-	-
Iceland: INo	10-Aug-1997	LA18982	PD-	Hordur Kristinsson	CE18	-	-

[a] INo, INv, IVe, IMi, IAu and ISu refer to corresponding area in Icelandic map below;



[b] Authentic herbarium specimens are marked in boldface;

[c] Spot testing/chemotype identification results are reported as PD+ (medullary red color after *p*-phenylendiamine staining) and PD- (no red color after *p*-phenylendiamine staining).

Table S2. Chromatographic and MS data of metabolites tentatively identified from acetone extracts of taxa in the *Cetraria islandica* species complex.

t_R (min)[a]	$[M\text{-}H]^-$ (m/z)[b]	Product ions (m/z)[c]	Mass error (ppm)[d]	Molecular formula	Compound[e]	Lichen[f]
2.48	373.0540	355.0498, 329.0706, 311.0576, 285.0798	-5.4	$C_{18}H_{14}O_9$	Protocetraric acid **1**	CI (PD+)
2.55	385.0650	341.0783, **329.2408**	-	-	Unidentified	CE
2.67	487.0986	373.0665, **355.0501**, 311.0594	-	-	Unidentified	CI (PD+)
2.75	473.0806	**355.0451**, 311. 0550	-0.8	$C_{22}H_{18}O_{12}$	Succinprotocetraric acid **2**	CI (PD+)
2.88	517.1052	401.0900, 369.0647, 325.0730	6.7	-	Unidentified	CI (PD+)
2.96	471.0536	**355.0467**, 311.0581	-1.4	$C_{22}H_{16}O_{12}$	Fumarprotocetraric acid **3**	CI (PD+)
3.07	489.3547	355.0500, **343.0474**, 311.0598, 299,0618	-	-	Unidentified	CI (PD+)
3.17	387.0728	355.0474, 343.0864, 311.0580, 299.0962	3.1	$C_{19}H_{16}O_9$	Unidentified	CI (PD+)
3.21	293.1744	236.1066, 221.1552	-3.1	$C_{17}H_{26}O_4$	Unidentified	CI, CE
3.34	431.3405	355.0503, 309.1720	7.4	$C_{24}H_{48}O_6$	Unidentified	CI (PD+)
3.57	357.0607	313.0723, 269.0848	-0.8	$C_{18}H_{14}O_8$	Virensic acid **4**	CI (PD+)
4.17	295.2257	277.2194, 171.1052	-5.4	$C_{18}H_{32}O_3$	Unidentified	CE
4.71	-	443.3083, **279.2310**, 250.1470	-5.0	-	Unidentified	CI, CE
4.94	-	297.2133, **279.2383**, 264.1647, 253.2214	-	-	Unidentified	CI, CE
5.03	279.2364	**251.2069**	-	-	Unidentified	CI, CE
5.09	295.1935	251.2062	8.8	$C_{17}H_{28}O_4$	Nephrosterinic acid **5**	CI, CE
5.44	-	311.2299, **281.2556**	-	-	Unidentified	CI, CE
5.77	323.2168	**279.2314**	-3.6	$C_{19}H_{32}O_4$	A stereoisomer of (+)-Protolichesterinic acid **7A**	CI, CE
5.77	325.2370	281.2527	-2.8	$C_{19}H_{34}O_4$	A stereoisomer of (+)-Roccellaric acid **6A**	CE
6.05	325.2405	281.2511	8.0	$C_{19}H_{34}O_4$	(+)-Roccellaric acid **6**	CI, CE
6.17	323.2234	**279.2322**	-0.7	$C_{19}H_{32}O_4$	(+)-Protolichesterinic acid **7**	CI, CE
6.23	323.2224	279.2372	0.6	$C_{19}H_{32}O_4$	Lichesterinic acid **8**	CI, CE
6.46	323.2218	**279.2336**	4.5	$C_{19}H_{32}O_4$	A stereoisomer of (+)-Protolichesterinic acid **7B**	CI, CE

[a] t_R means retention time;

[b] $[M\text{-}H]^-$ stands for deprotonated molecular ion;

[c] The product ion is marked in bold when it is the base peak in the MS spectrum;

[d] The mass error of the base peak is provided;

[e] Major compounds **1-8** are labelled corresponding to structures in Figure 2;

[f] The presence of lichen compounds in lichen taxa. CI (PD+): the PD+ chemotype of *Cetraria islandica*; CI: both PD+ and PD- chemotypes of *C. islandica*; CE: *C. ericetorum*.