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Testing the correlation between norstictic acid content and species evolution in the *Cetraria aculeata* group in Europe

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Abstract: Most lichen-forming fungi are characterized by the production of secondary metabolites. Differences in metabolite patterns have frequently served to distinguish lichen taxa with subsequent controversies about the rank of chemical variants (chemotype, variety, subspecies or species). Using a model system, we investigate whether production of norstictic acid within a group of lichenized ascomycetes is correlated with phylogenetic patterns, population differentiation or single and multi-locus haplotypes. Our study is based on DNA sequences of three gene loci (ITS, GPD, mtLSU) together with HPLC (311) and TLC (594) data from a total of 594 samples of three closely related fruticose lichens: *Cetraria aculeata* and *C. muricata* without norstictic acid, and *C. steppae* with norstictic acid. In nature, *C. aculeata* and *C. steppae* often occur together and the status of *C. steppae* as a separate species has been questioned. Our results show geographical but no phylogenetic structure of norstictic acid production and few significant associations between genetic clusters and the occurrence of norstictic acid. All frequently distributed haplotypes display differences in norstictic acid content. The few associations at the population level are most likely a by-product of spatial genetic structure, because norstictic acid was expressed only in individuals from the Mediterranean-Central Asian part of the study area. We conclude that the production of norstictic acid in the *C. aculeata* group is most likely triggered by the environment (climate, edaphic factors, associated symbionts). *Cetraria steppae* might be a different evolutionary lineage restricted to warm temperate regions but it is not uniquely characterized by the presence of norstictic acid.

Key words: chemical diversity, GPD, ITS, lichenized ascomycetes, mtLSU

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Introduction

To date, approximately 20 000 lichen species have been recognized, but this number probably grossly underestimates their real diversity (Lumbsch *et al.* 2011; Lücking *et al.* 2014). One major problem in detecting and describing lichen diversity has always been the identification of consistent diagnostic characters. Since differences in anatomical and morphological characters might be very subtle and subject to extreme morphological plasticity (Pintado *et al.* 1997; Pino-Bodas *et al.* 2011; Pérez-Ortega *et al.* 2012; Muggia *et al.* 2013), lichenologists are continually trying to widen the scope of taxonomically useful characters.

Ever since Nylander introduced simple spot tests to detect secondary metabolites, lichen systematics has also relied on chemical characters (Nylander 1866; see also reviews by Lumbsch 1998; Printzen 2010). At higher systematic levels, the widespread occurrence and consequent homoplasy of most secondary metabolites usually limits their usefulness (but see e.g. Schmitt & Lumbsch 2004; Lumbsch *et al.* 2006). At lower taxonomic

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levels, their value as distinctive phenotypic characters has also been questioned (Velmala *et al.* 2009; Leavitt *et al.* 2011; Pino-Bodas *et al.* 2011). Depending on the magnitude of the difference (e.g. substitution of biochemically related metabolites, chemosyndromic variation), some authors have either attributed specific (e.g. Culberson 1967), subspecific (e.g. Lumbsch *et al.* 1994) or varietal rank (e.g. Skult 1993) to chemical variants, while others avoided taxonomic conclusions altogether by treating them as chemotypes of the same species (e.g. Knoph *et al.* 1995). Recently, DNA sequence data have become an important additional tool to study the diversity, evolution and species delimitation of lichens (e.g. Hibbett *et al.* 2007; Schoch *et al.* 2009; Crespo & Lumbsch 2010; Wirtz *et al.* 2012). This not only raised hopes that single gene sequences could be used to identify existing and detect new lichen taxa (Hebert *et al.* 2003; Kelly *et al.* 2011; Lumbsch & Leavitt 2011), it also offered the opportunity to test existing species concepts. Most studies mapped characters on phylogenetic trees from DNA sequence data (e.g. Lohtander *et al.* 2009) and often found chemically uniform monophyletic groups of samples that were recognized as separate taxa (Kroken & Taylor 2001; Tehler & Källersjö 2001; Elix *et al.* 2009). Concurrently, some studies based on population genetic datasets demonstrated considerable infraspecific genetic variability in lichens (Zoller *et al.* 1999; Printzen *et al.* 2003; Palice & Printzen 2004; Werth & Sork 2008; Spribille *et al.* 2011). Some authors ascribed this high variability to the presence of cryptic lichen species (Divakar *et al.* 2006; Crespo & Pérez-Ortega 2009; Crespo & Lumbsch 2010; Leavitt *et al.* 2011; Parnmen *et al.* 2011) with the potential danger that infraspecific lineages might be misinterpreted as separate species. However, there is an alternative danger of failing to recognize species-level lineages. Lichens appear to retain ancestral polymorphisms (Printzen *et al.* 2003, Fernández-Mendoza & Printzen 2013). Unsorted polymorphisms between sibling taxa cause gene trees to deviate from the true species tree. In this case, chemically distinct lineages could appear para- or polyphyletic on phylogenetic trees and might be overlooked.

The interpretation of chemical patterns with the background of molecular genetic data is a complex issue. If variation of chemical characters has a genetic basis, it could be associated with 1) phylogenetic groups, 2) groups of individuals sharing a genealogical history and 3) certain genotypes that would not necessarily form monophyletic groups on phylogenetic trees. Alternatively, the production of certain secondary metabolites could be triggered by ecological factors or, in the case of lichens, features of the symbiotic lifestyle, such as association with certain photobiont or bacterial symbionts (Nützmann *et al.* 2011). In this latter case, no association with the genetically defined groups 1–3 would be observed. However, populations that occur along the same ecological gradient might demonstrate some commonality in the expression of secondary metabolites. In this case, clear genetic groupings might not automatically be evidence for a genetic basis for chemical differences.

In this study we use *Cetraria aculeata* and associated species as a model to test if there is a relationship between norstictic acid production and genetic groupings. We investigate whether the presence of norstictic acid can serve as a diagnostic character for the separation of genetic lineages that have been treated as two different taxa: *C. aculeata* (Schreb.) Fr. and *C. steppae* Savicz. Previous studies on this group have only considered morphological and ecological differences in relation to chemical variability (Nadyeina *et al.* 2013). The *C. aculeata* group is particularly well suited as a model group for this kind of study because it is widely distributed along a broad ecological gradient and its population genetics has been studied intensively in recent years (Fernández-Mendoza *et al.* 2011; Domaschke *et al.* 2012; Pérez-Ortega *et al.* 2012; Fernández-Mendoza & Printzen 2013; Nadyeina *et al.* 2013; Printzen *et al.* 2013; Lutsak *et al.* 2015).

Cetraria aculeata is a dark brown fruticose soil lichen that forms shrubby tufts 1–5 cm high (Kärnefelt 1986). It is rarely found

fertile, and seems to propagate mainly by thallus fragmentation (Heinken 1999). This species is frequent in open polar and boreal environments from the high Arctic southwards to the maritime Antarctic. At intermediate latitudes it is mostly found in high mountain ecosystems, but its distributional range also extends into forest gaps, woodland and steppe ecosystems, or coastal and riparian sand deposits of the Mediterranean and temperate zones.

The delimitation of *C. aculeata* from the rather similar *C. steppae* has always been challenging. *Cetraria steppae* was first described as a morph of *C. aculeata* occurring in steppes and short grass habitats of Ukraine, Russia and Kazakhstan (Mereschkowsky 1921; Savicz 1924). More recently, the presence of norstictic acid in the thallus of *C. steppae* has been regarded as a crucial character for its delimitation from *C. aculeata* (Kärnefelt 1986; Randlane & Saag 2006). In a recent study of Ukrainian populations, Nadyeina *et al.* (2013) showed that concentrations of norstictic acid found in individual thalli vary considerably among populations. They observed that the presence of norstictic acid depends on the size of the thallus and concluded that chemical and morphological differences between thalli represent ontogenetic rather than fixed phenotypic differences, and thus should not be considered for taxonomic purposes. Consequently, they suggested that *C. steppae* is conspecific with *C. aculeata*, which was essentially assumed in recent population genetic studies on *C. aculeata* (Fernández-Mendoza *et al.* 2011; Fernández-Mendoza & Printzen 2013). *Cetraria muricata*, a third species from the group, differs slightly from *C. aculeata* in morphological features, such as narrower, more evenly rounded branches and more scattered, rounded and superficial pseudocyphellae, but both species may be difficult to distinguish in the field (Poelt 1969; Kärnefelt 1986). Norstictic acid has never been reported from *C. muricata*. According to phylogenetic reconstructions, it appears as a sister clade to *C. aculeata* and its status as a separate species has usually not been doubted (Thell *et al.* 2002, 2009; Printzen *et al.* 2013).

In this study we use a broad sample of lichens from the *C. aculeata* group across Western Eurasia, and phylogenetic and population genetic methods to investigate whether norstictic acid production reflects evolutionary history or whether the presence of norstictic acid is correlated with genetic differences between individuals and populations of lichens.

Material and Methods

Sampling

A total of 594 specimens morphologically and chemically identified as *Cetraria aculeata* (435), *C. muricata* (61) and *C. steppae* (98) from 54 sampling localities were used for this study (Table 1). Specimens regarded as *C. muricata* were included in the dataset to test whether the production of norstictic acid is restricted to *C. aculeata*/*steppae*. Sampling covers a wide latitudinal and longitudinal gradient from Svalbard in the north to the Canary Islands in the south, and Greenland in the west to the Russian part of the Altai Mountains in the east. Species of the *C. aculeata* group disperse mostly by thallus fragmentation, consequently lichen thalli were sampled at intervals with a minimum distance of 0·5 m between samples within each population to avoid collecting clonal offspring. All specimens collected in one locality were considered as one population and were given names that refer to the collection localities.

DNA extraction and sequencing

Single branches of an average dry mass of 2 mg were cut off the thallus, cleaned from all visible contaminants, frozen in liquid nitrogen and ground manually. Total genomic DNA was extracted using the DNeasy™ Plant Mini Kit (Qiagen) following the manufacturer's protocol or the PrepEase DNA Isolation Kit (USB, Cleveland, Ohio, USA) using the plant leaf extraction protocol. As in previous studies, three fungal genetic markers were used: the internal transcribed spacer region of the nuclear ribosomal DNA (ITS), a partial sequence of the large subunit of the mitochondrial ribosomal DNA (mtLSU) and a fragment of the glyceraldehyde-3-phosphate dehydrogenase (GPD) gene. Amplification, purification and sequencing were performed following Fernández-Mendoza *et al.* (2011). Sequences were assembled, aligned and alignments manually refined using Geneious v.7.1.4 (Drummond *et al.* 2013). Sequences of each unique haplotype were deposited in GenBank (Accession numbers provided in Appendix I) and all alignments were submitted to the TreeBASE database under submission number 18951.

Chemistry

The secondary metabolite content of all samples was tested with TLC and HPLC using different thallus lobes.

TABLE 1. *Collection data for* Cetraria aculeata, C. muricata *and* C. steppae *from the 54 sampling localities used in this study.*

Country (abbreviation)	N	Population	nst +	*n*	Latitude (°)	Longitude (°)	Locality, *collector* and year
Austria (AUS)	6	Austria	0	6	47·132400 N	13·513700 E	Salzburg, *Türk*, 2008
Canary Islands (CAN)	21	Valleseco	1	10	28·012990 N	15·351590 W	Gran Canaria, above Valleseco, *Crespo et al.*, 2009
		Gran Canaria	1	5	27·594320 N	15·315450 W	Gran Canaria, Vega de San Mateo a Tenteniguada, *Crespo et al.*, 2009
		El Diablillo	1	4	28·241110 N	16·252260 W	Tenerife, El Diablillo, *Crespo*, 2009
		Ayosa	0	2	28·210000 N	16·266000 W	Tenerife, Ayosa, *Crespo*, 2009
Czech Republic (CZE)	12	Bohemia	0	12	50·210450 N	12·303530 E	Bohemia, Kraslice, *Palice*, 2011
France (FRA)	121	Cap Breton	0	24	43·441910 N	01·253537 W	Capbreton, *Fernandez-Mendoza*, 2014
		Galibier	0	20	45·490300 N	06·245176 E	Galibier, *Fernández-Mendoza*, 2014
		Lacanau	0	7	45·016060 N	01·115225 W	Lacanau, *Fernández-Mendoza*, 2014
		Massif Central	0	22	44·254744 N	03·455388 E	Massif Central, Les Thenes, *Fernández-Mendoza*, 2014
		Maures	0	24	43·252677 N	06·343060 E	Massif des Maures, *Fernández-Mendoza*, 2014
		Trois Pinions	0	24	48·223251 N	02·313017 E	Trois Pinion, *Fernández-Mendoza*, 2014
Germany (GER)	56	Brand	0	12	52·003331 N	13·433303 E	Brand, Aachen, *Fernández-Mendoza*, 2014
		Ehra	0	12	52·351855 N	10·473497 E	Ehra-Lessien, *Fernández-Mendoza*, 2014
		Friedensteich	0	17	53·002132 N	11·431855 E	Wittenberge, *Fernández-Mendoza*, 2014
		Fuchsberge	0	15	52·102419 N	12·412642 E	Brandenburg, *Fernández-Mendoza*, 2014
Greenland (GRE)	7	Hoim Bukt	0	7	72·295370 N	23·561720 W	Hoim Bukt, *Westergaard & Dahl*, 2007
Iceland (ICE)	19	Iceland 1	0	8	65·525500 N	18·030330 W	Syður-Pingeyjarsysla, *Domaschke & Ottich*, 2008
		Iceland 2	0	11	65·314700 N	19·310300 W	Skagafjarðarsysla, *Domaschke & I. Ottich*, 2008
Iran (IRA)	10	Iran	0	10	38·515320 N	46·580800 E	East Azarbaijan: Kalibar, *Sohrabi*, 2005
Ireland (IRE)	6	Malin Head	0	6	55·226180 N	07·224400 W	Co. Donegal, Malin Head, *Lutsak*, 2011
Italy (ITA)	8	Sardinia	0	8	40·757650 N	09·079400 E	Sardinia, *Sadowska-Des*, 2012
Kazakhstan (KAZ)	8	Kokchetav	8	8	53·164400 N	69·202040 E	Kokchetav area, *Wagner*, 2007
Portugal (POR)	17	Marofa	0	17	40·515300 N	06·592890 W	Marofa, *Fernández-Mendoza*, 2014
Russia (RUS)	12	Russia	0	12	49·380978 N	87·514206 E	Altai Mts, *Resl*, 2011
Scotland (SCO)	16	Skye	0	3	57·125519 N	05·501926 W	Isle of Skye, *Fernández-Mendoza*, 2010
		Cairngorm	1	13	57·075600 N	03·402200 W	Cairngorm, *Fernández-Mendoza*, 2010
Spain (SPA)	110	Canencia	2	22	40·523988 N	03·462742 W	Provincia de Madrid, Canencia, *Fernández-Mendoza*, 2014
		Gamoniteiro	0	24	43·111550 N	05·552481 W	Provincia de Asturias, Gamoniteiro, *Fernández-Mendoza*, 2014
		LaBaneza	1	2	42·206115 N	06·094154 W	Provincia de Leon, La Bañeza, *Fernández-Mendoza*, 2008
		Campillejo	2	10	41·034770 N	03·172380 W	Provincia de Guadalajara, Campillejo, *Fernández-Mendoza*, 2014
		Calatanazor	5	10	41·415856 N	02·490331 W	Provincia de Soria, Calatanazor, *Pérez-Ortega*, 2007
		Iruecha	3	9	41·062700 N	02·053684 W	Provincia de Soria, Iruecha, *Pérez-Ortega*, 2007
		Teruel	4	11	40·293000 N	01·360138 W	Provincia de Teruel, Sierra de Albarracín, *Perez-Ortega*, 2009
		Zaorejas	4	10	40·454300 N	02·120734 W	Provincia de Guadalajara, Zaorejas, *Pérez-Ortega*, 2007
		Despeñaperros	0	12	38·232821 N	03·302575 W	Provincia de Jaén, Despeñaperros, *Fernández-Mendoza*, 2014
Svalbard (SVA)	18	Svalbard 1	0	10	78·123400 N	15·353100 E	Longyearbyen, *Domaschke*, 2008
		Svalbard 2	0	8	78·104500 N	16·182400 E	Adventdalen, *Domaschke*, 2008
Switzerland (SWI)	20	Saint-Bernard	0	20	46·121972 N	05·594373 E	Grand Saint-Bernard, *Fernández-Mendoza*, 2014
Turkey (TUR)	30	Izmir	0	22	38·332300 N	27·263300 E	Izmir, *Şenkardeşler*, 2014
		Bolu	0	8	40·264900 N	31·450300 E	Bolu Province, *Spribille & Lembcke*, 2007

TABLE 1 (*continued*).

Country (abbreviation)	N	Population	nst +	*n*	Latitude (°)	Longitude (°)	Locality, *collector* and year
Ukraine (UKR)	107	Antracyt	1	6	48·010528 N	39·082273 E	Luhansk region, Antracyt district, *Vasyljuk*, 2011
		Arabat	5	5	45·296880 N	35·477430 E	Arabat Split Botanical Reserve, *Nadyeina*, 2011
		Chatyr-Dag	2	12	45·424540 N	36·482040 E	Crimea, Chatyr-Dag plateaus, *Nadyeina*, 2011
		Kamyani mogyly	9	13	44·457060 N	34·186690 E	Branch of Ukrainian Nature Steppe Reserve, *Nadyeina* 2011
		Kara-Dag	6	11	47·306944 N	37·077500 E	Crimea, Kara-Dag plateau, *Nadyeina*, 2012
		Kazantyp	7	9	44·562552 N	35·121890 E	Kazantyp Nature Reserve, *Nadyeina*, 2011
		Kerch	7	7	45·465460 N	35·828030 E	Crimea, Kerch peninsula, *Nadyeina*, 2011
		Novoplatonivka	8	8	49·438805 N	37·643570 E	Kharkiv Region, Borova district, *Nadyeina*, 2011
		Provallia	8	10	48·135002 N	39·801057 E	Branch of Luhansk Steppe Nature Reserve, *Nadyeina*, 2011
		Shatsk	0	13	51·475028 N	23·833731 E	Shatsk Lake National Nature Park, *Lutsak*, 2011
		Stanychno-Luhanske	9	10	48·450830 N	39·212550 E	Branch of Luhansk Steppe Nature Reserve, *Nadyeina*, 2011
		Trehizbenska	3	3	48·760148 N	38·961057 E	Branch of Luhansk Steppe Nature Reserve, *Nadyeina*, 2011

nst+ = number of individuals with detectable amounts of norstictic acid in each sampling locality; N = total number of specimens from each country; *n* = number of specimens per locality.

In a few cases we observed that HPLC could not detect norstictic acid, while TLC showed a clear spot. More rarely the opposite pattern was observed. In order to avoid false negatives we extracted secondary compounds from 2–3 branches (20 mg) for all further TLC analyses. Lichen compounds were extracted in 0·4 ml of acetone and run using the standard solvent system C (one volume of acetic acid in five volumes of toluene) following Orange *et al.* (2001). For 311 samples HPLC was used to double-check for the presence of secondary metabolites undetected by TLC. HPLC analyses were carried out using the Agilent 1260 Infinity System and the method described by Feige *et al.* (1993) on 1–6 mg of dry tissue. TLC proved to be the more reliable method as it detected norstictic acid in all samples that were positive in HPLC, and in some where HPLC failed to detect this substance.

Inference of admixture clusters

In order to reveal genetic clusters within the three loci sequence dataset, admixture fractions were inferred with STRUCTURE v.2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003) using these three loci and counting different haplotypes as alleles per locus (Fernández-Mendoza & Printzen 2013). To estimate the optimum number of admixture clusters we used the summary likelihood statistics ΔK proposed by Evanno *et al.* (2005). The analyses were based on ten serial runs for each number of clusters (K) between one and ten. Admixture models used a uniform alpha prior, independent allele frequencies and no prior population information. All analyses were run for 5×10^5 generations after a burn-in period of $2{\cdot}5 \times 10^5$ iterations. Using longer runs did not improve likelihood estimates or clustering solutions. The results were evaluated using a custom R script. Clustering solutions were summarized using CLUMPP (Jakobsson & Rosenberg 2007) and are presented as clustergrams in Fig. 1. Average admixture proportions per locality were mapped (Fig. 2) using R packages *rworldmaps* (South 2011) and *mapplots* (Gerritsen 2012), and later manually refined in Adobe Illustrator CS5.

Phylogenetic trees

Phylogenetic reconstructions were carried out for each locus independently using the Bayesian method implemented in BEAST v.1.8.1 (Drummond *et al.* 2012). For each locus datasets were collapsed to haplotypes. If members of the same haplotype differed in the content of norstictic acid, one individual with and one without norstictic acid were kept in the dataset (those are terminal nodes of the trees). The suitability of alternative nucleotide substitution models was approximated using jModelTest v.2 (Posada 2008); optimum models were chosen under a corrected Akaike information criterion (cAIC). The appropriateness of molecular clock models was tested in MEGA 5 (Tamura *et al.* 2011). We used a strict clock for GPD and mtLSU, but an exponential relaxed clock for ITS. Information about the final datasets and the selected models is

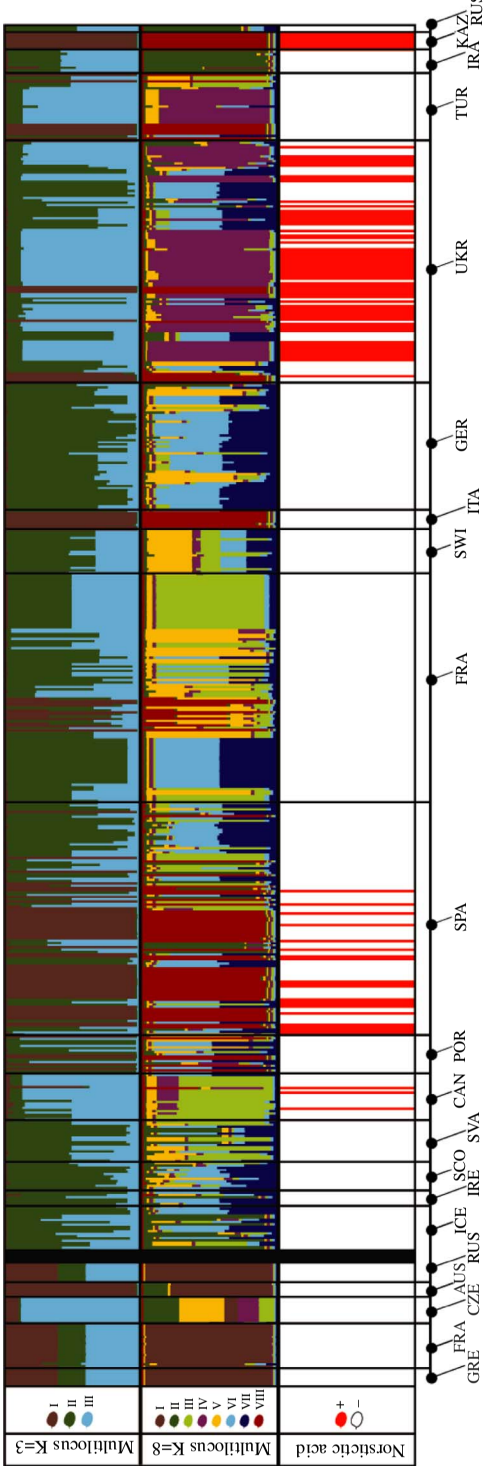

FIG. 1. Assignment of individuals to genetic clusters inferred from the datasets of three fungal loci using STRUCTURE. Each multicoloured bar represents an individual, the height of each colour corresponds to the estimated probability of the individual belonging to the respective genetic cluster (indicated by the colour legend and roman numerals on the left). K = 3 and K = 8 refer to the two clustering solutions. Populations are sorted geographically and arranged by country (see Table 1 for abbreviations), black vertical lines separate populations from different countries. The bold black vertical line separates the specimens identified as *Cetraria muricata* (on the left) from those of *C. aculeata* and *C. steppae* (on the right).

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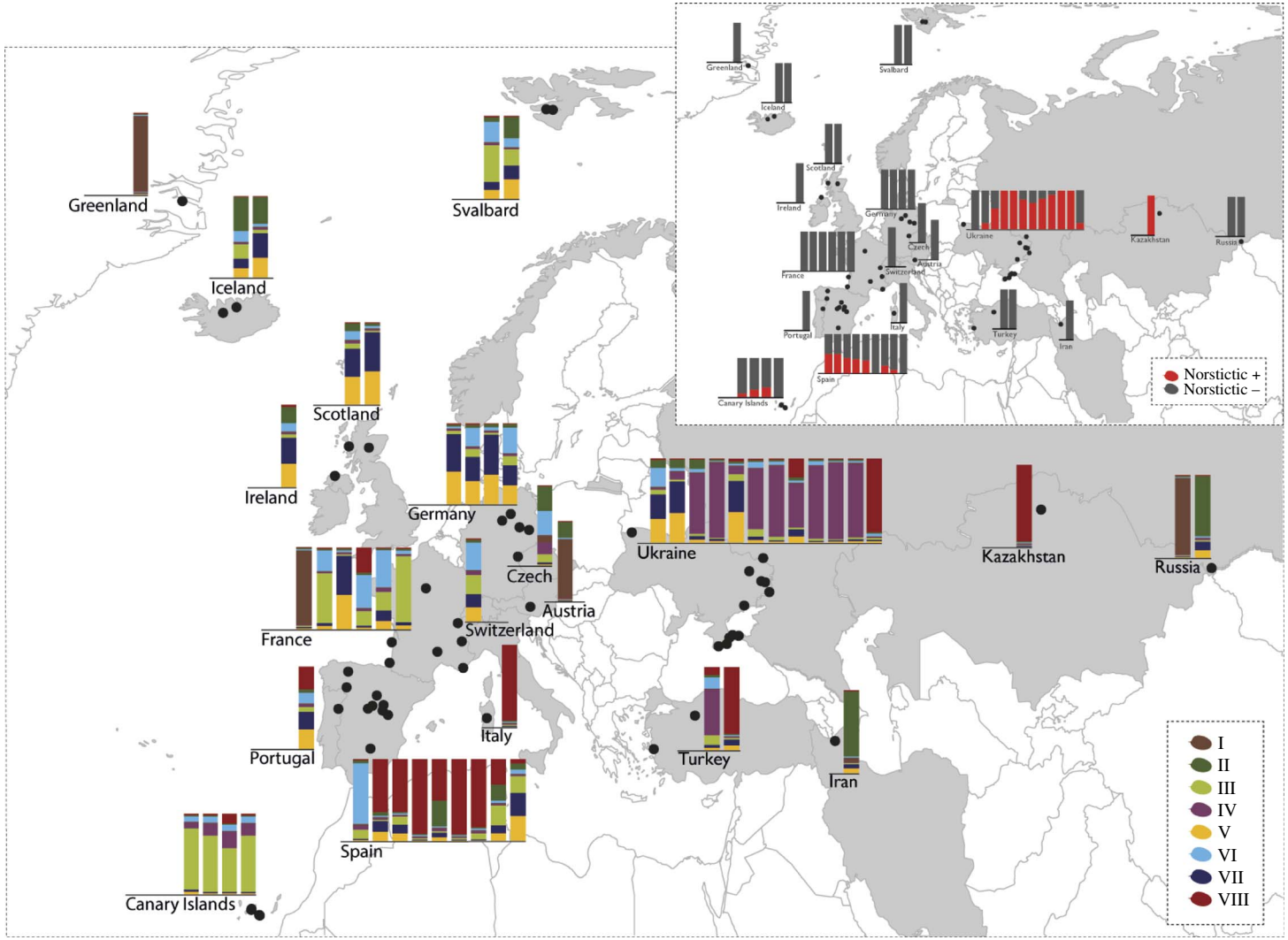

FIG. 2. Multilocus genetic clusters assessed with STRUCTURE per population together with their geographical distribution. Each bar plot represents a single population from the respective country; populations within countries are sorted from west to east. The height of each colour reflects the proportion of genetic clusters assignment. The inset shows the proportion of individuals with norstictic acid for each locality.

TABLE 2. *Summary statistics, PCR settings and optimum substitution models for the three gene loci used in this study.*

	Gene locus		
	ITS	mtLSU	GPD
Dataset			
Alignment length	471	798	796
Variable sites	71	22	51
Nucleotide diversity pi	0·01568	0·00603	0·01289
Number of haplotypes (tree tips)	68 (80)	19 (25)	34 (41)
PCR Settings			
Primers	ITS 1F-5' / ITS 4-3'	ML 3-A-5' / ML 4-A-3'	GPD LM-1-5' / GPD LM-2-3'
Reference	Gardes & Bruns 1993	Printzen 2002	Myllys *et al.* 2002
Denaturation (time)	94 °C (5 min)	95 °C (30 s)	95° C (5 min)
Amplification			
1st Phase (time)	5 cycles 94 °C (30 s) 54 °C (30 s) 72 °C (1 min)	5 cycles 95 °C (30 s) 63 °C (30 s, touchdown − 1 °C per cycle) 72 °C (1 min)	8 cycles 94 °C (1 min) 62 °C (1 min, touchdown − 1 °C per cycle) 72 °C (1 min)
2nd Phase (time)	33 cycles 94 °C (30 s) 48 °C (30 s) 72 °C (1 min)	37 cycles 95 °C (30 s) 58 °C (30 s) 72 °C (1 min)	30 cycles 95 °C (1 min) 52 °C (1 min) 72 °C (1 min)
Extension (time)	72 °C (10 min)	72 °C (10 min)	72 °C (10 min)
Phylogenetic reconstruction			
Substitution model	GTR	HKY+Γ	HKY+I+Γ
Molecular Clock	Exponential relaxed	Strict	Strict

summarized in Table 2. Two independent runs of 55 M generations sampling every 5000th tree were run for each locus. After convergence was checked in Tracer v.1.6.1 both runs were pooled using Logcombiner v.1.8.2 (Drummond *et al.* 2012). An 18% burn-in fraction was removed from the sample. Posterior tree distributions were summarized in maximum clade credibility trees also displaying the median of branch lengths at each node (Fig. 3).

Correlation of norstictic acid content with genetic structure

We tested the association between norstictic acid expression and the percentage of assignment to admixture clusters by means of a Welch's *t*-test, as implemented in the *Base* package of R. To avoid pseudoreplication, the dataset was collapsed to unique combinations of multilocus genotypes and norstictic acid phenotypes (see above). Graphical and statistical summaries of these tests are shown in Fig. 4. In order to test the phylogenetic signal in the expression of norstictic acid we used the D statistic developed by Fritz & Purvis (2010), implemented by the authors in function *phylo.d* of R package *caper*. We made a recursive use of this function to account for phylogenetic uncertainty in which the D statistic was calculated on a subsample of 1000 trees from the posterior tree distribution for each locus and processed to obtain average D-estimates and 95% confidence intervals (Table 3).

Results

Datasets

A total of 1782 gene sequences were used for this study, of which 1320 were newly generated, and 462 generated during previous studies (Fernández-Mendoza *et al.* 2011; Fernández-Mendoza & Printzen 2013). Information on aspects of the datasets is summarized in Table 2. Nucleotide diversity for the three markers ranged between 0·006 (mtLSU) and 0·016 (ITS), and haplotype richness varied from 19 (mtLSU, plus 4 haplotypes of *C. muricata)* to 68 (ITS, plus 12 haplotypes of *C. muricata*).

Geographical structure of norstictic acid production

Norstictic acid was detected in 98 out of 594 samples and exclusively in specimens identified as *C. aculeata/steppae*, and never in *C. muricata* from the temperate regions of the broader Mediterranean (populations from Spain, Canary Islands, Ukraine) and Central

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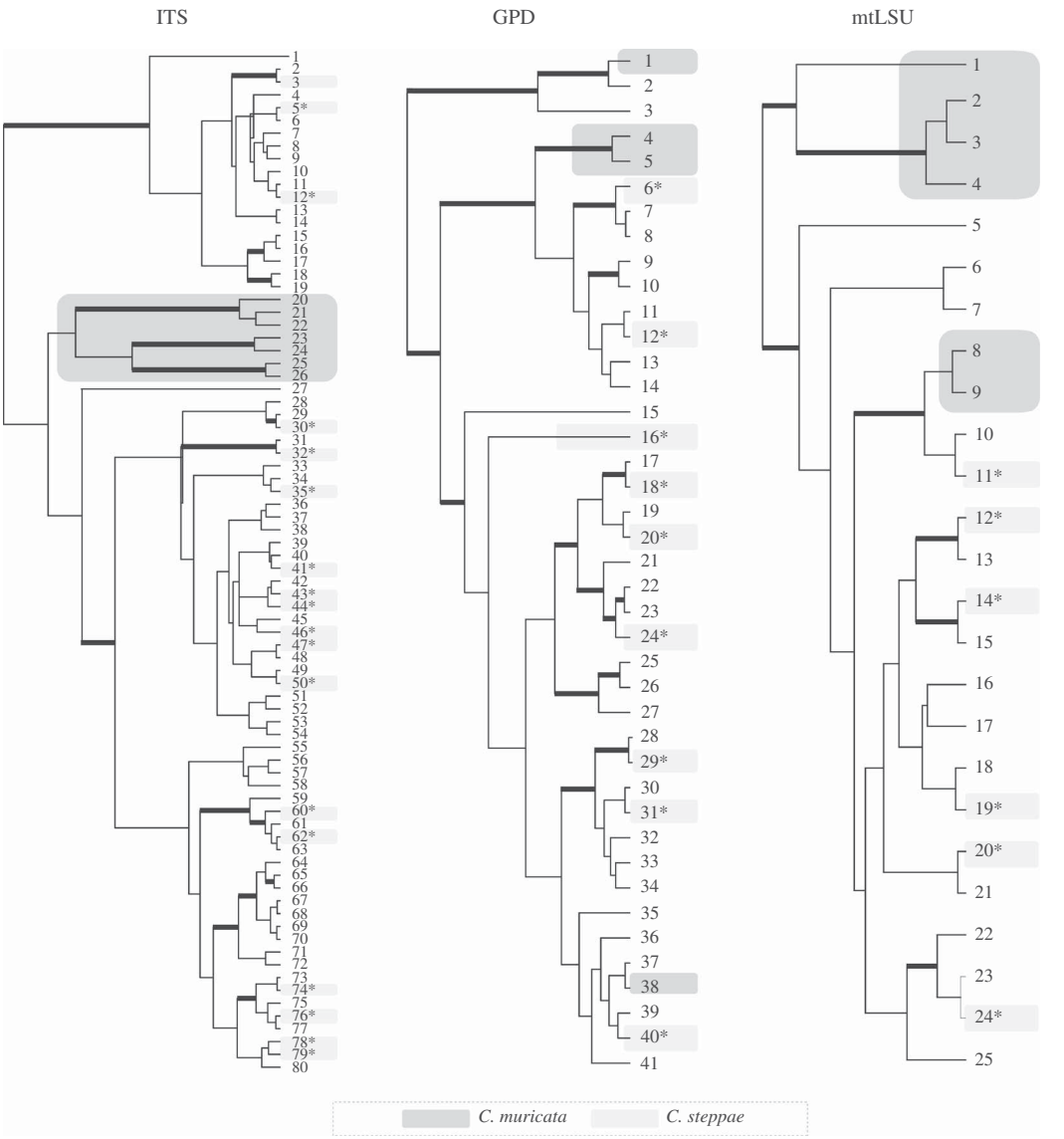

FIG. 3. Maximum clade credibility trees for the gene loci used in this study of the *Cetraria aculeata* group. Terminal nodes do not directly refer to haplotype numbers because several haplotypes are represented twice (once for each chemotype). Branches in bold received ≥0·95 posterior probability support. * = terminal nodes with norstictic acid

Asia (Kazakhstan, Figs 1 & 2). As observed previously (Nadyeina *et al.* 2013), specimens with and without norstictic acid were often found within the same populations. There were a few populations in Kazakhstan and Ukraine (Arabat, Kerch, Novoplatonivka, Trehizbenska) in which all individuals studied (3–8) contained norstictic acid. The population from Kazakhstan and one Ukrainian population (Arabat) turned out to be genetically homogeneous, with all samples belonging to the same single gene haplotypes. All the other populations were genetically heterogeneous.

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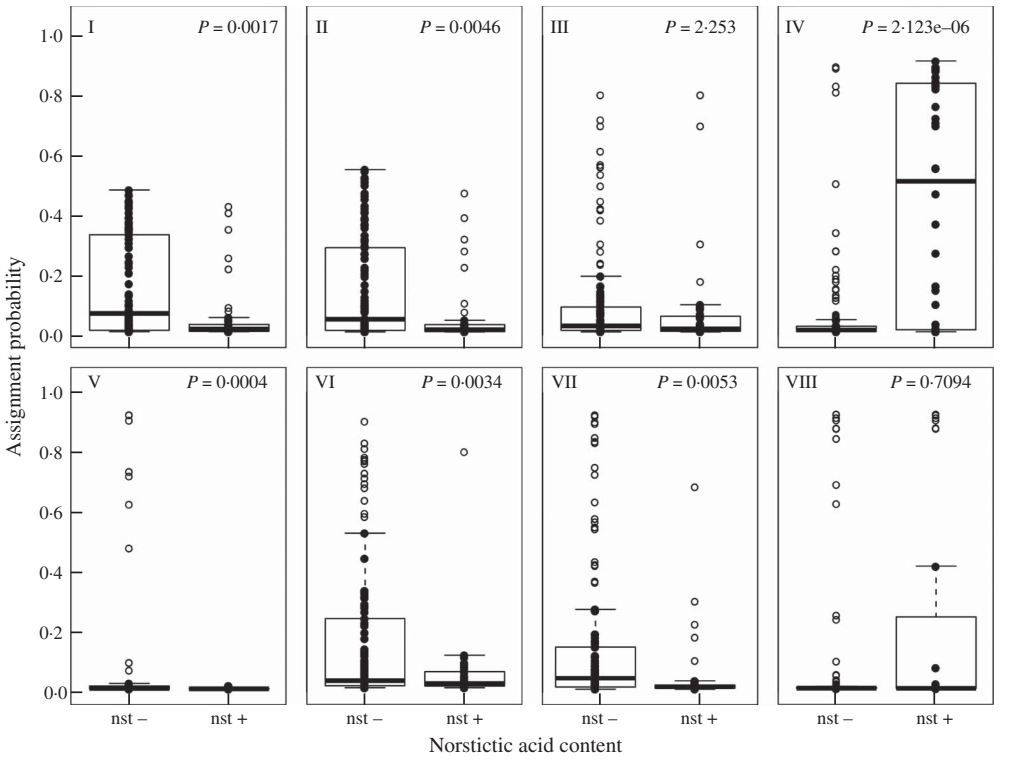

FIG. 4. Boxplots and summary of Welch's t-tests showing association between the inferred admixture clusters and the presence and absence of norstictic acid. The y-axis indicates the assignment probability of individuals to that admixture cluster. The bold line indicates the median; the box, the upper and lower quartile and whiskers extend to 1·5 times the interquartile range. Raw data points are denoted by filled black circles and those considered to be outliers by open black circles. *P* values are shown where the difference between presence and absence of norstictic acid was statistically significant.

Inference of multilocus clusters

Using the ΔK criterion of Evanno *et al.* (2005) we estimated two clustering solutions to be optimal for the three-gene dataset: with three and eight admixture clusters. The model with three clusters meant a steep increase in likelihood from the models with one and two populations, but did not render a reasonable clustering solution, especially regarding the specimens identified as *C. muricata*, which were misleadingly assigned equally to each of the clusters (multilocus K = 3, Fig. 1). Moreover, Mediterranean and Central Asian specimens are assigned to two different clusters that do not correlate with geographical location. For example, individuals from the Canary Islands are lumped together with most from Ukraine, and about half the specimens from Spain with those from Kazakhstan. For this reason, we preferred the maximum likelihood model with eight populations (Fig. 1), which also provided a clustering solution more compatible with our previous estimate (Fernández-Mendoza & Printzen 2013). The K = 8 model assigned all specimens of *C. muricata* to admixture cluster I, with the exception of the Czech population that could not be assigned to any of the clusters with certainty.

Correlation of norstictic acid production with genetic structure

The map (Fig. 2) shows a geographical pattern in the distribution of norstictic acid

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TABLE 3. *D-estimates and 95% confidence intervals for the ITS, GPD and mtLSU loci used in this study of the* Cetraria aculeata *group.*

	ITS	GPD	mtLSU
Estimated D	$1{\cdot}01 \pm 0{\cdot}053$	$1{\cdot}27 \pm 0{\cdot}066$	$1{\cdot}36 \pm 0{\cdot}071$
Probability of estimated D resulting from no (random) phylogenetic structure	$0{\cdot}52 \pm 0{\cdot}027$	$0{\cdot}88 \pm 0{\cdot}046$	$0{\cdot}85 \pm 0{\cdot}044$
Probability of estimated D resulting from Brownian phylogenetic structure	$3{\cdot}1 \times 10^{-5} \pm 1{\cdot}6 \times 10^{-6}$	$1{\cdot}3 \times 10^{-4} \pm 6{\cdot}6 \times 10^{-6}$	$2{\cdot}1 \times 10^{-3} \pm 1{\cdot}1 \times 10^{-4}$

The D value is a measure of phylogenetic signal in a binary trait, resulting from both random association and the clumping expected under a Brownian evolution threshold model. The value of D can be both smaller than 0 (highly conserved) and greater than 1 (overdispersed).

across the study area. This pattern largely coincides with the distribution of *C. steppae*, which Kärnefelt (1986) reported from Ukraine and Central Asia, and Crespo & Barreno (1978) from Spain. While the association between the norstictic acid chemotypes and multilocus clusters is generally weak, norstictic acid-containing specimens tended to be assigned to admixture cluster IV, and to a lesser extent cluster VIII. The Welch's *t*-test showed that only the association of norstictic acid chemotypes with cluster IV was statistically supported (Fig. 4). More than 50% of this cluster's samples were from Ukraine. The association with cluster VIII, containing specimens from Spain, Kazakhstan and Ukraine, was not significant. Statistical tests coupled with the D statistic showed a lack of phylogenetic signal in the expression of norstictic acid across the phylogenies studied (Table 3, Fig. 3).

Discussion

Since the 19th century, secondary metabolites have played an important role in delimiting lichen species. However, the question of whether chemical variants should be acknowledged taxonomic ranks, and if so at which levels, has never really abated (Lumbsch & Leavitt 2011). In the absence of genetic data and evidence from crossing experiments, lichenologists had to rely on indirect evidence to infer the taxonomic value of chemical characters that were not clearly associated with morphological traits. In such cases the presence of separate lineages was sometimes inferred based on differences in distributional ranges, as for example in *Cetraria ciliaris* (Hale 1963; Culberson & Culberson 1976; Brodo 1984). Phylogenetic trees based on molecular data facilitate the detection of evolutionary lineages. However, due to incomplete lineage sorting, hybridization or horizontal gene transfer, gene trees do not necessarily resemble the species phylogeny. At the level of sibling species, phylogenetic methods may therefore not be the most powerful analytical tools. And so, in this paper we attempted to assess the association between a chemical character (production of norstictic acid) and genetic groups from the population level to the level of phylogenetic lineages (admixture clusters and gene phylogenies).

One unambiguous result of our analyses is the finding that norstictic acid was detected exclusively in specimens identified as *C. aculeata*/*steppae* and never in *C. muricata*. Within *C. aculeata*/*steppae*, however, no clear phylogenetic pattern in the occurrence of norstictic acid was detected in any of the phylogenies. Instead, statistical tests support the idea of random occurrence of the trait across phylogenetic trees. Several recent studies have reported a similar picture with chemically different individuals appearing non-monophyletic on gene trees (Articus *et al.* 2002; Myllys *et al.* 2005; Nelsen & Gargas 2008, 2009; Lendemer *et al.* 2015). These authors concluded that chemotypes did not represent different evolutionary

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lineages. Reports of unsorted ancestral polymorphism in closely related lichens (Printzen *et al.* 2003; Wirtz *et al.* 2012), however, show that evolutionary lineages are not necessarily identical to monophyletic groups on trees.

Using niche modelling, Lendemer *et al.* (2015) showed that chemotypes of *Parmotrema subrigidum* with norstictic acid restricted to Florida occupied a different ecological niche than the genetically similar acid-deficient strain that is also found further north. Our dataset on *Cetraria aculeata* displays a very similar pattern in that norstictic acid was detected only in the southern part of the study area. In contrast to Lendemer *et al.* (2015), we observed a significant correlation of norstictic acid production with certain genetic groups, namely admixture clusters IV and VIII. However, these two groups are almost totally restricted to the Mediterranean and Central Asian range of *C. aculeata*/*steppae*. More than 50% of the samples from admixture cluster IV are from Ukraine; cluster VIII is restricted to Spain, France, Italy, Kazakhstan, Ukraine and Turkey. Hence, genetic and geographical structure co-vary in our dataset, and it remains unclear with which of these variables norstictic acid is actually correlated. The frequently observed disjunct or only partly overlapping ranges of lichen chemotypes leave room for various interpretations (e. g. Hale 1956; Culberson *et al.* 1977; Sheard 1977; Lumbsch *et al.* 1994; Lendemer *et al.* 2015). Sometimes, but not always, differences in distribution were interpreted as supporting evidence for the presence of different taxa. For example, Lumbsch *et al.* (1994) treated the three morphologically indistinguishable chemotypes of *Lecanora epibryon* as subspecies. Chemical differences were also regarded as sufficient to distinguish species within the *Parmotrema perforatum* group (Lendemer *et al.* 2015), whereas several chemotypes from the *Ramalina siliquosa* group displaying slightly different distributional ranges (Culberson *et al.* 1977) are not acknowledged at taxonomic rank (Fletcher *et al.* 2009).

The production of certain secondary metabolites has also been connected with ecological factors such as light and moisture regime, metal content, pH and the substratum preferences of lichens (Nash & Zavada 1977; Armaleo *et al.* 2008; Hauck *et al.* 2010). Those ecological factors that were studied in *C. aculeata* (altitude above sea level, bioclimatic zone, habitat and substratum type) showed no correlation with the production of norstictic acid (Nadyeina *et al.* 2013). Whether pH or metal content of substrata triggers the expression of norstictic acid is at least doubtful. Most populations studied by Nadyeina *et al.* (2013) were sampled on small patches of a few hundred square metres at most. Yet only four localities in Ukraine and one in Kazakhstan were fixed for norstictic acid. In all other temperate populations chemotypes were either mixed or only the acid-deficient chemotype was found (Fig. 2). Armaleo *et al.* (2008) showed that the norstictic acid content of *Parmotrema hypotropum* is negatively correlated with annual light levels and concluded that higher moisture levels trigger the production of norstictic acid. Our finding that norstictic acid is produced only in southern populations of *C. aculeata* runs counter to this and shows that correlations found in one species cannot be automatically transferred to another. Likewise, Asplund & Gauslaa (2007) found a positive correlation between thallus size and total concentration of secondary metabolites, including norstictic acid, in *Lobaria pulmonaria*. Yet Nadyeina *et al.* (2013) observed the opposite in Ukrainian populations of *C. aculeata*, where norstictic acid was more frequently found in smaller individuals with wider branches. Schroeckh *et al.* (2009) found that the production of orsellinic acid in *Aspergillus nidulans* was triggered by the co-cultivation of certain Actinomycetes. Whether the symbiosis with certain bacteria is a trigger for the production of secondary metabolites in the *C. aculeata* group would be worth studying.

At present, we can only speculate why norstictic acid is produced exclusively in the Mediterranean and Central Asian part of the study area. Our results show that the distinction of *Cetraria steppae* at species level based on the production of norstictic acid, as postulated by Kärnefelt (1986), has no

phylogenetic background. This is in line with the results from Nadyeina *et al.* (2013). We do not, however, fully agree with their conclusion that both names should be synonymized, as Savicz (1924) distinguished *C. steppae* using morphological differences. Our data, and that from previous studies, show that the Mediterranean and Central Asian populations are genetically differentiated from others (Fernández-Mendoza *et al.* 2011; Fernández-Mendoza & Printzen 2013) and might be interpreted as a separate evolutionary lineage. Genetic data with finer resolution is needed to assess whether this just reflects geographical structure within the wider species *C. aculeata* or whether gene flow between temperate populations and those in other regions is absent.

Conclusions

It was found that norstictic acid occurrence in lichens of the *Cetraria aculeata* group is associated with geographical, but not phylogenetic differences. The few positive associations of norstictic acid production with Mediterranean and Central Asian lineages at the population level probably reflect spatial genetic structure. We conclude that chemical differences observed in our dataset are most likely driven by the environment and do not delineate evolutionary lineages.

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Appendix 1. GenBank Accession numbers for all fungal multigene haplotypes of *Cetraria aculeata* s. lat. used in the study. One sample for each unique combination of the three single gene haplotypes (h) is listed.

Sample number	ITS (h)	GenBank Acc. no.	GPD (h)	GenBank Acc. no.	mtLSU (h)	GenBank Acc. no.	Sample number	ITS (h)	GenBank Acc. no.	GPD (h)	GenBank Acc. no.	mtLSU (h)	GenBank Acc. no.
1083	5	EU924121	1	HM573601	3	HM573622	2335	22	KU200363	2	HM573602	2	HM573621
1333	1	EU924114	1	HM573601	3	HM573622	2344	23	KT827712	10	JX840124	2	HM573621
1336	2	EU924127	2	HM573602	2	HM573621	2350	2	EU924127	2	HM573602	17	KU200400
1420	6	GQ375371	3	HM573606	2	HM573621	2356	2	EU924127	2	HM573602	14	KU200403
1421	7	GQ375372	4	HM573608	2	HM573621	2368	24	JX840082	1	HM573601	3	HM573622
1436	8	GU124725	2	HM573602	2	HM573621	2370	1	EU924114	2	HM573602	2	HM573621
1437	7	GQ375372	2	HM573602	2	HM573621	2371	25	JX840084	4	HM573608	13	JQ314494
1439	7	GQ375372	3	HM573606	2	HM573621	2373	14	GU124734	2	HM573602	13	JQ314494
1442	9	GQ375375	3	HM573606	2	HM573621	2377	26	JX840086	1	HM573601	3	HM573622
1445	10	GQ375380	3	HM573606	2	HM573621	2381	1	EU924114	12	JX840126	1	KU200401
1446	11	JX840106	3	HM573606	2	HM573621	2386	1	EU924114	3	HM573606	1	KU200401
1454	12	KU200360	3	HM573606	2	HM573621	2397	27	JX840088	1	HM573601	1	KU200401
1455	13	GQ375379	2	HM573602	2	HM573621	2548	28	KU200364	1	HM573601	3	HM573622
1641	2	EU924127	5	JX840121	2	HM573621	2551	1	EU924114	13	KU200414	3	HM573622
1643	8	GU124725	2	HM573602	15	HM573623	2554	1	EU924114	14	KU200415	1	KU200401
1649	14	GU124734	2	HM573602	2	HM573621	2555	7	GQ375372	15	KU200421	17	KU200400
1653	2	EU924127	2	HM573602	15	HM573623	2557	7	GQ375372	16	KU200416	7	KU200412
1689	10	GQ375380	2	HM573602	2	HM573621	2560	7	GQ375372	17	KU200417	17	KU200400
1690	15	JQ314489	6	HM573609	2	HM573621	2563	7	GQ375372	18	KU200418	2	HM573621
2002	16	KU200361	3	HM573606	16	KU200399	2578	29	KU200365	3	HM573606	8	KU200413
2003	17	KU200362	3	HM573606	16	KU200399	2581	30	KU200371	2	HM573602	19	KU200402
2199	2	EU924127	2	HM573602	13	JQ314494	2584	31	KU200395	3	HM573606	8	KU200413
2201	18	JQ314491	7	JX840133	3	HM573622	2594	32	KT827732	1	HM573601	3	HM573622
2203	19	JQ314490	1	HM573601	3	HM573622	2595	1	EU924114	19	KU200433	2	HM573621
2208	18	JQ314491	1	HM573601	3	HM573622	2598	32	KT827732	19	KU200433	2	HM573621
2210	1	EU924114	3	HM573606	2	HM573621	2600	33	KT827730	2	HM573602	1	KU200401
2212	20	EU924116	1	HM573601	3	HM573622	2818	35	KU200366	2	HM573602	1	KU200401
2218	21	JQ314492	1	HM573601	3	HM573622	2823	36	KT827723	2	HM573602	2	HM573621
2221	18	JQ314491	8	JX840134	3	HM573622	2824	37	KT827724	2	HM573602	2	HM573621
2222	18	JQ314491	9	JX840135	3	HM573622	2825	38	KU200367	2	HM573602	2	HM573621
2328	6	GQ375371	2	HM573602	2	HM573621	2827	39	KT827725	2	HM573602	2	HM573621
2330	2	EU924127	3	HM573606	2	HM573621	2830	40	KU200368	2	HM573602	1	KU200401
2331	1	EU924114	10	JX840124	2	HM573621	2831	40	KU200368	3	HM573606	1	KU200401
2332	2	EU924127	11	JX840123	2	HM573621	2832	35	KU200366	3	HM573606	1	KU200401
2333	2	EU924127	3	HM573606	14	KU200403	2834	2	EU924127	3	HM573606	1	KU200401
2835	41	KU200369	3	HM573606	1	KU200401	3090	52	KU200378	3	HM573606	2	HM573621
2837	33	KT827730	20	KU200419	1	KU200401	3092	53	KU200379	3	HM573606	2	HM573621
2838	2	EU924127	2	HM573602	1	KU200401	3098	54	KU200380	3	HM573606	2	HM573621
2839	41	KU200369	2	HM573602	1	KU200401	3576	55	KU200381	3	HM573606	2	HM573621
2841	42	KT827726	2	HM573602	2	HM573621	3602	14	GU124734	29	KU200427	2	HM573621
2844	14	GU124734	3	HM573606	1	KU200401	3603	2	EU924127	30	KU200428	2	HM573621
2849	14	GU124734	2	HM573602	1	KU200401	3604	56	KU200382	1	HM573601	3	HM573622

Appendix 1 (*continued*).

Sample number	ITS (h)	GenBank Acc. no.	GPD (h)	GenBank Acc. no.	mtLSU (h)	GenBank Acc. no.	Sample number	ITS (h)	GenBank Acc. no.	GPD (h)	GenBank Acc. no.	mtLSU (h)	GenBank Acc. no.
2851	33	KT827730	3	HM573606	1	KU200401	3606	5	EU924121	29	KU200427	2	HM573621
2852	14	GU124734	21	KU200431	1	KU200401	3615	1	EU924114	5	JX840121	2	HM573621
2865	1	EU924114	21	KU200431	1	KU200401	3622	2	EU924127	31	KU200429	2	HM573621
2886	2	EU924127	22	KU200420	2	HM573621	3624	57	KU200383	3	HM573606	2	HM573621
2887	7	GQ375372	21	KU200431	2	HM573621	3625	34	KU200384	2	HM573602	2	HM573621
2889	43	KU200370	2	HM573602	2	HM573621	3628	58	KU200387	2	HM573602	2	HM573621
2890	43	KU200370	21	KU200431	2	HM573621	3634	2	EU924127	10	JX840124	2	HM573621
2894	32	KT827732	2	HM573602	2	HM573621	3635	58	KU200387	10	JX840124	2	HM573621
2902	30	KU200371	15	KU200421	2	HM573621	3639	58	KU200387	6	HM573609	2	HM573621
2903	1	EU924114	23	KU200422	14	KU200403	3644	2	EU924127	32	KU200430	2	HM573621
2953	33	KT827730	24	KU200424	1	KU200401	3648	59	KU200385	1	HM573601	3	HM573622
2963	44	KU200372	2	HM573602	2	HM573621	3649	60	KU200386	21	KU200431	2	HM573621
2965	45	KU200390	2	HM573602	2	HM573621	3653	58	KU200387	32	KU200430	2	HM573621
2967	45	KU200390	20	KU200419	2	HM573621	3654	61	-	3	HM573606	2	HM573621
2969	43	KU200370	2	HM573602	1	KU200401	3656	17	KU200362	3	HM573606	2	HM573621
2970	43	KU200370	24	KU200424	2	HM573621	3687	50	KU200391	28	KU200435	4	KU200408
2971	43	KU200370	24	KU200424	1	KU200401	3688	50	KU200391	28	KU200435	2	HM573621
2973	2	EU924127	25	KU200423	1	KU200401	3694	50	KU200391	28	KU200435	11	KU200409
2975	33	KT827730	2	HM573602	18	KU200404	3699	50	KU200391	28	KU200435	9	KU200410
2981	14	GU124734	24	KU200424	2	HM573621	3702	50	KU200391	28	KU200435	10	KU200411
2982	46	KU200373	24	KU200424	2	HM573621	3712	62	KU200388	1	HM573601	3	HM573622
3064	47	KU200374	26	KU200425	12	KU200405	3713	63	-	3	HM573606	2	HM573621
3066	48	KU200375	26	KU200425	12	KU200405	3090	52	KU200378	3	HM573606	2	HM573621
3069	47	KU200374	27	KU200426	12	KU200405	3092	53	KU200379	3	HM573606	2	HM573621
3070	49	KU200376	28	KU200435	5	KU200406	3098	54	KU200380	3	HM573606	2	HM573621
3072	50	KU200391	28	KU200435	7	KU200412	3576	55	KU200381	3	HM573606	2	HM573621
3073	51	KU200377	28	KU200435	6	KU200407	3602	14	GU124734	29	KU200427	2	HM573621
3074	49	KU200376	28	KU200435	7	KU200412	3603	2	EU924127	30	KU200428	2	HM573621
3075	50	KU200391	28	KU200435	5	KU200406	3604	56	KU200382	1	HM573601	3	HM573622
3076	50	KU200391	28	KU200435	6	KU200407	3606	5	EU924121	29	KU200427	2	HM573621
3714	62	KU200388	3	HM573606	3	HM573622	4109	45	KU200390	3	HM573606	14	KU200403
3715	55	KU200381	1	HM573601	2	HM573621	4114	65	KU200392	2	HM573602	14	KU200403
3727	41	KU200369	3	HM573606	2	HM573621	4118	66	KU200393	3	HM573606	2	HM573621
3729	41	KU200369	33	KU200432	2	HM573621	4134	67	KU200394	2	HM573602	2	HM573621
3742	55	KU200381	19	KU200433	14	KU200403	4139	67	KU200394	2	HM573602	14	KU200403
3747	64	KU200389	3	HM573606	14	KU200403	4141	31	KU200395	2	HM573602	8	KU200413
3748	17	KU200362	19	KU200433	1	KU200401	4149	68	KU200396	2	HM573602	2	HM573621
3751	45	KU200390	3	HM573606	2	HM573621	4167	45	KU200390	19	KU200433	2	HM573621
4007	1	EU924114	34	KU200434	3	HM573622	4257	2	EU924127	21	KU200431	1	KU200401
4105	64	KU200389	2	HM573602	2	HM573621	4264	2	EU924127	21	KU200431	2	HM573621
4106	2	EU924127	19	KU200433	14	KU200403	4273	4	KU200397	21	KU200431	2	HM573621
4107	64	KU200389	3	HM573606	2	HM573621	4274	3	KU200398	3	HM573606	2	HM573621