

# *Authentication of Iceland Moss (Cetraria islandica) by UPLC-QToF-MS chemical profiling and DNA barcoding*

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**Authentication of Iceland Moss by UPLC-QToF-MS chemical profiling  
and DNA barcoding**

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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

Taxonically, *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) (Ingolfsson, 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).

31 Untargeted chemical profiling is especially useful in the distinction of  
 32 closely-related plant species, where certain genetic markers may not be  
 33 informative (Messina, Callahan, Walsh, Hoebee, & Green, 2014). That  
 34 approach has been successfully applied to the lichen *Ramalina siliquosa*  
 35 complex using liquid chromatography-mass spectrometry (LC-MS) (Parrot,  
 36 Jan, Baert, Guyot, & Tomasi, 2013).  
 37  
 38 Recently, DNA barcoding has emerged as an effective tool in the  
 39 identification of plant and animal materials using defined species-specific  
 40 DNA markers. It has found wide application in the authentication and  
 41 traceability of food materials (Galimberti et al., 2013). This approach has  
 42 been extended to the authentication of multiple ingredients samples using a  
 43 more advanced DNA metabarcoding approach, which involves next  
 44 generation sequencing (Staats et al., 2016). Practically, DNA barcoding has  
 45 been applied for authentication of fungi-based dietary products (Raja, Baker,  
 46 Little, & Oberlies, 2017). Furthermore, identification of lichenized fungi  
 47 using DNA barcoding has been successfully performed using the fungal  
 48 nuclear ribosomal internal transcribed spacer region (nrITS) (Kelly et al.,  
 49 2011), which has been proposed as the universal DNA barcode for fungi  
 50 (Schoch et al., 2012).  
 51  
 52 Two DNA barcodes, nrITS and RPB2 (the second largest subunit of  
 53 ribosomal polymerase II) were selected for this study on the *C. islandica*  
 54 lichen materials. Although the widely used nrITS region is known to  
 55 provide a sufficient amount of variation to distinguish between most fungal  
 56 species and is represented by many reference sequences in public databases,  
 57 some drawbacks for DNA barcoding and especially DNA metabarcoding  
 58 have been reported (Větrovský, Kolařík, Žifčáková, Zelenka, & Baldrian,  
 59 2016). While nrITS can identify species, its multi-copy nature of the ITS  
 60 region may render problems with relative quantification of species in mixed

61 samples. In other cases, intra-individual polymorphism including multiple  
62 functional genes, putative pseudo genes or recombinants hamper  
63 identifications (Mark, Cornejo, Keller, & Flück, 2016). The single-copy  
64 RPB2 gene has been proposed as an alternative to the nrITS region, which  
65 could overcome some of these challenges (Větrovský et al., 2016). The  
66 performance of RPB2 will be compared to that of nrITS in our study.

67

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

75

## 76 2. Materials and Methods

77

### 78 2.1 Sampling and chemotype identification

79

80 *Cetraria islandica* (L.) Ach. (English: Iceland Moss; Icelandic: fjallagrös)  
81 and *Cetraria ericetorum* Opiz specimens were collected in Iceland.  
82 Authentic specimens of *C. islandica* are provide by lichenologists from the  
83 Icelandic Institute of Natural History, Akureyri, Iceland (AMNH). Collected  
84 voucher specimens are deposited at the AMNH herbarium. Intraspecific  
85 morphological variation of Iceland Moss (*C. islandica*) as well as the  
86 interspecific similarity between the two species (*C. islandica* and *C.*  
87 *ericetorum*) can be seen in Figure 1. Additional *C. ericetorum* type  
88 specimens were kindly provided by Dr. Stefan Ekman, Museum of  
89 Evolution, Uppsala University, Uppsala, Sweden. The chemotypes of *C.*  
90 *islandica* and *C. ericetorum* were tested using a conventional spot testing

91 method (Kristinsson, 1969). Briefly, a small fragment was cut from a thallus  
92 with a blade, and drops of *p*-phenyldiamine (PD) solution (ca. 2% in  
93 ethanol) were added to lichen medulla on a white paper with a glass  
94 capillary tube. Then the medullary color reactions were visualized under a  
95 stereoscope. Specimens showing a red medullary color after spot testing  
96 were assigned as PD+, while the ones without color change as PD-. PD spot  
97 testing was carried out on fragments from three different parts of the thallus,  
98 to make sure the chemotype. Fragments were discarded immediately after  
99 testing. Voucher information and gene accession numbers are provided in  
100 Online Resource (see Table S1). In total, 30 specimens of PD+ *C. islandica*,  
101 15 specimens of PD- *C. islandica* and 18 specimens of uniformly PD- *C.*  
102 *ericetorum* were identified.

103

## 104 2.2 Chemical profiling

105

### 106 2.2.1 LC-MS analysis

107

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

117

118 The UPLC system was equipped with a binary solvent delivery system and  
119 autosampler. Chromatographic separation of lichen compounds was  
120 conducted on an ACQUITY UPLC BEH C18 column (2.1 mm x 100 mm,



1.7  $\mu\text{m}$ ; Waters corp., Milford, MA, USA). The column oven was kept at 40°C and the autosampler was maintained at 6.0°C. The mobile phase consisted of solvent A: H<sub>2</sub>O 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  $\mu\text{L}$ .

130

The Synapt G1 QToF-MS mass spectrometer was operated in negative electrospray ionization mode (capillary voltage 3.0 kV, source temperature 120°C, desolvation temperature 400°C, 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).

139

#### 2.2.2 Chemometric data analysis

141

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

150 with those from isolated pure compounds, previously published data and  
151 public databases (Metlin and ChemSpider).

152

### 153 2.3 Molecular analysis

154

#### 155 2.3.1 DNA extraction, PCR and sequencing

156

157 Air-dried lichen residues after acetone maceration were used for total DNA  
158 extraction following the CTAB protocol (Cubero, Crespo, Fatehi, & Bridge,  
159 1999). Lichen DNA extracts were stored in TE buffer (pH 8.0) at -20°C  
160 until use. Polymerase chain reactions (PCRs) were performed to amplify the  
161 fungal nuclear ribosomal internal transcribed spacer (nr ITS) and the second  
162 largest subunit of RNA polymerase II (RPB2). Each reaction (25 µL)  
163 contained 1×standard Taq reaction buffer, 200 µM dNTPs, 0.2 µM forward  
164 and reverse primer, 1.25 units of Taq DNA polymerase (New England  
165 Biolabs), 1 µL DNA template, and PCR-grade water. The fungi-specific  
166 primers used for the amplification of nr ITS region were: ITS1F (5'-  
167 CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4  
168 (5'-TCC CCGCTTATTGATATGC-3') (White, Bruns, Lee, & Taylor,  
169 1990), while the primers for the RPB2 regions were: RPB2-6F (5'-  
170 TGGGGKWTGGTYTGYCCTGC-3') (Liu, Whelen, & Hall, 1999) and  
171 fRPB2-7cR (5'-CCCATRGCTTGYTTRCCCAT-3') (Liu et al., 1999). PCR  
172 amplification was carried out in a Perkin-Elmer Gene Amp PCR system  
173 9700 thermal cycler. The PCR cycling conditions for nrITS were: initial  
174 denaturation at 94°C for 3 min, 34 cycles of 94°C for 40 s, 54°C for 40 s,  
175 68°C for 1 min, then final extension at 68°C for 5 min before cooling down  
176 to 4°C. A touchdown PCR program was used for RPB2 region: 94°C for 4  
177 min, followed by 6 cycles of 94°C for 1min, 55-50°C (decrease 1°C per  
178 cycle) for 1min and 68°C for 1min, then 32 cycles of 94°C for 1min, 50°C  
179 for 1min and 68°C for 1min, and final extension at 68°C for 7min, before

180 cooling down at 4°C. Amplicons were visualized in 1.3% agarose gel (gel  
181 picture refers to Online Resource Figure S1), purified using ExoSAP  
182 (Fermentas) and sent for Sanger sequencing by Marogen Inc. using the same  
183 set of primers as used in PCRs.

184

### 185 2.3.2 DNA barcoding analysis

186

187 The discriminatory power of the nrITS and RPB2 barcodes was assessed  
188 according to the monophyly criterion and the DNA barcoding gap concept  
189 (Meyer & Paulay, 2005). The sequences representing each barcode were  
190 aligned using MAFFT v7.215 (Katoh & Standley, 2013) with default  
191 parameters and trimmed if necessary. Phylogenetic trees using the Genbank  
192 sequences of *C. sepincola* (accession number KC990137.1 for nrITS) as  
193 outgroups were built with RAxML v. 8.0.26 (Stamatakis, 2014) with 100  
194 rapid bootstrap replicates under the GTRGAMMA model. The DNA  
195 barcode gap analysis was conducted on *C. islandica* and its sister species *C.*  
196 *ericetorum* using the R package SPIDER (Brown et al., 2012), using the  
197 best-fitting substitution models to measure pair-wise distances. Best-fitting  
198 substitution models for each region (TIM2+G for nrITS; TIM2+I for RPB2)  
199 were chosen by using the Akaike Information Criterion (AIC) in  
200 jModelTest 2 (Darriba, Taboada, Doallo, & Posada, 2012). Because the  
201 TIM2+G and TIM2+I models were not available in the R package APE  
202 (Paradis, Claude, & Strimmer, 2004), which was used to calculate pairwise  
203 distances, the next best-fitting model for both alignments, TrN+G, was used.  
204  
205 The number of false positive and false negative identifications along a DNA  
206 divergence threshold were calculated and plotted using the R package  
207 SPIDER (Brown et al., 2012). Additionally, the minimum interspecific and  
208 maximum intraspecific divergence for each sequence was calculated and  
209 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 & Ingólfssdóttir, 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 fluoruous 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 & Ingólfssdóttir, 1997). MS<sup>2</sup> 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

240 3. The stereochemical diversity of (+)-protolichesterinic acid **7** seems to be  
 241 largely underestimated before, since two compounds (i.e. **7A** and **7B**) were  
 242 detected with the same molecular formula, molecular ions and  
 243 fragmentation patterns (Figure S4) with compound **7** (Table S2 and Figure  
 244 3). In *C. ericetorum*, an additional unknown compound **6A** in the peak  
 245 eluting out at 5.77 min (Figure 3c) was detected having the same mass to  
 246 charge ratio as well as fragmentation pattern as (+)-roccellaric acid **6**, which  
 247 suggests that **6A** could be a stereoisomer of compound **6** (Figure S4). Up to  
 248 now, only one stereochemical form of roccellaric acid has been reported in  
 249 nature, namely (+)-roccellaric acid **6** in *C. islandica* (Horhant et al., 2007).  
 250 Three additional stereochemical forms have been synthesized by Mulzer et  
 251 al. (Mulzer, Salimi, & Hartl, 1993). Minor compounds **1** and **3** eluted quite  
 252 early ( $t_R$  = 2.49 and 2.96 min, respectively) under the chromatographic  
 253 conditions used, reflecting that they are more water-soluble than compounds  
 254 **5-8**. Fumarprotocetraric acid **3** (50 mg) is reported to be moderately soluble  
 255 in 30 mL phosphate buffer at pH 7.4 (Syers, 1969), while the solubility of  
 256 compound **3** is low (1 mg/L) in 90% acetonitrile with 1% phosphoric acid  
 257 (Gudjónsdóttir & Ingólfssdóttir, 1997). Thus, polarity and pH of the  
 258 extraction solvent can be expected to have considerable influence on the  
 259 extraction efficiency of these lichen acids (i.e. compounds **1-4**).  
 260  
 261 As a conventional diagnostic tool, the PD spot test was used to check the  
 262 chemotype and the presence of compound **3** in *C. islandica* (Kristinsson,  
 263 1969). From LC-MS chromatograms shown in Figure 3, the red color  
 264 reaction by PD spot testing was found to correlate with the presence of  
 265 compounds **1** and **3**, while these compounds were absent in the PD- *C.*  
 266 *islandica* chemotype and *C. ericetorum*. The presence of the aliphatic lichen  
 267 acids (i.e. compounds **5-8**) did not result in a red color reaction. The co-  
 268 occurrence of compounds **1** and **3** in organic extracts of *C. islandica* has been  
 269 found in literature (Fernández-Moriano et al., 2015).

270

271 The two-component PCA score plot (Figure 4) of the UPLC-QToF-MS data

272 provides the visualization of how different chemical groups relate to each

273 other. Three chemical groups were formed based on their chemical profiles,

274 representing the PD- (CI PD-) and PD+ chemotypes (CI PD+) of *C.*

275 *islandica* and *C. ericetorum* (CE). The first component explains 43.6%

276 chemical variations, mainly interspecific differences between CE and CI.

277 The secondary component accounts for 10.4% variations, mainly

278 intraspecific differences between CI PD+ and CI PD-. PCA is a useful tool

279 in summarizing metabolite data and revealing groupings of food ingredients

280 from different biological origins (Azilawati, Hashim, Jamilah, & Amin,

281 2015; Cubero-Leon et al., 2014). From the Figure 4, the lichen *C. islandica*

282 shows high intraspecific chemical variations in Iceland with two

283 chemotypes recognized as reported before (Kristinsson, 1969), while

284 Icelandic *C. ericetorum* shows relatively less variation, even when

285 compared with non-Icelandic *C. ericetorum* specimens. This could partly be

286 explained by the limited distribution of CE, resulting in less variation. CE

287 has a restricted geographic distribution in north and east Iceland, whereas CI

288 has a rather wide distribution around Iceland (Thell & Moberg, 2011).

289

290 3.2 DNA barcoding

291

292 Sizes of PCR products were ca. ~600-900 bp for fungal nrITS and ca. ~900

293 bp for fungal RPB2 (Online Resource Figure S1). The variation of the

294 fungal nrITS PCR products was due to the presence of a group I intron

295 sequence in the longer amplicons, and absence in the short ones. In total 97

296 new sequence were obtained, including 48 for RPB2 and 49 for fungal

297 nrITS. PCRs of a few old herbarium reference specimens were not

298 successful (Online Resource Table S1).

299

300 The phylogenetic tree based on the ITS region (Figure 5a) shows that *C.*  
 301 *ericetorum* is paraphyletic and nested within the *C. islandica* clade, whereas  
 302 both *C. ericetorum* and *C. islandica* are monophyletic in the RPB2 tree  
 303 (Figure 5b). Therefore the phylogenetic analyses support RPB2 as a barcode  
 304 with power to discriminate between the two species of *Cetraria*, but shows  
 305 that the nrITS barcode does not discriminate the species under study. The  
 306 pairwise distance analysis supports this interpretation, revealing that RPB2  
 307 is able to distinguish the two taxa, whereas the nrITS region always yields  
 308 false positive or negative identifications (Figure 6). There are other cases  
 309 where the nrITS region fails to discriminate lichenized fungi (Kelly et al.,  
 310 2011; Pino-Bodas, Martín, Burgaz, & Lumbsch, 2013), though the nrITS  
 311 region revealed interspecific barcoding gaps among most species of genera  
 312 *Melanelia* and *Montanelia* (Leavitt et al., 2014; Pino-Bodas et al., 2013).  
 313 The failure of the nrITS region in this case might be attributed to  
 314 intragenomic polymorphism in the ITS region, a phenomenon described in  
 315 other lichen-forming fungi (Kelly et al., 2011; Mark et al., 2016). It has also  
 316 been reported that the nrITS region alone is not suitable to estimate the  
 317 phylogenetic relationships within the *C. islandica* group (Thell et al., 2000).  
 318  
 319 The limited application of RPB2 as a barcoding region has been explained  
 320 by difficult PCR amplification and sequencing (Schoch et al., 2012).  
 321 Specimens stored for over 3 years have shown problems in PCR  
 322 amplification (Kelly et al., 2011). We have, however, not encountered a  
 323 PCR amplification problem for either locus even with specimens which are  
 324 15 years old. Successful PCR amplification of the RPB2 region using even  
 325 older specimens (*Cladonia* sp.) of about 30 years has also been recorded  
 326 (Pino-Bodas et al., 2013). Age-dependent problems with PCR amplification  
 327 may be taxon-specific, as well as influenced by the DNA extraction method  
 328 in use. We noted that the sequence alignment of RPB2 is much simpler. In  
 329 contrast to the hypervariability of the nrITS region, RPB2 is also

330 recommended as an alternative marker for phylogenetic analysis (Větrovský  
 331 et al., 2016). We therefore reject the nrITS region and propose the RPB2  
 332 region as an efficient DNA barcode for testing medicinal products  
 333 containing Iceland Moss, at least in terms of discriminating between *C.*  
 334 *ericetorum* and *C. islandica*.  
 335  
 336 Although the RPB2 region is effective for discriminating between species,  
 337 chemotypes of *C. islandica* are not discriminated (Figure 5). In Figure 5b, *C.*  
 338 *islandica* specimens from Iceland show two strongly supported clades, I and  
 339 II. All of the *C. islandica* PD- chemotype specimens fall into clade I, but are  
 340 interspersed with PD+ chemotypes, while clade II contains exclusively PD+  
 341 *C. islandica* specimens. Some lichen chemotypes have been shown to be  
 342 monophyletic (Fehrer, Slavíková-Bayerová, & Orange, 2008), but they can  
 343 also be not (Lutsak, Fernández-Mendoza, Nadyeina, Şenkardeşler, &  
 344 Printzen, 2017).  
 345  
 346 Domestically, *Cetraria islandica* is sold as whole lichen-thalli food  
 347 ingredients or tea in Iceland. Accurate identification is generally not  
 348 difficult for taxonomic experts, but it may prove intractable to identify  
 349 powdered lichen materials, which lack morphological or sometimes  
 350 chemical characters. DNA barcoding as outlined here could substantially  
 351 facilitate identification by comparing new sequence data with reference data  
 352 generated from expertly identified voucher specimens.  
 353  
 354 The current study focused on the authentication of natural lichen materials  
 355 without downstream processing. In case of highly processed herbal  
 356 materials where DNA may undergo considerable degradation, an alternative  
 357 method could be double gene targeting PCR, which amplifies selected  
 358 shorter regions (e.g. 70-150 bp) (Hossain et al., 2016, 2017).  
 359



360           3.3 The advantage of the integrative approach for authentication of  
361           Iceland Moss  
362

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

385

386 Although chemical profiling does not have an independent role in lichen  
387 species identification/Iceland Moss authentication, it is indispensable for the  
388 quality control of marker or health-beneficial components. It can provide  
389 both qualitative and quantitative information on phytochemical composition

390 during extraction and downstream processing. Coupled to chemometric  
391 tools, chemical profiling could also be used in differentiation of intraspecific  
392 chemical variants, which is superior to DNA barcoding.

393

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

404

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406

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415

416 **Conflicts of interest:** none

417

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593 Figure captions  
594

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

605

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

613

614  
615 **Figure 3.** MS chromatograms of the PD+ (**a**) and PD- (**b**)  
616 chemotypes of *Cetraria islandica* and PD- *C. ericetorum* (**c**)  
617 and thallus color reaction by PD staining of PD+ (**d**) and PD- (**e**)  
618 chemotypes of *Cetraria islandica* and PD- *C. ericetorum* (**f**).

619 Major secondary metabolites are labelled corresponding to  
620 structures **1-8** in Figure 2. Compounds **7A** and **7B** were  
621 tentatively identified as stereoisomers of (+)-protolichesterinic  
622 acid **7**, and **6A** a stereoisomer of (+)-roccellaric acid **6**. Scale =  
623 0.5 mm.

624

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

632

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

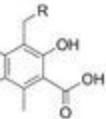
641

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

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







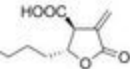
1  
H<sub>2</sub>O, 2  
H<sub>2</sub>O, 3



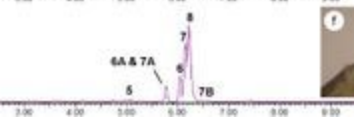
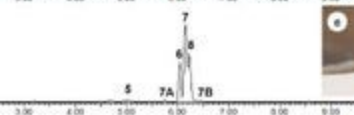
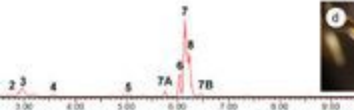
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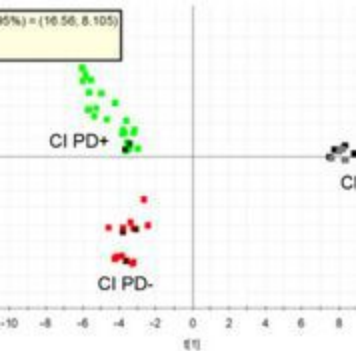
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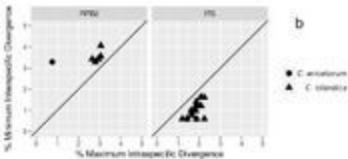
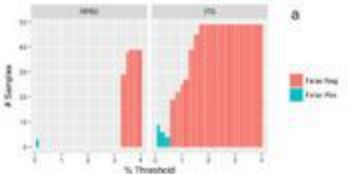
8

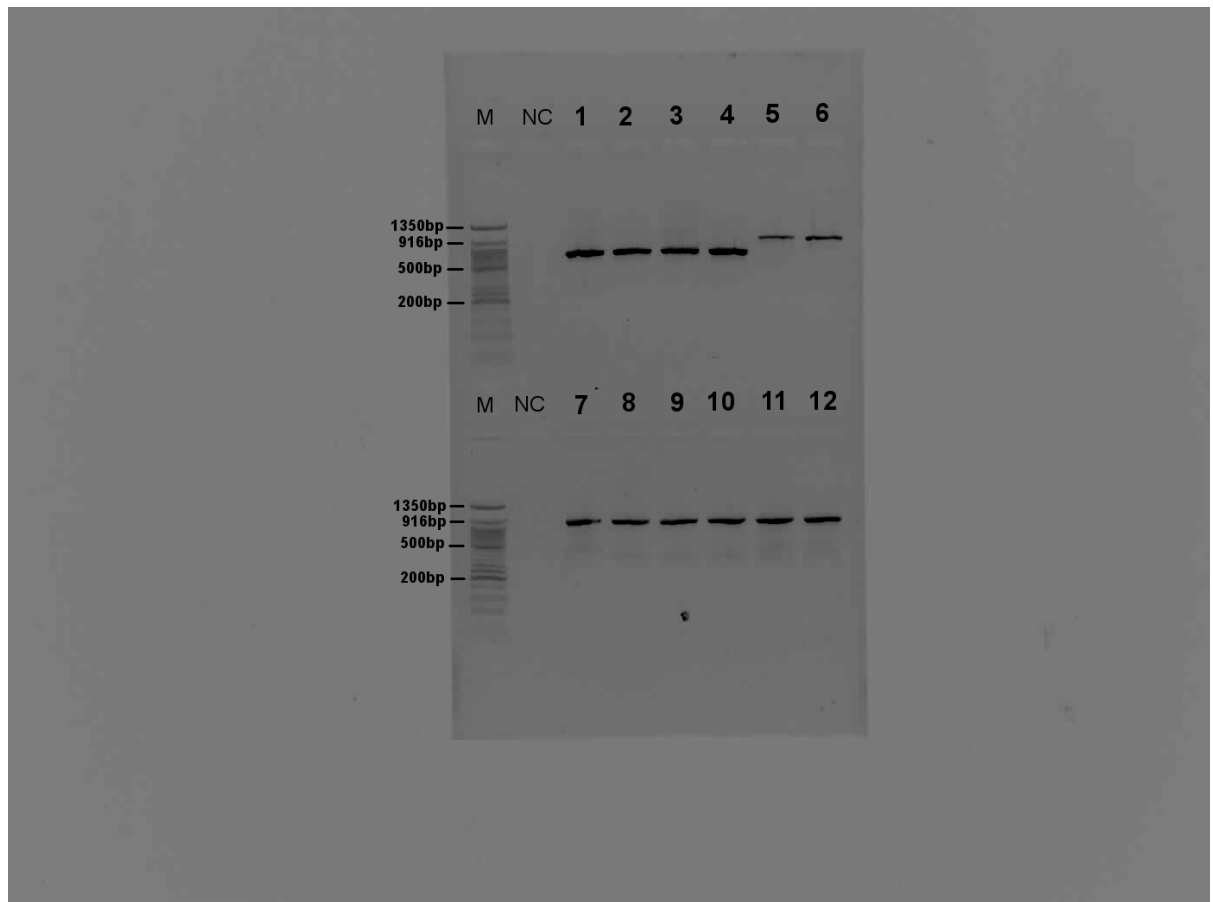


95%) = (10.56, 8.105)



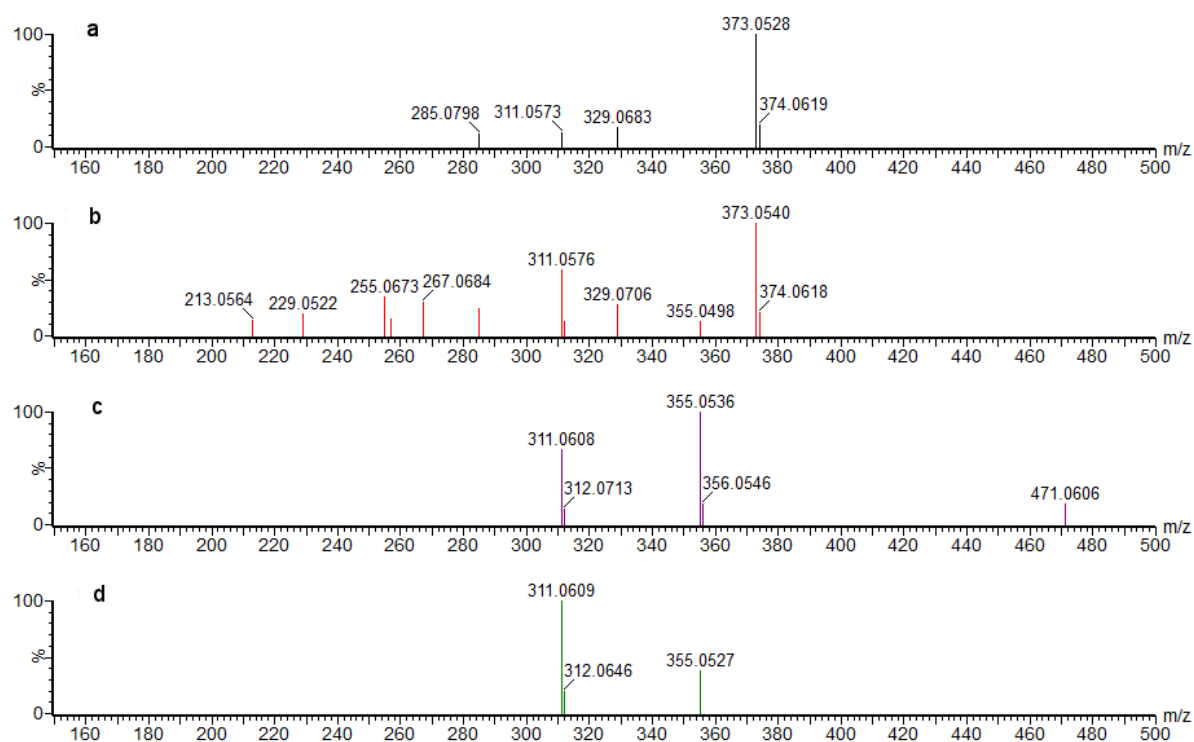






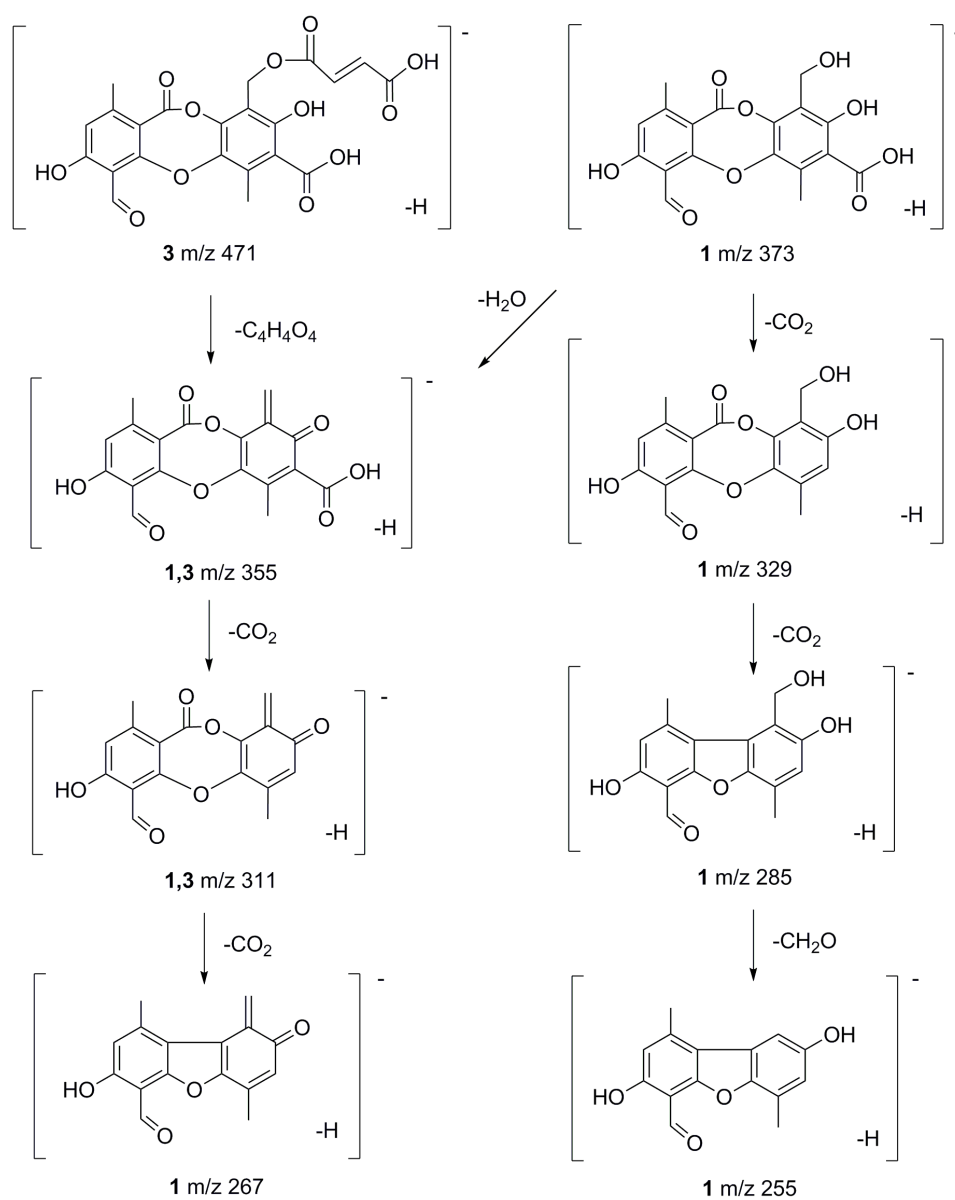
**Figure S1.** Agarose gel electrophoresis of PCR products from fungal nrITS and RPB2 loci. Lane 1-6: PCR products of fungal nrITS locus, ranging from 600 (intron-absent) to 900 bp (intron-present). Lane 7-12: PCR products of fungal RPB2 locus of ca. 900 bp. M: ladder. NC: negative control.

Figure S2

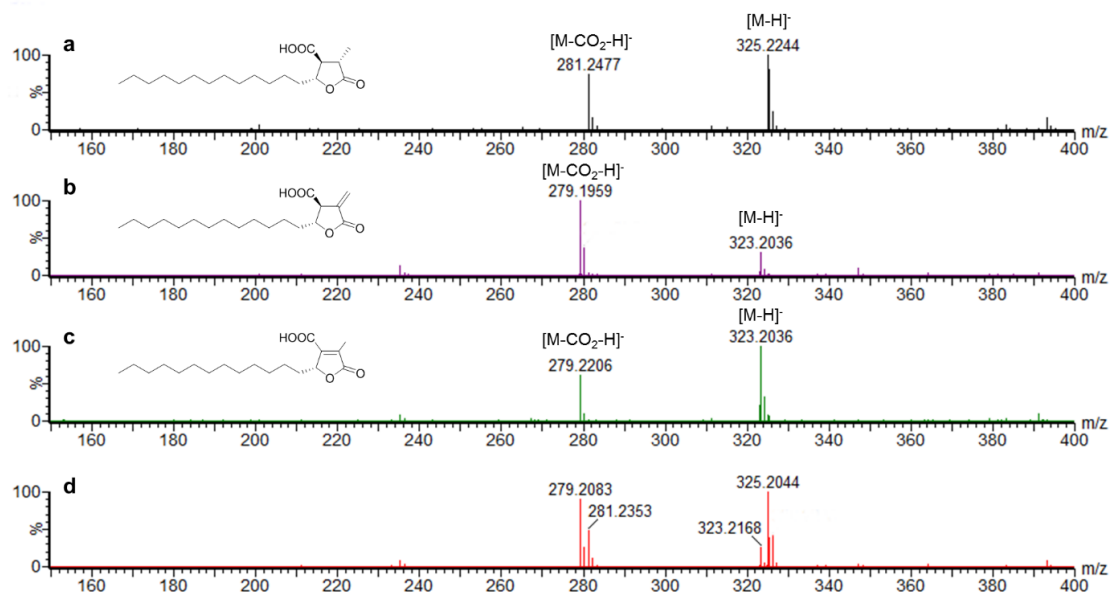


**Figure S2.** MS spectra of major depsidones in the PD+ *Cetraria islandica* chemotype. MS (a) and MS<sup>2</sup> (b) spectra for protocetraric acid **1**; MS (c) and MS<sup>2</sup> (d) spectra for fumarprotocetraric acid **3**.





**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 <sup>a</sup>	Collection date	Specimen voucher <sup>b</sup>	Spot test <sup>c</sup>	Collector	DNA Isolate	GenBank accession number	
						RPB2	nrITS
<i>Cetraria islandica</i>							
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	<b>LA30017</b>	PD-	Hordur Kristinsson	CI115	KY768985	KY765007
Iceland: INo	14-Aug-2012	LA31900	PD-	Starri Heidmarsson	CI117a	KY768986	KY765008
Iceland: INo	10-Aug-2006	<b>LA31128</b>	PD-	Hordur Kristinsson	CI113	-	-
Iceland: INo	10-Jul-1998	<b>LA17549</b>	PD-	Hordur Kristinsson	CI36	-	-
Iceland: INo	5-Jul-1998	<b>LA17221</b>	PD-	Hordur Kristinsson	CI37	-	-
<b><i>Cetraria ericetorum</i></b>							
Iceland:IAu	10-Aug-1997	LA18976	PD-	Hordur Kristinsson	CE1	-	KY765009
Finland: Sodankylä	21-Aug-2003	<b>NO2530</b>	PD-	Beata Krewicka	CE6	KY768987	KY765010
Sweden: Uppsala	20-May-2002	<b>NO23002</b>	PD-	Leif Tibell	CE8	KY768988	KY765011
Sweden: Uppsala	18-Oct-2015	<b>NO5626</b>	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	<b>NO501</b>	PD-	Ake Agren	CE3	-	-
Russian: Komi	6-Jul-2000	<b>L135019</b>	PD-	-	CE4	-	-
Canada: Quebec	2-Jul-1999	<b>NO5021</b>	PD-	Jan-Eric Mattsson	CE5	-	-
Poland: Silesia	19-Jul-1998	<b>KO2101</b>	PD-	-	CE7	-	-
Russia: Komi	2-Jul-1997	<b>NO7971</b>	PD-	Björn Larsson	CE9	-	-
Iceland: INo	31-Jul-1996	<b>NO720</b>	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	-	-

<sup>a</sup> INo, INv, IVe, IMi, IAU and ISu refer to corresponding area in Icelandic map below;



<sup>b</sup> Authentic herbarium specimens are marked in boldface;

<sup>c</sup> 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) <sup>a</sup>	$[M-H]^-$ (m/z) <sup>b</sup>	Product ions (m/z) <sup>c</sup>	Mass error (ppm) <sup>d</sup>	Molecular formula	Compound <sup>e</sup>	Lichen <sup>f</sup>
2.48	373.0540	355.0498, 329.0706, 311.0576, 285.0798	-5.4	C <sub>18</sub> H <sub>14</sub> O <sub>9</sub>	Protocetraric acid <b>1</b>	CI (PD+)
2.55	385.0650	341.0783, <b>329.2408</b>	-	-	Unidentified	CE
2.67	487.0986	373.0665, <b>355.0501</b> , 311.0594	-	-	Unidentified	CI (PD+)
2.75	473.0806	<b>355.0451</b> , 311.0550	-0.8	C <sub>22</sub> H <sub>18</sub> O <sub>12</sub>	Succinprotocetraric acid <b>2</b>	CI (PD+)
2.88	517.1052	401.0900, 369.0647, 325.0730	6.7	-	Unidentified	CI (PD+)
2.96	471.0536	<b>355.0467</b> , 311.0581	-1.4	C <sub>22</sub> H <sub>16</sub> O <sub>12</sub>	Fumarprotocetraric acid <b>3</b>	CI (PD+)
3.07	489.3547	355.0500, <b>343.0474</b> , 311.0598, 299.0618	-	-	Unidentified	CI (PD+)
3.17	387.0728	355.0474, 343.0864, 311.0580, 299.0962	3.1	C <sub>19</sub> H <sub>16</sub> O <sub>9</sub>	Unidentified	CI (PD+)
3.21	293.1744	236.1066, 221.1552	-3.1	C <sub>17</sub> H <sub>26</sub> O <sub>4</sub>	Unidentified	CI, CE
3.34	431.3405	355.0503, 309.1720	7.4	C <sub>24</sub> H <sub>48</sub> O <sub>6</sub>	Unidentified	CI (PD+)
3.57	357.0607	313.0723, 269.0848	-0.8	C <sub>18</sub> H <sub>14</sub> O <sub>8</sub>	Virensic acid <b>4</b>	CI (PD+)
4.17	295.2257	277.2194, 171.1052	-5.4	C <sub>18</sub> H <sub>32</sub> O <sub>3</sub>	Unidentified	CE
4.71	-	443.3083, <b>279.2310</b> , 250.1470	-5.0	-	Unidentified	CI, CE
4.94	-	297.2133, <b>279.2383</b> , 264.1647, 253.2214	-	-	Unidentified	CI, CE
5.03	279.2364	<b>251.2069</b>	-	-	Unidentified	CI, CE
5.09	295.1935	251.2062	8.8	C <sub>17</sub> H <sub>28</sub> O <sub>4</sub>	Nephrosterinic acid <b>5</b>	CI, CE
5.44	-	311.2299, <b>281.2556</b>	-	-	Unidentified	CI, CE
5.77	323.2168	<b>279.2314</b>	-3.6	C <sub>19</sub> H <sub>32</sub> O <sub>4</sub>	A stereoisomer of (+)-Protolichesterinic acid <b>7A</b>	CI, CE
5.77	325.2370	281.2527	-2.8	C <sub>19</sub> H <sub>34</sub> O <sub>4</sub>	A stereoisomer of (+)-Roccellaric acid <b>6A</b>	CE
6.05	325.2405	281.2511	8.0	C <sub>19</sub> H <sub>34</sub> O <sub>4</sub>	(+)-Roccellaric acid <b>6</b>	CI, CE
6.17	323.2234	<b>279.2322</b>	-0.7	C <sub>19</sub> H <sub>32</sub> O <sub>4</sub>	(+)-Protolichesterinic acid <b>7</b>	CI, CE
6.23	323.2224	279.2372	0.6	C <sub>19</sub> H <sub>32</sub> O <sub>4</sub>	Lichesterinic acid <b>8</b>	CI, CE
6.46	323.2218	<b>279.2336</b>	4.5	C <sub>19</sub> H <sub>32</sub> O <sub>4</sub>	A stereoisomer of (+)-Protolichesterinic acid <b>7B</b>	CI, CE

<sup>a</sup>  $t_R$  means retention time;<sup>b</sup>  $[M-H]^-$  stands for deprotonated molecular ion;<sup>c</sup> The product ion is marked in bold when it is the base peak in the MS spectrum;<sup>d</sup> The mass error of the base peak is provided;<sup>e</sup> Major compounds **1-8** are labelled corresponding to structures in Figure 2;<sup>f</sup> 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*.