

# Intraspecific variation of some brown *Parmeliae* (in Poland) – a comparison of ITS rDNA and non-molecular characters

Katarzyna Szczepańska<sup>1</sup>, Beata Guzow-Krzemińska<sup>2</sup>, Jacek Urbaniak<sup>1</sup>

**1** Department of Botany and Plant Ecology, Wrocław University of Environmental and Life Sciences, pl. Grunwaldzki 24a, PL-50-363 Wrocław, Poland **2** Department of Plant Taxonomy and Nature Conservation, Faculty of Biology, University of Gdańsk, Wita Stwosza 59, PL-80-308 Gdańsk, Poland

Corresponding author: Katarzyna Szczepańska ([katarzyna.szczepanska@upwr.edu.pl](mailto:katarzyna.szczepanska@upwr.edu.pl))

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## Abstract

Intraspecific variation of the ITS rDNA region of some brown *Parmeliae* occurring in Poland is studied and compared with non-molecular characters. Haplotype networks are used to illustrate the variability within the species. Both newly-produced sequences from Central Europe and from all over the world, downloaded from the GenBank, are used.

The number of haplotypes found for each taxon ranged from five in *Melanelia stygia* to 12 in *Melanelia hepatizon* and *Montanelia disjuncta*; however, their numbers correlate with the number of specimens tested. New haplotypes for *Melanelia agnata*, *M. hepatizon* and *Cetraria commixta* are found. Based on our 169-sample dataset, we could not infer any geographical correlation, either locally or world-wide. Many of the analysed haplotypes were widely distributed and the same haplotype was often shared between temperate and polar populations. A comparison of molecular, morphological, anatomical and chemical characters also shows no correlation.

## Keywords

Cryptic species, haplotype, lichenised fungi, Parmeliaceae, phylogeny, taxonomy

## Introduction

The brown *Parmeliae* (Esslinger 1977) have been an object of numerous studies (Guzow-Krzemińska and Węgrzyn 2003; Blanco et al. 2005; Crespo et al. 2010, 2011; Nelsen et al. 2011; Divakar et al. 2012; Thell et al. 2012; Leavitt et al. 2014, 2015) and, due to this exceptional attention, they are one of the best-studied assemblages in the family Parmeliaceae. These lichens are a polyphyletic group possessing foliose, a dark to medium brown thallus and usually lacking atranorin or usnic acid in the cortex (Esslinger 1977; Blanco et al. 2004).

For many years, one of the largest genera within this group was *Melanelia* Essl., segregated from *Parmelia* Ach. by Esslinger (1978) to accommodate species with brown, foliose thalli and an N– cortex layer. However, during the following years, it has been demonstrated that the genus *Melanelia* s. lat. was polyphyletic and several new genera were distinguished within it, such as *Melanelixia* O. Blanco et al., *Melanohalea* O. Blanco et al. (Blanco et al. 2004) and *Montanelia* Divakar et al. (Divakar et al. 2012). In traditional terms, brown *Parmeliae* includes other genera, such as *Allantoparmelia* (Vain.) Essl., *Pleurosticta* Petr. and some species of *Xanthoparmelia* (Vain.) Hale. Moreover, due to the historical taxonomic approach (Thell 1995; Rico et al. 2005) and the similarity in the morphological and anatomical features of thalli, *Cetraria commixta* is also referred to this group.

Our studies have focused on the saxicolous species of *Melanelia* and *Montanelia* genera. According to Otte et al. (2005), species of these genera are arctic-alpine, circumpolar and occur on silicate rocks in the mountain areas of the Northern Hemisphere, including Arctic Regions (Divakar et al. 2012). Nowadays, *Melanelia* s. str. is restricted to a small clade of saxicolous, cetrarioid lichens and includes four species: *M. agnata* (Nyl.) A. Thell, *M. hepatizon* (Ach.) A. Thell, *M. pseudoglabra* (Essl.) Essl. and *M. stygia* (L.) Essl. According to Thell (1995), these species are characterised by broadly clavate asci with a small tholus and a broad axial body, a thick, paraplectenchymatous cortex and dumb-bell-shaped pycnoconidia. *Montanelia*, representing the parmelioid clade, includes eight species: *M. disjuncta* (Erichsen) Divakar, A. Crespo, Wedin & Essl., *M. occultipanniformis* S.D. Leav., Essl., Divakar, A. Crespo & Lumbsch, *M. panniformis* (Nyl.) Divakar, A. Crespo, Wedin & Essl., *M. predisjuncta* (Essl.) Divakar, A. Crespo, Wedin & Essl., *M. saximontana* (R.A. Anderson & W.A. Weber) S.D. Leav., Essl., Divakar, A. Crespo & Lumbsch, *M. secwepemc* S.D. Leav., Essl., Divakar, A. Crespo & Lumbsch, *M. sorediata* (Ach.) Divakar, A. Crespo, Wedin & Essl. and *M. tominii* (Oxner) Divakar, A. Crespo, Wedin & Essl. (Divakar et al. 2012; Leavitt et al. 2015; Leavitt et al. 2016). The characteristic features of the *Montanelia* genus are short and narrow lobes, with flat to convex lobe margins, a non-pored epicortex, cylindrical to fusiform conidia, a medulla containing orcinol depsides and flat, effigurate pseudocyphellae (absent only in *M. sorediata*; Divakar et al. 2012). Three of these species (*M. disjuncta*, *M. panniformis* and *M. sorediata*) have broad, intercontinental distributions, with no evidence of phylogeographic substructure (Leavitt et al. 2015).

The genera *Melanelia* and *Montanelia* have been the subject of a critical revision in Poland and data concerning their distribution, ecology and morphological, anatomical and chemical features are presented in previous papers (Szczepańska et al. 2015;

Szczepańska and Kossowska 2017). However, recent molecular studies imply that both genera may include previously unrecognised species-level diversity (Divakar et al. 2012; Leavitt et al. 2014), especially within Icelandic populations of *M. stygia* (Xu et al. 2017).

One of the goals of this study was to assess the intraspecific internal transcribed spacer (ITS) rDNA variability in brown *Parmeliae* species. Investigations of genetic variation in lichen-forming symbionts have advanced considerably in recent years and resulted in interesting conclusions (Palice and Printzen 2004; Lindblom and Ekman 2006; Domaschke et al. 2012; Starosta and Svoboda 2020). Although brown *Parmeliae* appear to be well studied in taxonomic terms, there are insufficient molecular data to estimate their genetic variation. Most of the available data concern samples collected in a few regions of the world, such as Europe and North America. The North American species of this group were studied in Greenland and Canada (Leavitt et al. 2014; Leavitt et al. 2015), while samples from Europe originated mainly from the north – Iceland, Finland, Norway and Sweden (Blanco et al. 2004; Divakar et al. 2012; Xu et al. 2017). Therefore, we decided to fill in the gap in sampling and focused our study on samples collected in Central Europe. We have used phylogenetic trees and haplotype networks to investigate the extent of molecular differences between newly-generated sequences from samples collected in Central Europe (Austria, Czech Republic, Germany, Poland and Slovakia) and others originating from different geographical regions. Due to additional samplings from previously unexplored areas, it was possible to evaluate and compare the genetic variability of the studied specimens in Central Europe with samples from other regions of the world and to identify areas with the greatest haplotype diversity. In addition, we analysed morphological, anatomical and chemical characters of collected specimens to find a potential correlation between phenotypic characters and genetic variation of the studied taxa. By analysing genetic diversity and geographical distribution of identified haplotypes, as well as phenotypic characters of collected samples, we tried to better define and designate the species boundaries within analysed taxa. Special emphasis was placed on analysis of European, Greenlandic and Icelandic samples of *M. agnata* and *M. stygia* to revise the hypothesis assuming a semi-cryptic or cryptic nature of their potential species-level diversity.

## Materials and methods

### Taxon sampling

The study is based on collections from the AMNH, C and WRSL Herbaria, as well as the private material of Dr Maria Kossowska (hb. Kossowska). Our sampling focused on saxicolous representatives of the Parmeliaceae family occurring in Poland, with brown, foliose thalli, such as *Cetraria commixta*, *Melanelia agnata*, *M. hepatizon*, *M. stygia*, *Montanelia disjuncta* and *M. sorediata*. We also included the holotype of *Melanelia agnata* (*Platysma agnatum*; Austria, Tirol, Gerölle unter dem Gneissfelsen zum wilden see. Auf dem Kraxentrag, Tirol, Brenner 225, Aug 1871, H-NYL 36086), borrowed from Herbarium of W. Nylander in Helsinki in our analyses.

Specimens for molecular study were selected after detailed morphological and chemical analyses. Due to DNA degradation, it was not possible to use samples collected more than three years prior to the DNA extraction procedure in most cases. As the *Melanelia agnata* and *M. stygia* specimens from Greenland and Iceland were collected more than 10 years ago, we had to limit our phylogenetic analyses to ITS rDNA markers and used the sequences stored in GenBank. Before phylogenetic analysis, newly-obtained ITS rDNA sequences were subjected to a BLAST search (Altschul et al. 1997). The final ITS dataset used in this study includes 52 sequences newly generated and 117 sequences downloaded from GenBank (Table 1).

## Morphology and chemistry

The morphology and anatomy of the specimens were studied in detail with dissecting and light microscopes, following routine techniques. All specimens were examined for the assessment of the morphological characters, such as lobe width and morphology (flat/convex), the appearance of the upper surface (dull/glossy), the appearance of the lower surface (light/dark), apothecia morphology (sessile/constricted), appearance and position of pycnidia (marginal/laminal), appearance and position of the pseudocyphellae (marginal/laminal), size and shape of conidia (bacilliform/bifusiform), as well as ascospore size. For light microscopy, vertical sections of apothecia were cut by hand using a razor blade and mounted in water. Hymenium and conidia measurements were made in water and ascospore measurements were made in 10% potassium hydroxide (KOH). At least ten measurements of morphological variables and measurements of 20 spores and conidia were made for each sample and their minimum and maximum values were calculated.

The TLC analyses were undertaken in A and C solvent systems using the standardised method of Culberson (1972) and following Orange et al. (2001).

## DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted from specimens after cell disruption in a Mixer Mill MM400 (Retsch, Haan, Germany) using a CTAB method according to the standard protocol of isolation (Doyle and Doyle 1987). The quality of the isolated DNA was determined using 1% TBE agarose electrophoresis. PCR reactions were performed in 20 µl reaction tubes that contained a Dream Taq reaction buffer containing MgCl<sub>2</sub>, a 0.2 mM dNTP mix, 1u DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 mM each ITS1 and ITS4 primers and 0.8 µl of total genomic DNA. The adequate annealing temperature was determined using the gradient method. The PCR programme consisted of an initial denaturation at 95 °C for 6 min, according to a previous study (Szczepańska et al. 2020), followed by 30 cycles at 95 °C for 30 sec, 51.2 °C for 45 sec, 72 °C for 45 sec, with a final extension at 72 °C for 10 min. While performing PCR, the Veriti Thermal Cycler (Life Technologies, Carlsbad, CA, USA) was used. Amplification products were separated in 1% agarose gel, photographed and compared with the DNA mass ruler (Thermo Fisher Scientific Waltham, MA, USA).

**Table 1.** The species and specimens used in the phylogenetic analyses and/or haplotype network analyses, sequences newly generated for this study are in bold.

Species	Year of collection	Isolate	Locality	Collector (-s)	Voucher specimens (herbarium)	GenBank no. (ITS)
<i>Cetrariella commixta</i>	2007	36	Poland, Sudety Mts	Kossowska, M.	Kossowska 107 (personal herbarium)	MZ029708
<i>Cetrariella commixta</i>	2008	37	Poland, Sudety Mts	Kossowska, M.	Kossowska 231 (personal herbarium)	MZ029709
<i>Cetrariella commixta</i>	2016	97	Poland, Sudety Mts	Szczepeńska, K.	Szczepeńska 1137 (WRSL)	MZ029733
<i>Cetrariella commixta</i>	2016	124	Poland, Sudety Mts	Szczepeńska, K.	Szczepeńska 1184 (WRSL)	MZ029753
<i>Cetrariella commixta</i>	2018	129	Germany, Bayerischer Wald	Szczepeńska, K.	Szczepeńska 1267 (WRSL)	MZ029758
<i>Cetrariella commixta</i>			Finland	Haikonen, V.	Haikonen 19093 (H)	AF451796
<i>Cetrariella commixta</i>	1996		Canada, British Columbia	Miao, V. & Taylor, T.		AF451797
<i>Cetrariella commixta</i>			Sweden	Wedin, M.	Wedin 8143 (UPS)	GU994554
<i>Cetrariella commixta</i>			Spain, Segovia	Rico, V. J.	15555 (MAF)	GU994555
<i>Cetrariella commixta</i>	2004	CCO 01	Sweden, Lule Lappmark		1273926 (LD)	KC990132
<i>Cetrariella commixta</i>		6543	Greenland, SEm, Tasilaq	Hansen, E. S.	Hansen ESH-10B.139 (C)	KF257934
<i>Cetrariella commixta</i>		6547	Greenland, SWm, Qeqertaq	Hansen, E. S.	Hansen ESH-09.087 (C)	KF257935
<i>Cetrariella commixta</i>		6567	Greenland, S, Igaliku	Hansen, E. S.	Hansen ESH-08.173 (C)	KF257936
<i>Cetrariella commixta</i>		6570	Greenland, SWm, Midgard	Hansen, E. S.	Hansen ES-09.030 (C)	KF257937
<i>Cetrariella commixta</i>		6572	Greenland, S, Aappilattoq	Hansen, E. S.	Hansen ES-04.070 (C)	KF257938
<i>Cetrariella commixta</i>		6573	Greenland, SWm, Qeqertaq	Hansen, E. S.	Hansen ES-09.064 (C)	KF257939
<i>Cetrariella commixta</i>	2014		Norway, Finnmark	Westberg, M.	O-L-195926	KY266843
<b><i>Melanelia agnata</i></b>	2016	102	Poland, Karpaty Mts	Szczepeńska, K.	Szczepeńska 1151 (WRSL)	MZ029737
<b><i>Melanelia agnata</i></b>	2016	103	Poland, Karpaty Mts	Szczepeńska, K.	Szczepeńska 1150 (WRSL)	MZ029738
<i>Melanelia agnata</i>	2009	6549	Greenland, SW m, Jensens Nunatakker	Hansen, E. S.	Hansen ESH-09.478 (C)	KF257940
<i>Melanelia agnata</i>	2009	6553	Greenland, SW m, Jensens Nunatakker	Hansen, E. S.	Hansen ESH-09.435 (C)	KF257941
<i>Melanelia agnata</i>	2007	6563	Greenland, N, Constable Bugt	Hansen, E. S.	Hansen ESH-07.464 (C)	KF257942
<i>Melanelia agnata</i>	2002	MX_MS2	Iceland, Imi	Heiðmarsson, S.	LA29683 (AMHN)	KY508672
<i>Melanelia agnata</i>	2005	MX_MS3	Iceland, Ino	Kristinsson, H.	LA27562 (AMHN)	KY963373
<i>Melanelia agnata</i>	2008	MX_MS4	Iceland, Isu	Hjaltdóttir, A.	LA30974 (AMHN)	KY508673
<i>Melanelia agnata</i>	2012	MX_MS5	Iceland, Ino	Heiðmarsson, S.	LA31859 (AMHN)	KY963374
<i>Melanelia agnata</i>	2014		Norway, Sor-Trondelag	Timdal, E.	O-L-196376	MK812394
<i>Melanelia culbersonii</i>			USA	Lendemer, J.	Lendemer 13821 (NY)	KR995286
<b><i>Melanelia hepatizon</i></b>	2016	83	Poland, Sudety Mts	Szczepeńska, K.	Szczepeńska 1051 (WRSL)	MZ029723
<b><i>Melanelia hepatizon</i></b>	2016	91	Poland, Sudety Mts	Szczepeńska, K.	Szczepeńska 1120 (WRSL)	MZ029717
<b><i>Melanelia hepatizon</i></b>	2016	95	Poland, Sudety Mts	Szczepeńska, K.	Szczepeńska 1136A (WRSL)	MZ029731
<b><i>Melanelia hepatizon</i></b>	2016	96	Poland, Sudety Mts	Szczepeńska, K.	Szczepeńska 1136B (WRSL)	MZ029732

Species	Year of collection	Isolate	Locality	Collector (-s)	Voucher specimens (herbarium)	GenBank no. (ITS)
<i>Melanelia hepaticozona</i>	2016	98	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1138 (WRSL)	MZ029734
<i>Melanelia hepaticozona</i>	2016	109	Poland, Karpaty Mts	Szczepańska, K.	Szczepańska 1153 (WRSL)	MZ029741
<i>Melanelia hepaticozona</i>	2016	110	Poland, Karpaty Mts	Szczepańska, K.	Szczepańska 1154A (WRSL)	MZ029730
<i>Melanelia hepaticozona</i>	2016	111	Poland, Karpaty Mts	Szczepańska, K.	Szczepańska 1154B (WRSL)	MZ029743
<i>Melanelia hepaticozona</i>	2016	113	Poland, Karpaty Mts	Szczepańska, K.	Szczepańska 1144 (WRSL)	MZ029745
<i>Melanelia hepaticozona</i>	2016	116	Slovakia, Karpaty Mts	Szczepańska, K.	Szczepańska 1146 (WRSL)	MZ029746
<i>Melanelia hepaticozona</i>	2016	117	Slovakia, Karpaty Mts	Szczepańska, K.	Szczepańska 1147 (WRSL)	MZ029747
<i>Melanelia hepaticozona</i>	2016	119	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1180 (WRSL)	MZ029748
<i>Melanelia hepaticozona</i>	2016	122	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1182 (WRSL)	MZ029751
<i>Melanelia hepaticozona</i>	2018	128	Germany, Bayerischer Wald	Szczepańska, K.	Szczepańska 1269 (WRSL)	MZ029757
<i>Melanelia hepaticozona</i>	1996		Canada, British Columbia		Thell & Veer BC-9677 (LD)	AF141369
<i>Melanelia hepaticozona</i>	2001	DNA-AT934	Italy, Trentino-Alto Adige (south Tirolia)	Feuerer T. & Thell A. s. n.	LD, HBG	AF451776
<i>Melanelia hepaticozona</i>			Sweden	Wedin, M.	Wedin 6812 (UPS)	DQ980016
<i>Melanelia hepaticozona</i>			Greenland, NWn, Siorapuluk	Hansen, E. S.	Hansen ESH-09B.164 (C)	KF257943
<i>Melanelia hepaticozona</i>			Greenland, NWn, Qaanaaq	Hansen, E. S.	Hansen ESH-09B.026 (C)	KF257944
<i>Melanelia hepaticozona</i>			Greenland, SEm, Tasilaq	Hansen, E. S.	Hansen ESH-10B.014 (C)	KF257945
<i>Melanelia hepaticozona</i>			Greenland, SWm, Nuuk	Hansen, E. S.	Hansen ESH-10A.019 (C)	KF257946
<i>Melanelia hepaticozona</i>			Greenland, S, Qaqortoq	Hansen, E. S.	Hansen ESH-08.036 (C)	KF257947
<i>Melanelia hepaticozona</i>			Greenland, S, Igaliku	Hansen, E. S.	Hansen ESH-08.170 (C)	KF257948
<i>Melanelia hepaticozona</i>			Greenland, S, Narssarsuag	Hansen, E. S.	Hansen ESH-08.263 (C)	KF257949
<i>Melanelia hepaticozona</i>			Greenland, S, Igaliku	Hansen, E. S.	Hansen ESH-08.215 (C)	KF257950
<i>Melanelia hepaticozona</i>			Greenland, SWm, Midgard	Hansen, E. S.	Hansen ESH-09.386 (C)	KF257951
<i>Melanelia hepaticozona</i>			Greenland, SWm, Frederikshab Isblink	Hansen, E. S.	Hansen ESH-09.324 (C)	KF257952
<i>Melanelia hepaticozona</i>			Greenland, S, Igaliku	Hansen, E. S.	Hansen ESH-08.477 (C)	KF257953
<i>Melanelia hepaticozona</i>	2014		Norway, Finnmark	Westberg, M.	O-L-195864	KY266879
<i>Melanelia hepaticozona</i>	2003	MH1	Iceland, IAu		LA30501 (AMHN)	KY508674
<i>Melanelia hepaticozona</i>	2007	MH3	Iceland, IVe		LA30676 (AMHN)	KY508675
<i>Melanelia hepaticozona</i>	2007	MH4	Iceland, IVe		LA30674 (AMHN)	KY508676
<i>Melanelia hepaticozona</i>	2007	MH5	Iceland, IVe		LA30675 (AMHN)	KY508677
<i>Melanelia hepaticozona</i>	2007	MH6	Iceland, IVe		LA30673 (AMHN)	KY508678
<i>Melanelia hepaticozona</i>	2014	MH9	Iceland, INo		LA20781 (AMHN)	KY508679
<i>Melanelia hepaticozona</i>	2013	MH10	Iceland, INv		LA30117 (AMHN)	KY508680
<i>Melanelia hepaticozona</i>	2012	MH11	Iceland, Inv		LA31861 (AMHN)	KY963376



Species	Year of collection	Isolate	Locality	Collector (-s)	Voucher specimens (herbarium)	GenBank no. (ITS)
<i>Melanelia hepaticon</i>	2014		Norway, Hordaland	Timdal, E.	O-L-195807	MK812512
<i>Melanelia hepaticon</i>	2015		Norway, Nord-Trøndelag	Bendiksby, M. et al.	O-L-201254	MK812070
<i>Melanelia hepaticon</i>	2013		Norway, Buskerud	Rui, S. & Timdal, E.	O-L-184723	MK812188
<i>Melanelia stygia</i>	2007	40	Poland, Sudety Mts	Kossowska, M.	Kossowska 123 (personal herbarium)	MZ029710
<i>Melanelia stygia</i>	2009	42	Austria, Tyrol	Szczepańska, K.	Szczepańska 737 (WRSL)	MZ029712
<i>Melanelia stygia</i>	2016	94	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1134 (WRSL)	MZ029719
<i>Melanelia stygia</i>	2016	104	Poland, Karpaty Mts	Szczepańska, K.	Szczepańska 1152 (WRSL)	MZ029739
<i>Melanelia stygia</i>	2016	108	Poland, Karpaty Mts	Szczepańska, K.	Szczepańska 1149 (WRSL)	MZ029740
<i>Melanelia stygia</i>	2016	112	Poland, Karpaty Mts	Szczepańska, K.	Szczepańska 1160 (WRSL)	MZ029744
<i>Melanelia stygia</i>	2018	127	Czech Republic, Šumava	Szczepańska, K.	Szczepańska 1265 (WRSL)	MZ029756
<i>Melanelia stygia</i>			Finland, Nyland	Kuusinen, M.	FIN-9714 (LD)	AF115763
<i>Melanelia stygia</i>			Italy	Feurerer, T & Thell, A.	DNA-AT922 (LD)	AF451775
<i>Melanelia stygia</i>			Finland, Enonkoski	Haikonen, V.	Haikonen 20365	AY611097
<i>Melanelia stygia</i>			Austria, Steiermark	Hafellner, J.	Hafellner 51658	AY611121
<i>Melanelia stygia</i>	2008	6551	Greenland, S, Qaqortoq	Hansen, E. S.	Hansen ESH-08.036 (C)	KF257954
<i>Melanelia stygia</i>	2008	6569	Greenland, S, Igaliku	Hansen, E. S.	Hansen ESH-08.478 (C)	KF257955
<i>Melanelia stygia</i>	1998	MX_MS1	Iceland, IAU	Kristinsson, H.	LA19972 (AMHN)	KY508681
<i>Melanelia stygia</i>	2014	MX_MS3	Iceland, IAU	Kristinsson, H.	LA20775 (AMHN)	KY508682
<i>Melanelia stygia</i>	2013	MX_MS4	Iceland, IAU	Kristinsson, H.	LA16894 (AMHN)	KY508683
<i>Melanelia stygia</i>	2000	MX_MS2	Iceland, IAU	Kristinsson, H.	LA28243 (AMHN)	KY963375
<i>Melanelia stygia</i>	2013		Norway, Buskerud	Rui, S. & Timdal, E.	O-L-184736	MK812608
<i>Melanelia stygia</i>	2014		Norway, Sor-Trøndelag	Timdal, E.	O-L-196377	MK812312
<i>Montanelia disjuncta</i>	2013	50	Poland, Sudety Forelands	Szczepańska, K.	Szczepańska 969 (WRSL)	MZ029713
<i>Montanelia disjuncta</i>	2014	51	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 989 (WRSL)	MZ029714
<i>Montanelia disjuncta</i>	2015	57	Poland, Sudety Foothills	Szczepańska, K.	Szczepańska 1023 (WRSL)	MZ029715
<i>Montanelia disjuncta</i>	2015	78	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1034 (WRSL)	MZ029716
<i>Montanelia disjuncta</i>	2015	79	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1038 (WRSL)	MZ029711
<i>Montanelia disjuncta</i>	2015	80	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1039 (WRSL)	MZ029720
<i>Montanelia disjuncta</i>	2016	81	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1047 (WRSL)	MZ029721
<i>Montanelia disjuncta</i>	2016	82	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1048 (WRSL)	MZ029722
<i>Montanelia disjuncta</i>	2016	85	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1054 (WRSL)	MZ029724
<i>Montanelia disjuncta</i>	2016	86	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1081 (WRSL)	MZ029725
<i>Montanelia disjuncta</i>	2016	87	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1082 (WRSL)	MZ029726

Species	Year of collection	Isolate	Locality	Collector (-s)	Voucher specimens (herbarium)	GenBank no. (ITS)
<i>Montanelia disjuncta</i>	2016	88	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1110 (WRSL)	MZ029727
<i>Montanelia disjuncta</i>	2016	89	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1111 (WRSL)	MZ029728
<i>Montanelia disjuncta</i>	2016	90	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1119 (WRSL)	MZ029729
<i>Montanelia disjuncta</i>	2016	92	Poland, Sudety Foothills	Szczepańska, K.	Szczepańska 1127 (WRSL)	MZ029755
<i>Montanelia disjuncta</i>	2016	93	Poland, Sudety Foothills	Szczepańska, K.	Szczepańska 1128 (WRSL)	MZ029718
<i>Montanelia disjuncta</i>	2016	120	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1181A (WRSL)	MZ029749
<i>Montanelia disjuncta</i>	2016	121	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1181B (WRSL)	MZ029750
<i>Montanelia disjuncta</i>	2016	123	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1183 (WRSL)	MZ029752
<i>Montanelia disjuncta</i>	2016	125	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1185 (WRSL)	MZ029754
<i>Montanelia disjuncta</i>	2016	126	Poland, Sudety Mts	Szczepańska, K.	Szczepańska 1230 (WRSL)	MZ029742
<i>Montanelia disjuncta</i>	2018	130	Czech Republic, Šumava	Szczepańska, K.	Szczepańska 1271 (WRSL)	MZ029759
<i>Montanelia disjuncta</i>			Austria, Steiermark		Mayrhofer 13743	AY611077
<i>Montanelia disjuncta</i>			India		MAF-Lich 15512	GU994556
<i>Montanelia disjuncta</i>			United Kingdom		Coppins 637	JX974654
<i>Montanelia disjuncta</i>			Greenland, NWN, Siorapaluk	Hansen, E. S.	Hansen ESH-09B.363 (C)	KF257957
<i>Montanelia disjuncta</i>		3921	Canada, Yukon Territory	Spribile, T.	Spribile s.n.	KP771824
<i>Montanelia disjuncta</i>		3963	Greenland, Northwest	Hansen, E. S.	Hansen ESH-09B.051 (C)	KP771827
<i>Montanelia disjuncta</i>		3995	USA, Maine	Harris, R.	Harris 52938 (NY)	KP771828
<i>Montanelia disjuncta</i>		4503	Norway, Tromsø	Bjerke, J.W.	Bjerke WP286-2 (TLE)	KP771829
<i>Montanelia disjuncta</i>		4851	Canada, Yukon Territory	Esslinger, T. L.	Esslinger BP94-3 (TLE)	KP771830
<i>Montanelia disjuncta</i>		5970	USA, Alaska	Esslinger, T. L.	Esslinger 19403 (TLE)	KP771831
<i>Montanelia disjuncta</i>		6575	Greenland, Northwest, Siorapaluk	Hansen, E. S.	Hansen ESH-09B.323 (C)	KP771833
<i>Montanelia disjuncta</i>		MDISJUNCT	Sweden, Lycksele Lappmark	Wedin, M.	Wedin 7143 (UPS)	KP771834
<i>Montanelia disjuncta</i>		MEDI637	United Kingdom, Scotland	Coppins, B.	Coppins s.n (MAF)	KP771835
<i>Montanelia disjuncta</i>		MESO773	India, Uttaranchal	Divakar, P. K.	MAF-Lich 15512	KP771837
<i>Montanelia disjuncta</i>	2014		Norway, Finnmark, Vadso	Haugan, R.	O-L-198675	KY266910
<i>Montanelia disjuncta</i>	2007	MD8	Iceland, INo		LA30657 (AMHN)	KY508686
<i>Montanelia disjuncta</i>			Sweden	Wedin, M.	Wedin 7143 (UPS)	DQ980015
<i>Montanelia disjuncta</i>			USA	Lumbsch, H. T.	Lumbsch 2010/M7 (F)	JX126181
<i>Montanelia disjuncta</i>			USA, Maine		Harris 55589 (NY)	KF257960
<i>Montanelia disjuncta</i>			USA, Alaska		Esslinger 19403 (TLE)	KF257968
<i>Montanelia disjuncta</i>			Canada		Goward 08	JX974658
<i>Montanelia disjuncta</i>			Canada, Yukon		Spribile s.n. (GZU)	KF257956
<i>Montanelia disjuncta</i>			Canada, Alberta		Holzinger 1061 (UBC)	KF257962
<i>Montanelia disjuncta</i>			Canada, British Columbia		Esslinger BP109-1 (TLE)	KF257964
<i>Montanelia disjuncta</i>			Canada, British Columbia		Esslinger BP97-01 (TLE)	KF257965



Species	Year of collection	Isolate	Locality	Collector (-s)	Voucher specimens (herbarium)	GenBank no. (ITS)
<i>Montanelia disjuncta</i>			Canada, Yukon		Esslinger BP94-2 (TLE)	KF257966
<i>Montanelia disjuncta</i>			Canada, Yukon		Esslinger BP94-3 (TLE)	KF257967
<i>Montanelia disjuncta</i>			Canada, New Brunswick		McMullin 7483 (TLE)	KF257969
<i>Montanelia disjuncta</i>			Canada, British Columbia		Goward 2008 (MAF)	KP771836
<i>Montanelia disjuncta</i>			Greenland, S, Igaliku	Hansen, E. S.	Hansen ESH-08.304 (C)	KF257958
<i>Montanelia disjuncta</i>			Greenland, NWn, Qaanaaq	Hansen, E. S.	Hansen ESH-09B.051 (C)	KF257959
<i>Montanelia disjuncta</i>			Greenland, S, Igaliku	Hansen, E. S.	Hansen ESH-08.216 (C)	KF257970
<i>Montanelia disjuncta</i>			Greenland, NWn, Siorapuluk	Hansen, E. S.	Hansen ESH-09B.323 (C)	KF257971
<i>Montanelia disjuncta</i>		3956	Greenland, Northwest	Hansen, E. S.	Hansen ESH-09B.363 (C)	KP771825
<i>Montanelia disjuncta</i>		3957	Greenland, South	Hansen, E. S.	Hansen ESH-08.304 (C)	KP771826
<i>Montanelia disjuncta</i>		6574	Greenland, South, Igaliku	Hansen, E. S.	Hansen ESH-08.216 (C)	KP771832
<i>Montanelia disjuncta</i>			Norway, Tromso		Bjerke WP286-2 (TLE)	KF257961
<i>Montanelia disjuncta</i>			India, Uttar Pradesh		Divakar 15512 (MAF-Lich)	KF257972
<i>Montanelia disjuncta</i>	2000	MD2	Iceland, Iau		LA28245 (AMHN)	KY963377
<i>Montanelia disjuncta</i>	2009	MD5	Iceland, Ino		LA31552 (AMHN)	KY963378
<i>Montanelia disjuncta</i>	2007	MD3	Iceland, Ino		LA30617 (AMHN)	KY508684
<i>Montanelia disjuncta</i>			Canada, British Columbia		Goward 10-19 (UBC)	KF257963
<i>Montanelia disjuncta</i>	2014		Norway, Sor-Trondelag	Timdal, E.	O-L-196357	MK811711
<i>Montanelia disjuncta</i>	2014		Norway, Finnmark	Timdal, E.	O-L-195590	MK811852
<i>Montanelia disjuncta</i>	2006	MD4	Iceland, Ino		LA27588	KY508685
<b><i>Montanelia soredata</i></b>	<b>2016</b>	<b>100</b>	<b>Poland, Karpaty Mts</b>	<b>Szczepeńska, K.</b>	<b>Szczepeńska 1156 (WRSL)</b>	<b>MZ029735</b>
<b><i>Montanelia soredata</i></b>	<b>2016</b>	<b>101</b>	<b>Poland, Karpaty Mts</b>	<b>Szczepeńska, K.</b>	<b>Szczepeńska 1155 (WRSL)</b>	<b>MZ029736</b>
<i>Montanelia soredata</i>		4001	USA, Pennsylvania	Lendemer, J.	Lendemer 13329 (NY)	KF257978
<i>Montanelia soredata</i>		4824	Canada, British Columbia	Esslinger, T.L.	Esslinger BP111-1 (TLE)	KF257979
<i>Montanelia soredata</i>		4884	USA, Alaska	Esslinger, T.L.	Esslinger BP73-6 (TLE)	KF257980
<i>Montanelia soredata</i>		5981	Russia, Khabarovskiy Krai	Spribile, T.	Spribile 31972 (GZU)	KF257981
<i>Montanelia soredata</i>		6380	Canada, Ontario	McMullin, T.	McMullin 8139 (TLE)	KF257982
<i>Montanelia soredata</i>		B_8600	Japan, Mt. Ohyama	Ohmura, Y.	Ohmura 9666 (TNS)	KM386101
<i>Montanelia soredata</i>		MESO778	Sweden, Vasterbotten	Wedin, M.	Wedin 6862 (UPS)	KP771845
<i>Montanelia soredata</i>		4001	USA, Pennsylvania	Lendemer, J.	Lendemer 13329 (NY)	KP771846
<i>Montanelia soredata</i>		5981	Russia, Khabarovskiy Krai	Spribile, T.	Spribile 31972 (GZU)	KP771847
<i>Montanelia soredata</i>	2014		Norway, Telemark	Timdal, E.	O-L-195791	MK811963
<i>Montanelia soredata</i>	2014		Norway, Troms	Timdal, E.	O-L-195658	MK811965
<i>Montanelia soredata</i>	2016		Norway, Buskerud	Dahl, M. S., Kistenich, S. D., Timdal, E., Toreskaas, A. K.	O-L-204941	MK811977
<i>Montanelia soredata</i>		C_4670	Canada, British Columbia	Bjork, C.	Bjork 15153 (UBC)	KM386102

Bands corresponding to the ITS region were excised from the agarose gel and then purified by ethanol precipitation. Cleaned samples were sent to a sequencing service (Genomed, Warszawa, Poland). All laboratory analyses were performed at the Department of Botany and Plant Ecology at the Wrocław University of Environmental and Life Sciences.

## Sequence alignment and phylogenetic analysis

The newly-generated sequences and selected representatives of brown saxicolous Parmeliaceae were aligned using the Guidance 2 server (Landan and Graur 2008; Penn et al. 2010; Sela et al. 2015) employing the MAFFT algorithm (Katoh et al. 2002) followed by elimination of terminal ends. The final alignment consisted of 117 sequences of 535 sites. Further, we used Partition Finder 2 (Lanfear et al. 2016) implemented at the CIPRES Science Gateway (Miller et al. 2010). Two different models were found for partitions: GTR+G for ITS1 and ITS2 and K80+G for the 18S and 5.8S regions.

Moreover, phylogenetic analysis of all *Melanelia* sequences was also performed. Newly-generated sequences and these downloaded from GenBank, together with representatives of *Cetraria commixta*, which were further used as an outgroup, were aligned using the Guidance 2 server (Landan and Graur 2008; Penn et al. 2010; Sela et al. 2015) employing the MAFFT algorithm (Katoh et al. 2002) followed by elimination of unreliable columns. The final alignment consisted of 76 sequences of 803 sites. Further, we used jModeltest 2.1 (Darriba et al. 2012) implemented at the CIPRES Science Gateway (Miller et al. 2010) and the K80+G model was selected.

Bayesian analysis was carried out using a Markov Chain Monte Carlo (MCMC) method, in MrBayes v. 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) on the CIPRES Web Portal (Miller et al. 2010) using best models. Two parallel MCMC runs were performed, each using four independent chains and four million generations, sampling every 1000<sup>th</sup> tree. Posterior probabilities (PP) were determined by calculating a majority-rule consensus tree after discarding the initial 25% trees of each chain as the burn-in.

A Maximum Likelihood (ML) analysis was performed using RAxML-HPC2 v.8.2.10 (Stamatakis 2014) with 1000 ML bootstrap iterations (BS) and the GTR-GAMMAI model for both analyses. Phylogenetic trees were visualised using FigTree v. 1.4.2 (Rambaut 2012) and modified in Inkscape (<https://inkscape.org/>).

## Haplotype networks

Newly-generated sequences of the ITS rDNA marker, together with sequences downloaded from GenBank from specimens of *Cetraria commixta*, *Melanelia agnata*, *M. hepatizon*, *M. stygia*, *Montanelia disjuncta* and *M. sorediata*, were aligned separately for each species using Seaview software (Galtier et al. 1996; Gouy et al. 2010). TCS networks (Clement et al. 2002) were created as implemented in PopART software (<http://popart.otago.ac.nz>). Nucleotide diversity per site was calculated using DnaSP v.6 software (Rozas et al. 2017).

## Results

### Phylogeny and haplotype networks

A total of 169 sequences were analysed in this study.

The RAxML tree did not contradict the Bayesian trees topologies for the strongly-supported branches and only the latter is shown with posterior probabilities. The bootstrap support values  $BS \geq 70$  and  $PP \geq 0.95$  were considered to be significant and are shown near the branches. In Fig. S1, three main, highly supported lineages representing *Melanelia* spp. (i.e. *M. agnata*, *M. hepatizon* and *M. stygia*), *Montanelia* spp. (i.e. *M. disjuncta* and *M. sore-diata*) and *Cetraria commixta* were distinguished. The newly-sequenced specimens clustered together with other representatives of the species downloaded from GenBank. Amongst them, *Melanelia stygia* is not monophyletic, but forms two separate well-supported clades.

Moreover, to better understand phylogenetic relationships in the *Melanelia*, we performed additional analysis for all available ITS rDNA sequences from representatives of this genus. The Bayesian tree is presented in Fig. 1 with posterior probabilities and the bootstrap support values presented near the branches and with *Cetraria commixta* as an outgroup. In this tree, *Melanelia stygia* also forms two separate, highly-supported clades.

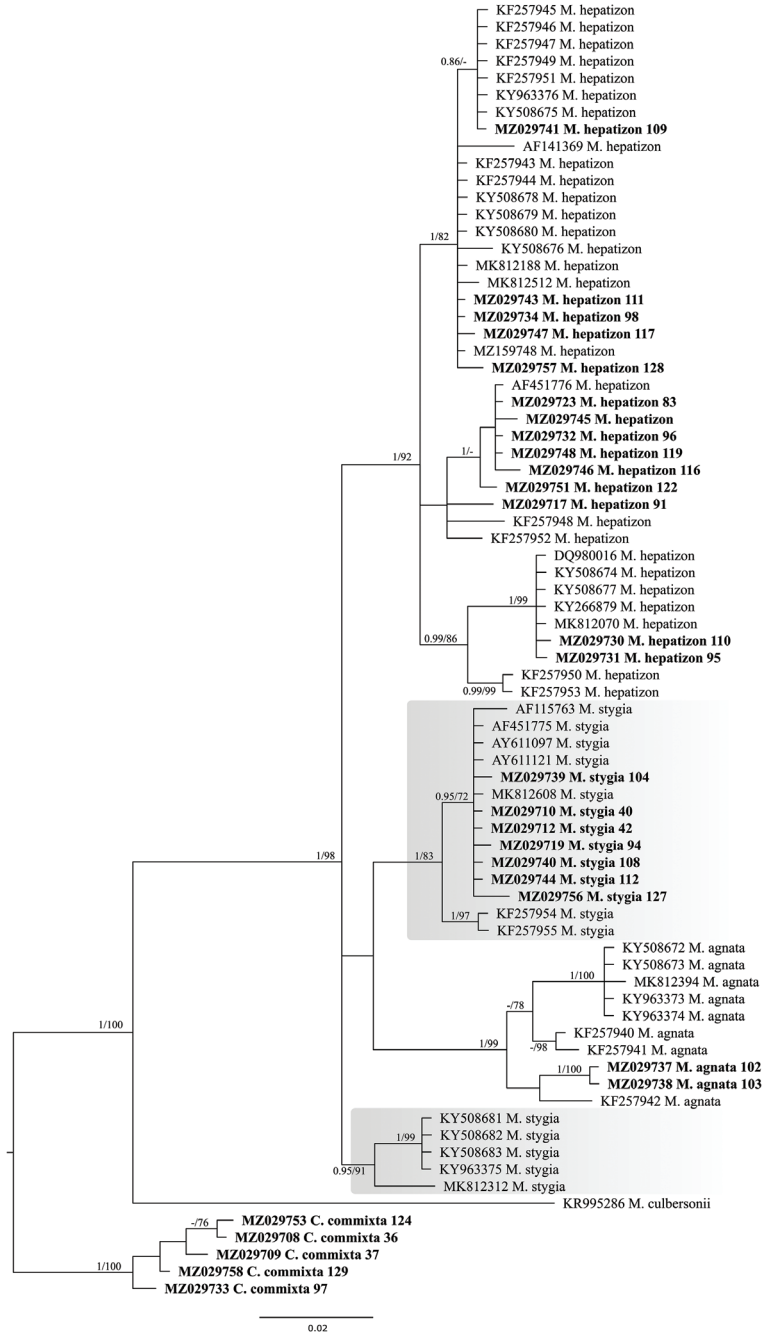
We constructed haplotype networks (Figs 2–7) to assess genetic variability within ITS rDNA marker for each species, including newly-collected specimens and data were downloaded from GenBank. The number of haplotypes found for each taxon ranged from five (in *Melanelia stygia*) to 12 (in *Melanelia hepatizon* and *Montanelia disjuncta*); however, their numbers seem to be correlated with the abundance of specimens tested, which ranged from 10 (in *Melanelia agnata*) to 67 (in *Montanelia disjuncta*). Moreover, we also calculated nucleotide diversity for each dataset and found lower values for *Montanelia disjuncta* and *Cetraria commixta* (0.00380 and 0.00405, respectively) and higher values for *Melanelia agnata*, *M. hepatizon* and *M. stygia* (0.01552, 0.01421 and 0.01418, respectively) (Table 2).

### Characteristics of the studied species

#### *Cetraria commixta* (Nyl.) Th. Fr.

Lichenographia Scandinavica 1:109 (1871)  $\equiv$  *Platysma commixtum* Nyl., Synopsis methodica lichenum 1:310 (1860)  $\equiv$  *Melanelia commixta* (Nyl.) A. Thell, Nova Hedwigia 60:417 (1995)  $\equiv$  *Cetrariella commixta* (Nyl.) A. Thell & Kärnefelt, Mycological Progress 3:309 (2004).

**Description.** *C. commixta* is a foliose species with elongated, smooth and flat lobes, 0.25–2.5 mm broad, which are thick on the margins and rounded at the ends (Szczepańska and Kossowska 2017). Its upper surface is glossy, olive-brown to dark brown or almost black. The lower surface is pale brown, but darker in the centre, with single, dark rhizines. *C. commixta* possess rounded or slightly elongated pseudocyphellae, which are present only on the margins and edges of lobes and cylindrical, marginal pycnidia, producing



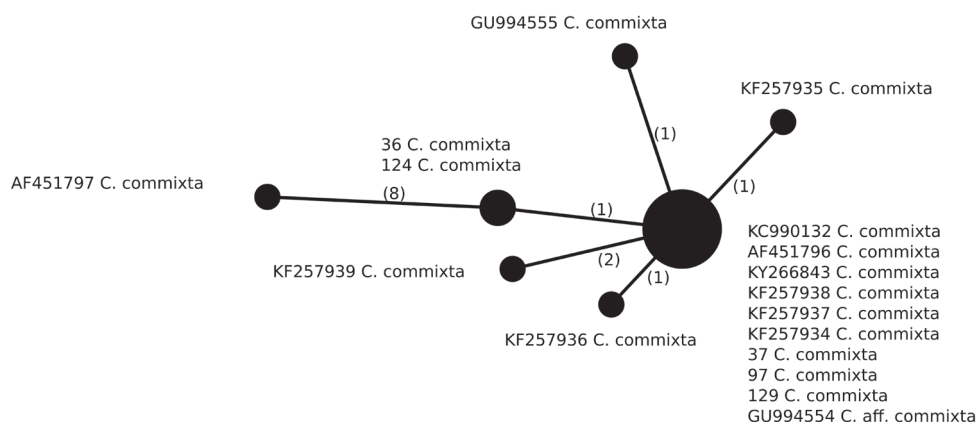
**Figure 1.** Phylogenetic relationships of *Melanelia* spp., based on Bayesian analysis of the ITS rDNA dataset. Posterior probabilities and Maximum Likelihood bootstrap values are shown near the internal branches. Newly-generated sequences are additionally described with isolate numbers following the species names and are marked in bold. GenBank accession numbers of sequences downloaded from GenBank are listed on the tree with species names.

hyaline, citriform conidia ( $3\text{--}4 \times 1\text{--}1.5 \mu\text{m}$ ). Apothecia are marginal, constricted at base, 0.2–7 mm diam., with hyaline, ellipsoid to oblong-ellipsoid ascospores ( $6\text{--}8 \times 4\text{--}6 \mu\text{m}$ ).

**Chemistry.**  $\alpha$ -collatolic acid (chemotype I) or no substances (chemotype III).

**Distribution.** *C. commixta* is a circumpolar and arctic-alpine species (Otte et al. 2005), growing mainly in mountain sites, in open places with high precipitation, on natural acid, siliceous rocks in North America and Europe. Available molecular data concern samples collected in North America (Canada, Greenland), as well as North (Finland, Norway, Sweden) and West (Spain) Europe.

**Haplotypes differentiation.** We identified seven different haplotypes (Fig. 2, Table 2) within *C. commixta* ( $n = 17$ ) that differ from each other in one or two positions, except for a single Canadian sample that differs in at least eight positions. The most common haplotype was found in ten specimens occurring in Greenland and North and Central Europe, amongst them being three newly-sequenced specimens (samples 37 and 97 from Poland and sample 129 from Germany). Moreover, two Polish specimens (samples 36 and 124 from the Sudety Mountains) represent a unique haplotype that differs from the most common one in a single position. Five haplotypes identified in our dataset were represented by single specimens originating from Greenland (3 haplotypes), Canada or Spain.



**Figure 2.** Haplotype network, based on ITS rDNA sequences from specimens of *Cetraria commixta*. Newly-generated sequences are described with isolate numbers preceding the species names. Sequences downloaded from GenBank are described with their accession numbers. Mutational changes are presented as numbers in brackets near lines between haplotypes.

### *Melanelia agnata* (Nyl.) A. Thell

Nova Hedwigia 60:416 (1995)  $\equiv$  *Platysma agnatum* Nyl., Flora, Jena 60:562 (1877)  $\equiv$  *Cetraria agnata* (Nyl.) Kristinsson, Lichenologist 6:144 (1974).

**Description.** *M. agnata* has foliose thallus with flat, smooth, 0.25–2 mm broad lobes which are thicker on the margins and rounded at the ends (Szczepańska and Kossowska

**Table 2.** List of haplotypes identified in this study and their geographical distribution. Nucleotide diversity for each species is also presented, and the newly generated sequences are in bold.

Haplotypes number	North America	North Europe	Central Europe	West Europe	Asia	Nucleotide diversity
<i>Cetraria commixta</i>						
1	KF257934 Greenland KF257937 Greenland KF257938 Greenland	AF451796 Finland KY266843 Norway KC990132 Sweden GU994554 Sweden	<b>37 Poland</b> <b>97 Poland</b> <b>129 Germany</b>			0.00405
2			<b>36 Poland</b> <b>124 Poland</b>			
3	AF451797 Canada					
4	KF257939 Greenland					
5	KF257936 Greenland					
6	KF257935 Greenland					
7				GU994555 Spain		
<i>Melanelia agnata</i>						
1		KY508672 Iceland KY508673 Iceland KY963373 Iceland KY963374 Iceland				0.01552
2			<b>102 Poland</b> <b>103 Poland</b>			
3	KF257940 Greenland					
4	KF257941 Greenland					
5	KF257942 Greenland					
6		MK257942 Norway				
<i>Melanelia hepaticozona</i>						
1	KF257943 Greenland KF257944 Greenland	KY508678 Iceland KY508680 Iceland KY508679 Norway MK812188 Norway	<b>98 Poland</b> <b>111 Poland</b> <b>128 Germany</b>			0.01421
2	KF257945 Greenland KF257946 Greenland KF257947 Greenland KF257949 Greenland KF257951 Greenland	KY508675 Iceland KY508676 Iceland	<b>109 Poland</b>			
3		KY508674 Iceland KY508677 Iceland KY266879 Iceland KY266879 Norway DQ980016 Sweden	<b>95 Poland</b> <b>110 Poland</b>			
4			<b>83 Poland</b> <b>96 Poland</b> <b>113 Poland</b> <b>116 Slovakia</b> <b>119 Poland</b> <b>122 Poland</b>	AF451776 Italy		
5	KF257950 Greenland KF257953 Greenland					
6	KF257952 Greenland					
7	KF257948 Greenland					
8	AF141369 Canada					
9		KY963376 Iceland				
10		MK812512 Norway				
11			<b>91 Poland</b>			
12			<b>117 Slovakia</b>			



Haplotypes number	North America	North Europe	Central Europe	West Europe	Asia	Nucleotide diversity
<i>Melanelia stygia</i>						
1		AY611097 Finland MK812608 Norway	AY611121 Austria <b>40 Poland</b> <b>42 Austria</b> <b>94 Poland</b> <b>104 Poland</b> <b>108 Poland</b> <b>112 Poland</b> <b>127 Czech Republic</b>	AF451775 Italy		0.01418
2		KY508681 Island KY508682 Island KY508683 Island KY963375 Island				
3	KF257954 Greenland KF257955 Greenland					
4		AF115763 Finland				
5		MK812312 Norway				
<i>Montanelia disjuncta</i>						
1	KF257964 Canada KF257967 Canada KF257969 Canada KP771830 Canada JX126181 USA	KY963378 Iceland KF257961 Norway KP771829 Norway KP771834 Sweden	AY611077 Austria <b>50 Poland</b> <b>51 Poland</b> <b>57 Poland</b> <b>80 Poland</b> <b>81 Poland</b> <b>82 Poland</b> <b>85 Poland</b> <b>86 Poland</b> <b>87 Poland</b> <b>88 Poland</b> <b>93 Poland</b> <b>121 Poland</b> <b>125 Poland</b> <b>126 Poland</b> <b>130 Czech Republic</b>		GU994556 India KF257972 India KP771837 India	0.00380
2	KF257962 Canada KF257965 Canada KF257966 Canada KP771832 Greenland KF257958 Greenland KF257970 Greenland KP771826 Greenland	KY963377 Iceland KY266910 Norway DQ980015 Sweden	<b>90 Poland</b> <b>120 Poland</b>			
3	KF257957 Greenland KF257971 Greenland KP771825 Greenland KP771833 Greenland	KY508684 Iceland KY508685 Iceland KY508686 Iceland				
4	-	JX974654 United Kingdom KP771835 United Kingdom	<b>78 Poland</b> <b>79 Poland</b>  <b>89 Poland</b>  <b>92 Poland</b> <b>123 Poland</b>			

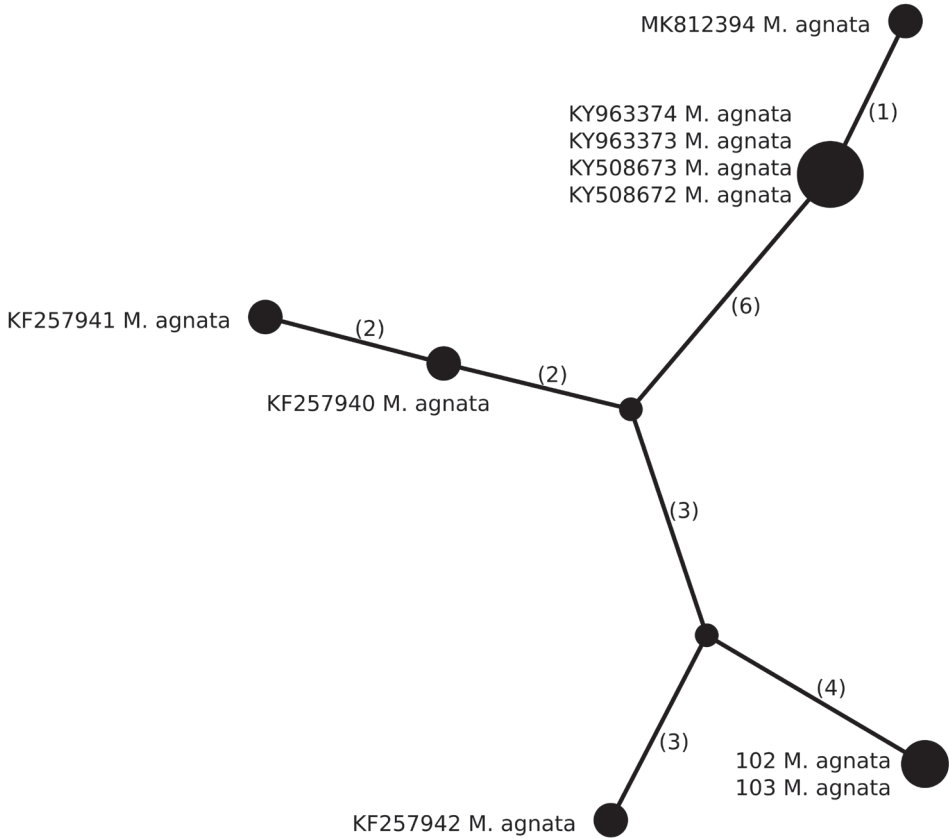
Haplotypes number	North America	North Europe	Central Europe	West Europe	Asia	Nucleotide diversity	
5	KF257956 Canada KP771824 Canada						
6	JX974658 Canada KP771836 Canada						
7	KF257963 Canada						
8	KF257959 Greenland KP771827 Greenland						
9	KF257968 USA KP771831 USA						
10	KF257960 USA KP771828 USA						
11		MK811852 Norway					
12		MK811711 Norway					
<i>Montanelia soreliata</i>							
1		MK811977 Norway MK811965 Norway GU994557 Sweden KP771845 Sweden	<b>100 Poland</b>				0.00830
2	KF257978 USA KP771846 USA				KF257981 Russia KP771847 Russia KM386101 Japan		
3	KF257980 USA		<b>101 Poland</b>				
4	KM386102 Canada KF257982 Canada						
5	KF257979 Canada						
6		MK811963 Norway					

2017). The upper surface is glossy, olive-brown to dark brown. The lower surface is pale brown to dark brown in the centre, with single, dark rhizines. *M. agnata* possess pseudocypheae which are larger on the lobe margins and smaller, punctiform on the upper surface of the lobes. Pycnidia are mainly marginal to laminal, partially immersed and globose with hyaline bacilliform conidia ( $4.5\text{--}5.5 \times 1 \mu\text{m}$ ). Apothecia are not seen in examined material.

**Chemistry.** No secondary metabolites were detected by TLC.

**Distribution.** *M. agnata* is a rare taxon occurring in arctic and boreal regions in North America and Europe, growing in open stands on siliceous and basalt rocks (Otte et al. 2005). Available molecular data concern samples collected only in North America (Greenland) and North Europe (Iceland, Norway).

**Haplotypes differentiation.** Six different haplotypes were identified in *M. agnata* ( $n = 10$ ), of which two Polish specimens, collected in the Karpaty Mountains, have the same, not previously known, haplotype (Fig. 3, Table 2). It differs from other haplotypes in at least seven positions. However, the remaining specimens originate from Greenland, Iceland or Norway and no other samples from Central Europe have been sequenced until now. Four Icelandic specimens have the same haplotype, which is similar to the haplotype from Norwegian specimens. In contrast, Icelandic haplotypes differ from Greenlandic haplotypes in at least eight positions. Whether their genetic diversity supports conclusions from previous papers suggesting potentially unrecognised species lineages in the *M. agnata* genus (Leavitt et al. 2014; Xu et al. 2017) remains unresolved and should be further studied.



**Figure 3.** Haplotype network, based on ITS rDNA sequences from specimens of *Melanelia agnata*. Newly-generated sequences are described with isolate numbers preceding the species names. Sequences downloaded from GenBank are described with their accession numbers. Mutational changes are presented as numbers in brackets near lines between haplotypes.

### *Melanelia hepaticum* (Ach.) A. Thell

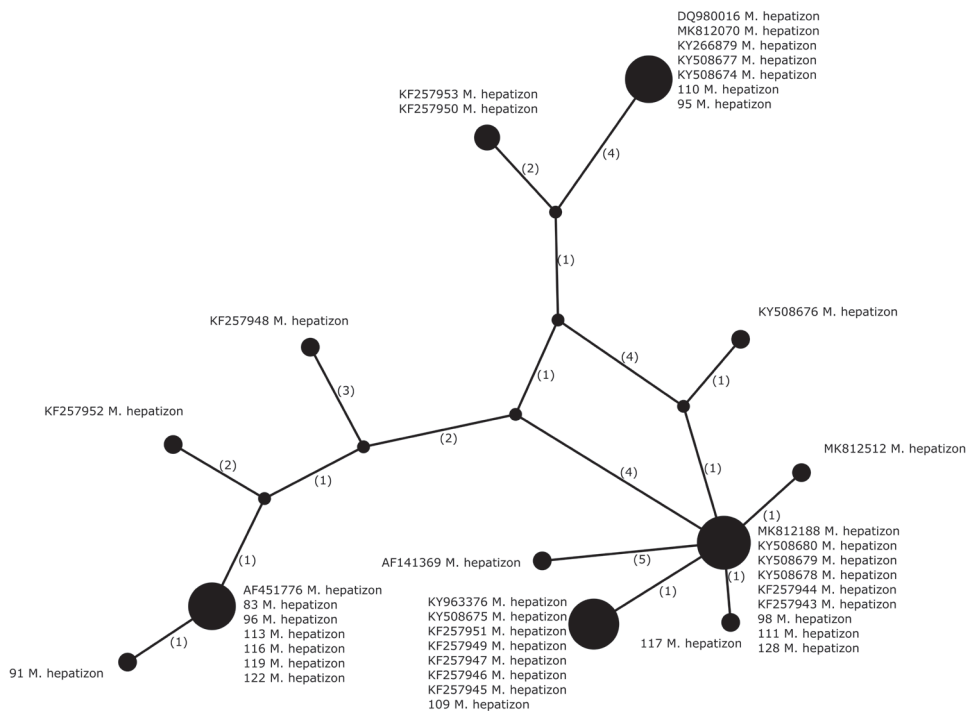
Nova Hedwigia 60:419 (1995)  $\equiv$  *Lichen hepaticum* Ach., Lichenographiae Sueciae Prodomus 110 (1798)  $\equiv$  *Cetraria hepaticum* (Ach.) Vain., Termesztetrajzi Füzetek 22:278 (1899).

**Description.** *M. hepaticum* is foliose species with flat lobes that are 0.25–2.5 mm broad and thick at the margins (Szczepeńska and Kossowska 2017). Its upper surface is glossy, brown to almost black. The lower surface is dark brown to black, paler near the margins, with single, dark rhizines. Pseudocyphellae are mainly present on the margins and edges of lobes. Pycnidia are marginal, but sometimes also laminal, sessile, globose to stalked, slightly elongated or cylindrical with hyaline, bacilliform conidia (3–5  $\times$  1  $\mu$ m). Apothecia are marginal to laminal, sessile, with hyaline, ellipsoid to oblong-ellipsoid ascospores (6–8  $\times$  4–6  $\mu$ m).

**Chemistry.** Stictic and norstictic acids.

**Distribution.** *M. hepaticum* is a circumpolar and arctic-alpine species occurring from oceanic to continental sites on siliceous rocks in North America and Europe (Otte et al. 2005). Available molecular data concern samples collected in North America (Canada, Greenland) as well as North (Iceland, Norway, Sweden) and West (Italy) Europe.

**Haplotypes differentiation.** A higher number of haplotypes was detected in *M. hepaticum* ( $n = 40$ ), in which we identified 12 haplotypes (Fig. 4, Table 2). Amongst newly-sequenced specimens, we identified six haplotypes. Some are more common and were previously found in Greenland, Iceland, Italy, Norway or Sweden. In contrast, others were only found in newly-sequenced specimens, such as sample 91 from the Sudety Mountains in Poland and sample 117 from the Karpaty Mountains in Slovakia. However, no geographic pattern was found in the dataset.



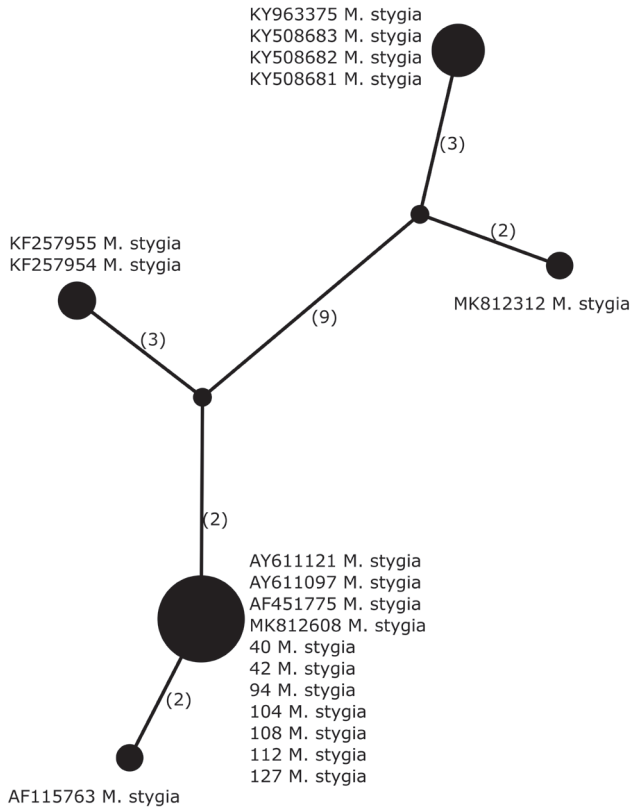
**Figure 4.** Haplotype network, based on ITS rDNA sequences from specimens of *Melanelia hepaticum*. Newly-generated sequences are described with isolate numbers preceding the species names. Sequences downloaded from GenBank are described with their accession numbers. Mutational changes are presented as numbers in brackets near lines between haplotypes.

### *Melanelia stygia* (L.) Essl.

Mycotaxon 7:47 (1978)  $\equiv$  *Lichen stygius* L., Species Plantarum 2:1143 (1753).

**Description.** *M. stygia* has foliose thallus, composed of 0.25–1.5 mm broad, smooth and usually distinctly convex lobes (Szczepańska and Kossowska 2017). The upper

surface is glossy, dark brown to almost black. The lower surface is dark brown to black, paler near the margins, with single, dark rhizines. Pseudocyphellae in this species are numerous, rounded or slightly elongated and laminal – clearly visible on the upper surface of the lobes. Pycnidia are also common, globose, laminal and immersed with hyaline, bacilliform conidia ( $3.5\text{--}5 \times 1 \mu\text{m}$ ). Apothecia are laminal, constricted at the



**Figure 5.** Haplotype network, based on ITS rDNA sequences from specimens of *Melanelia stygia*. Newly-generated sequences are described with isolate numbers preceding the species names. Sequences downloaded from GenBank are described with their accession numbers. Mutational changes are presented as numbers in brackets near lines between haplotypes.

base and 0.5–6 mm in diameter. Ascospores are hyaline, ellipsoid to oblong-ellipsoid,  $6\text{--}8 \times 4\text{--}6 \mu\text{m}$  in size.

**Chemistry.** Protocetraric and fumarprotocetraric acids (Race 1) or no substances detected (Race 6).

**Distribution.** *M. stygia* is a circumpolar and arctic-alpine species occurring mainly on siliceous rocks in upper mountain areas in North America and Europe (Otte et al. 2005). Available molecular data concern only a few samples collected in North America (Greenland) and North (Iceland, Finland, Norway) and West (Italy) Europe.

**Haplotypes differentiation.** Amongst five identified haplotypes in *Melanelia stygia* (n = 19), all newly-sequenced specimens (five from Poland, one from Austria and one from the Czech Republic) have the same haplotype, previously reported from Austria, Finland, Italy and Norway (Fig. 5, Table 2). It differs from the haplotype identified in another Finnish specimen in two positions. Two Greenlandic specimens have the same haplotype that differs from the most common one in five positions. Four Icelandic samples have an identical haplotype that differs from the Norwegian sample in five positions; however, these samples differ in at least 13 positions from other haplotypes of *M. stygia*. Moreover, these Icelandic and one Norwegian samples form a separate clade shown in Fig. 1, in contrast to the remaining specimens of *M. stygia*. These molecular data suggest that these lineages may represent phenotypically indistinguishable cryptic species.

***Montanelia disjuncta* (Erichsen) Divakar, A. Crespo, Wedin & Essl.**

American Journal of Botany 99:2022 (2012)  $\equiv$  *Parmelia disjuncta* Erichsen, Annales Mycologici 37:78 (1939)  $\equiv$  *Melanelia disjuncta* (Erichsen) Essl., Mycotaxon 7:46 (1978).

**Description.** *M. disjuncta* possess foliose thallus composed of 0.6–1.2 mm broad, flat to slightly convex and glossy lobes (Szczepańska et al. 2015). Its upper surface is smooth, olive-brown to dark brown. Pseudocyphellae are small, rather indistinct and submarginal. Its characteristic feature is the presence of the soralia (0.2–0.5 mm in diameter), which are punctiform, irregular, usually capitate and arise on the surface or at the margins of the lobes. Soredia are granular to isidioid, dark, but appearing white when abraded. Pycnidia are rare, conidia are 6–7  $\times$  1  $\mu$ m. Apothecia are not seen in the examined material.

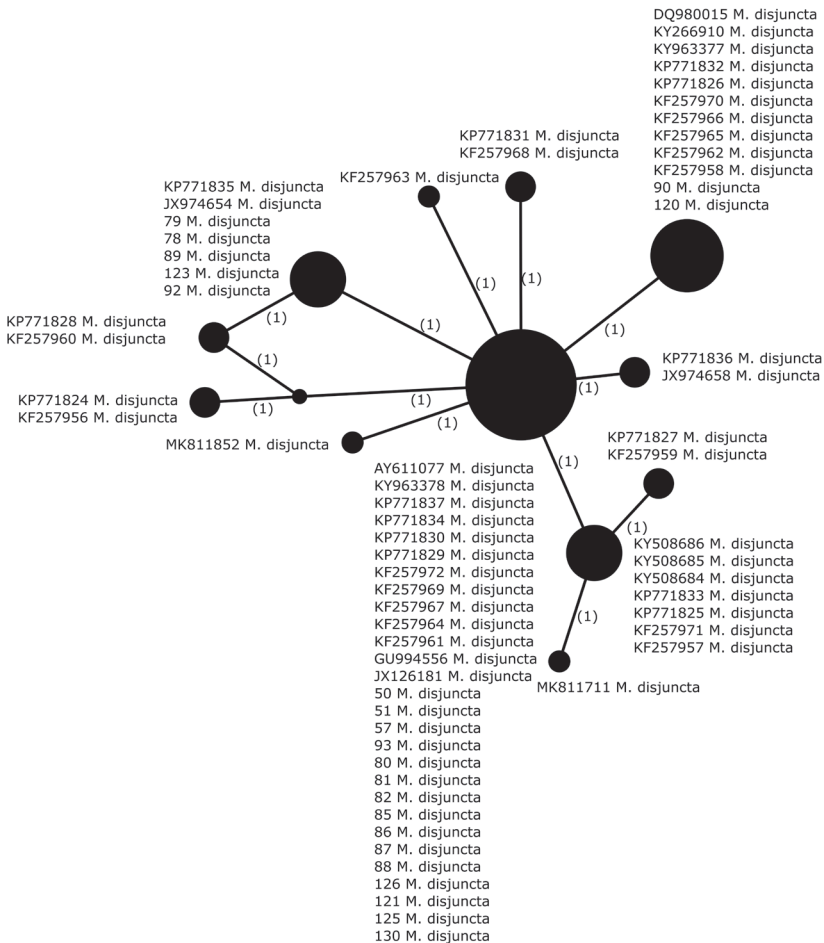
**Chemistry.** Perlatolic and stenosporic acids.

**Distribution.** *M. disjuncta* is a circumpolar species growing mainly on siliceous rocks. The geographical range of this species consists of both continental and oceanic areas of Europe and North America (Esslinger 1977; Otte et al. 2005; Hansen 2013). Available molecular data concern samples collected in North America (Canada, Greenland, USA), North (Iceland, Norway, Sweden, United Kingdom) and Central (Austria) Europe, as well as Asia (India).

**Haplotypes differentiation.** Twelve different haplotypes were identified in *M. disjuncta* (n = 67), of which the most common haplotype occurs in Europe, North America and Asia (Fig. 6, Table 2). The highest diversity was observed in North America (Canada, Greenland, USA), for which a total of nine different haplotypes were found, including six that were exclusive for this region. We identified three different haplotypes amongst the newly-collected samples (n = 22). The most common one also occurs in other European countries, Asia and North America. The second most common also occurs in Northern Europe and North America, while the third haplotype was previously identified in specimens collected in the



United Kingdom. Moreover, four different haplotypes were identified amongst specimens collected in Norway, while five haplotypes were identified in Canadian samples, of which three are unique to Canada. Three haplotypes were identified in samples from both Iceland and Greenland, two of which are common for these areas and one haplotype is unique to Greenland. Some haplotypes are represented by more than one sample originating from particular areas, such as Alaska and Maine (USA), the Yukon Territory (Canada) or Greenland. The haplotypes identified in our dataset originated from different geographical areas and two of the most common haplotypes are widely distributed in the Northern Hemisphere. Based on the presented sampling, we could not indicate any geographical pattern, neither locally nor worldwide.



**Figure 6.** Haplotype network, based on ITS rDNA sequences from specimens of *Montanelia disjuncta*. Newly-generated sequences are described with isolate numbers preceding the species names. Sequences downloaded from GenBank are described with their accession numbers. Mutational changes are presented as numbers in brackets near lines between haplotypes.

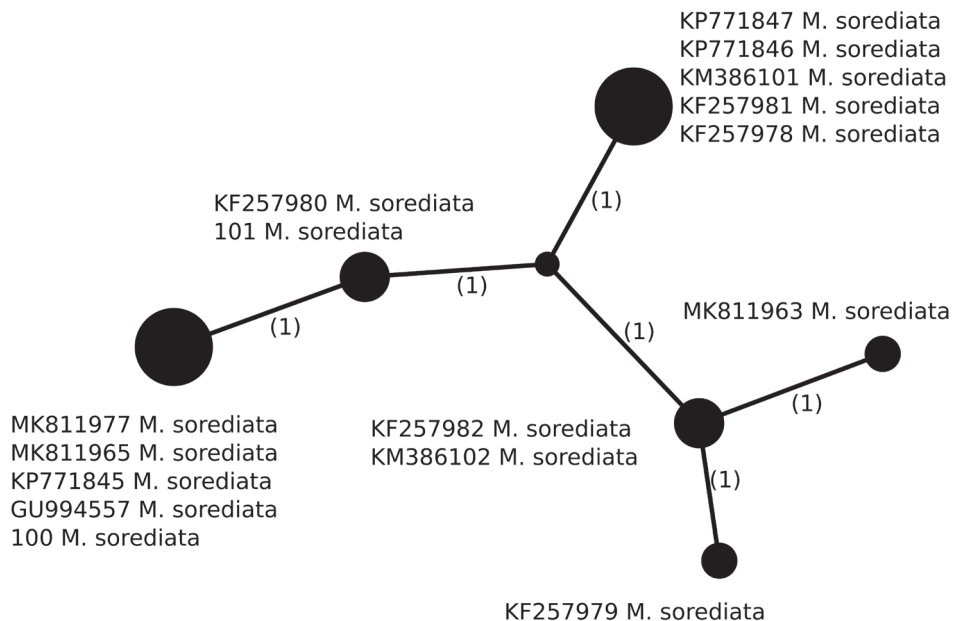
***Montanelia sorediata* (Ach.) Divakar, A. Crespo, Wedin & Essl.**

American Journal of Botany 99:2023 (2012)  $\equiv$  *Parmelia stygia* var. *sorediata* Ach., Lichenographia Universalis 471 (1810)  $\equiv$  *Melanelia sorediosa* (Almb) Essl., Mycotaxon 7:47 (1978)  $\equiv$  *Melanelia sorediata* (Ach.) Goward & Ahti, Mycotaxon 28:94 (1987).

**Description.** *M. sorediata* is a foliose species. Its lobes are flat to slightly convex, 0.2–0.6 mm broad, distinctly rugged and pitted at the ends (Szczepańska et al. 2017). The upper surface is smooth, dull, olive brown to dark brown. Characteristic soralia arise on the ends of the main lobes or on the smaller, erect side lobes. They are usually distinctly convex and capitate with granular to isidioid, dark soredia. Pseudocyphellae and pycnidia are absent. Apothecia are not seen in the examined material.

**Chemistry.** Perlatolic and stenosporic acids.

**Distribution.** *M. sorediata* is a probably circumpolar species that prefers siliceous substrates, usually in open and well-lit places. The species is mentioned as occurring



**Figure 7.** Haplotype network, based on ITS rDNA sequences from specimens of *Montanelia sorediata*. Newly-generated sequences are described with isolate numbers preceding the species names. Sequences downloaded from GenBank are described with their accession numbers. Mutational changes are presented as numbers in brackets near lines between haplotypes.

in North America and Europe (Esslinger 1977; Otte et al. 2005). Available molecular data concern only a few samples collected in North America (Canada, USA), North Europe (Norway, Sweden) and Asia (India).

**Haplotypes differentiation.** Six different haplotypes were identified in *M. sorediata* (n = 16), of which two Polish specimens, collected in the Karpaty Mountains, have two different haplotypes that differ in a single position (Fig. 7, Table 2). Interestingly, sample 101 has the same haplotype as the specimen collected in Alaska (KF257980), while sample 100 has the same haplotype as four Scandinavian specimens collected in Norway and Sweden. Another of the most common haplotypes is represented by specimens collected in Japan, Russia and the USA. Therefore, no specific geographic pattern was observed in the dataset.

## Discussion

Although several studies focused on the phylogeny of brown *Parmeliae*, in the analysed datasets, there was an evident lack of molecular data concerning this group from Central Europe. The available data included only North America (mainly Greenland), Northern Europe (Scandinavian countries) and single sequences from specimens collected in Western Europe (Spain, Italy) and Asia (India, Russia). Having the opportunity to collect data from Poland, we focused on taxa occurring in this country, such as *Cetraria commixta*, *Melanelia agnata*, *M. hepatizon*, *M. stygia*, *Montanelia disjuncta* and *M. sorediata*. Additionally, in analyses, we also included newly-generated sequences from samples collected in Austria, Czech Republic, Germany and Slovakia. By supplementing the dataset with new sequences from a previously-unexplored area, we wanted to study the intraspecific internal transcribed spacer (ITS) rDNA variability of mentioned species and analyse distribution patterns of individual haplotypes. Previously, Leavitt et al. (2014) reported mean genetic distance (given as the number of nucleotide substitutions per site) in brown *Parmeliae* and found higher values in the case of *Melanelia agnata* and *M. hepatizon* (0.013) in contrast to *Cetraria commixta* and *M. stygia* (0.002 and 0.007, respectively). In this study, we found the highest nucleotide diversity in *Melanelia agnata* and *M. hepatizon* (0.01552 and 0.01421, respectively), but also in *M. stygia* (0.01418) as a result of additional sampling.

In our study, the haplotype networks illustrated that single-locus haplotypes and clades have no geographic clustering and cannot be useful in defining the species boundaries within brown *Parmeliae*. Haplotypes are dispersed amongst the sites and clades do not show apparent association with spatial location, as reported in literature data (Werth and Sork 2008; Starosta and Svoboda 2020). In addition, many of the analysed haplotypes of brown *Parmeliae* are widely distributed and, in many cases, the same haplotypes are shared between temperate and polar populations. What is more, all taxa, except *Melanelia stygia*, seem to be monophyletic and newly-sequenced specimens cluster together with other representatives of the species downloaded from

GenBank. The extremely wide geographical distribution of mycobiont haplotypes has been observed in some other species, such as *Cavernularia hultenii* (Printzen et al. 2003), *Cetraria aculeata* (Fernández-Mendoza et al. 2011) and *Cladonia subcervicornis* (Printzen and Ekman 2003). In the first two cases, this phenomenon is assigned to lichens characterised by vegetative propagation and interpreted as evidence for ancestral polymorphisms and slow genetic drift (Printzen et al. 2003). This finding conforms well with the results of our study on Parmeliaceae, which are usually sterile species, reproducing by soredia (*Montanelia*) and conidia (*Cetraria*, *Melanelia*).

Although representatives of brown *Parmeliae* are known from both Hemispheres (Otte et al. 2005), all species studied in this paper represent circumpolar distribution and occur only on northern continents. The specimens used for the analyses originated mainly from mountain areas of Poland, both the Carpathians and the Sudetes; however, the range of sampling seems to be representative for this part of Europe. The number of analysed haplotypes representing different geographical regions was comparable for each taxon; nevertheless, the number of *Melanelia agnata* and *Montanelia soredata* samples remain very small. Due to the newly-generated molecular data covering Central Europe, we were able to compare the haplotype distribution in this area with other parts of the world. Unfortunately, the data available for discussed lichens taxa include, almost exclusively, specimens from North America and Northern Europe; the data concerning Asia and Southern Europe are not sufficient to make a reliable comparison possible. In almost all analysed taxa, stronger genetic differentiation was found amongst North American populations, with a few haplotypes unique for this part of the world, especially for Greenland. Specimens occurring in Central Europe have lower haplotype diversification and many of these haplotypes have wide geographical distribution (Table 2). Nevertheless, it seems that the number of analysed sequences is still insufficient to indicate high diversity areas (hotspots), species speciation centres or glacial refugia. Although the numbers of haplotypes correlated with the number of specimens tested, two species occurring in Poland (*Melanelia agnata* and *M. stygia*) clearly indicate a very low level of genetic diversity. Both species are rare in Poland and their distribution is limited to the high mountain regions (Szczepańska and Kossowska 2017). Low genetic diversity and limited occurrence suggest considering both taxa as critically endangered in Poland.

In recent years, it has been proved that cryptic species-level lineages are very common amongst lichen-forming fungi (Crespo and Pérez-Ortega 2009; Crespo and Lumbsch 2010; Lumbsch and Leavitt 2011). At the same time, it has been shown that phenotypic variation is not always ‘sensitive’ enough for delimitation and description of new taxa. Modern methods of genetic analysis are recommended as an additional tool for this purpose (Molina et al. 2011; de Paz et al. 2012; Leavitt et al. 2013; Renner 2016). At the same time, it is necessary to include other evidence, such as chemistry, ecology, geography and morphology, for the proper delimitation of lichenised fungi species (Hawksworth 1976; Dayrat 2005; Crespo and Pérez-Ortega 2009). Such careful and versatile analysis of distinct phylogenetic lineages may lead to recognising some previously-overlooked characteristics (Kroken and Taylor 2001; del Prado et al. 2007;

Frolov et al. 2016; Leavitt et al. 2016; Szczepańska et al. 2020). In the recent review paper, Lücking et al. (2021) proposed a detailed protocol for consistent taxonomy of lichen-forming fungi. The integrative taxonomy employing phylogeny, reproductive biology and phenotype should be used to delimit species (Lücking et al. 2020). Aime et al. (2021) recommended circumscription of new taxa, based on an appropriate sampling of multiple representatives from different collections for which multi-loci analyses should be performed. They also noted that description of a new species, based on single-locus phylogenetic analyses, could only be done in exceptional cases. The errors caused by contaminant sequences, laboratory mix-ups and chimeric sequences should be avoided for proper establishment of novel taxa, based on molecular data only (Lücking et al. 2021). Therefore, it is crucial to employ unlinked loci from different parts of the genome, even though the ITS rDNA marker is widely used in DNA barcoding of fungal taxa.

We analysed phenotypic diversity of samples representing individual haplotypes in our studies. However, in morphological, anatomical and chemical analyses, we observed that phenotypic characters of individuals representing different haplotypes are homogeneous and no visible distinctive features for samples with different geographic distribution were recognised. Recent molecular studies of one of the analysed genus – *Melanelia*, suggested previously unrecognised species-level diversity within this taxon (Divakar et al. 2012; Leavitt et al. 2014; Xu et al. 2017). However, the authors based their assumptions primarily on phylogenetic analyses without considering phenotypic features. Therefore, we have decided to analyse differences in morphology, anatomy and chemistry of *M. stygia* and *M. agnata* specimens originating from different geographic regions (Greenland, Iceland and Central Europe).

*Melanelia agnata* is a rare lichen recorded in North America and some European countries, such as Austria, Iceland, Norway, Poland, Russia, Sweden, Switzerland and Slovakia (Westberg et al. 2004; Hawksworth et al. 2008; Szczepańska and Kosowska 2017). The analysed holotype of *Melanelia agnata* is characterised by small (ca. 3.0 cm in diam.), foliose, olive-brown to dark-brown thallus, composed of flat, shiny, 0.25–2 mm broad, smooth lobes with thicker margins (Fig. 8A). Its lower surface is pale brown with single, dark rhizines. Polish (Figs. 8G and H) and Greenlandic (Fig. 8E and F) specimens comply with the type. However, Icelandic material differs in a larger thallus size (up to 10 cm in diam.) and the appearance of the lobes, which are more convex than flat, 1–5 mm broad and distinctly wrinkled (Fig. 8C). Thell (1995) made an interesting taxonomic description of *M. agnata*, in which he noted that its thallus could reach up to 10 cm diam. However, in his research, Thell (1995) analysed only a few specimens, including one from Iceland (Kristinsson 14781, GZU, LD) and treated them all as a single taxon. A similar situation applies to conidia, reaching 5–7.5  $\mu\text{m}$  in *M. agnata*, according to Thell (1995). Pycnidia observed in Icelandic specimens are usually marginal (Fig. 8D), very often double and produce bifusiform conidia, 4.5–6  $\times$  1  $\mu\text{m}$ , in contrast to the type specimen, which pycnidia are simple, marginal to laminal (Fig. 8B) with smaller conidia, at 3.5–5  $\times$  1  $\mu\text{m}$ . Pseudocyphellae are always whitish, rounded or irregular, marginal

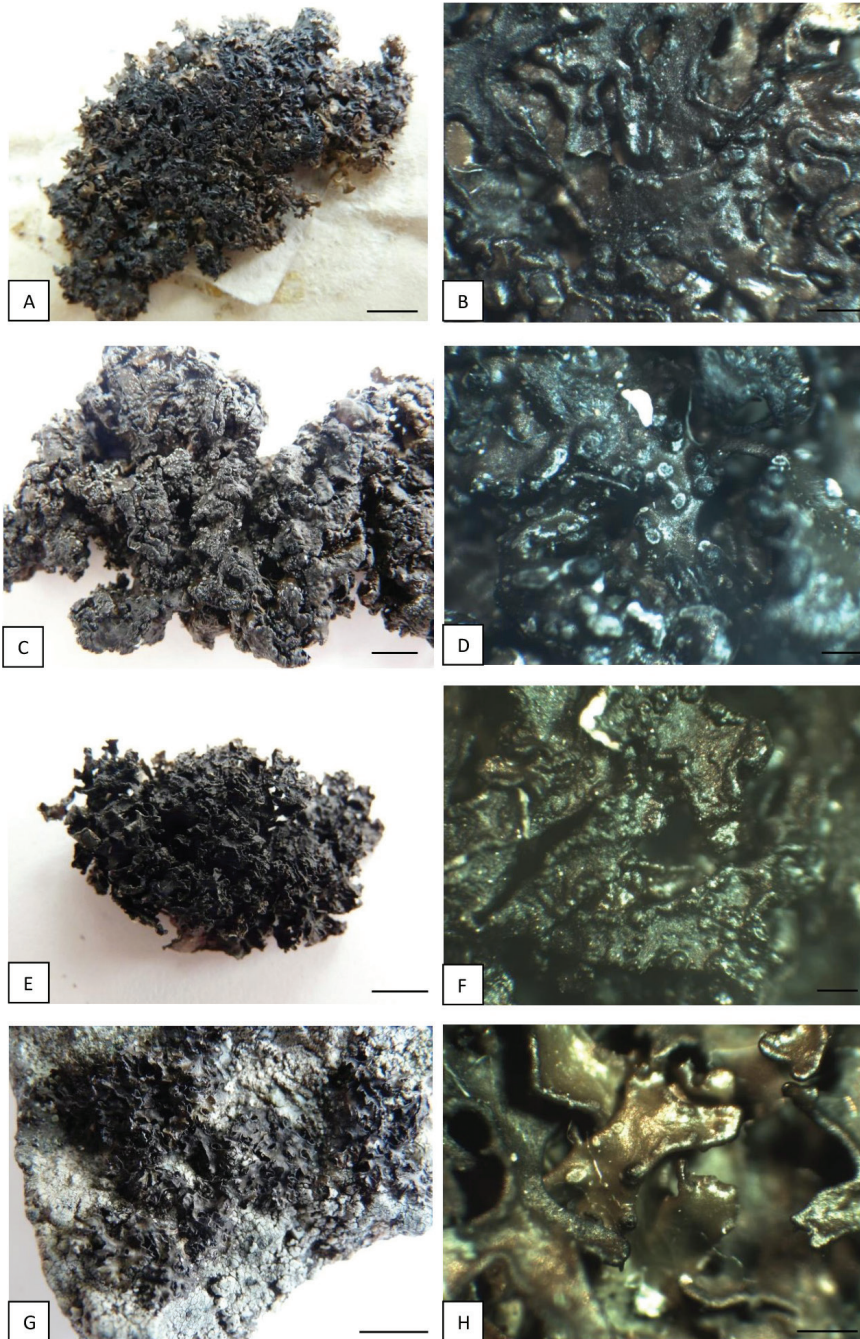
and laminal in all analysed material; they are much more abundant in specimens from Iceland (Fig. 8D). None of the Icelandic specimens had apothecia, so their anatomical analysis was impossible. All material was chemically homogeneous and no secondary metabolites were detected by thin-layer chromatography (TLC), which is consistent with other descriptions (Thell 1995; Xu et al. 2017).

*Melanelia stygia* is a much more common species than *M. agnata*. In Europe, it was recorded in the upper mountain areas of Austria, the Czech Republic, Germany, Great Britain, Poland, Romania, Russia, Slovakia, Switzerland and Ukraine (Hawksworth et al. 2008).

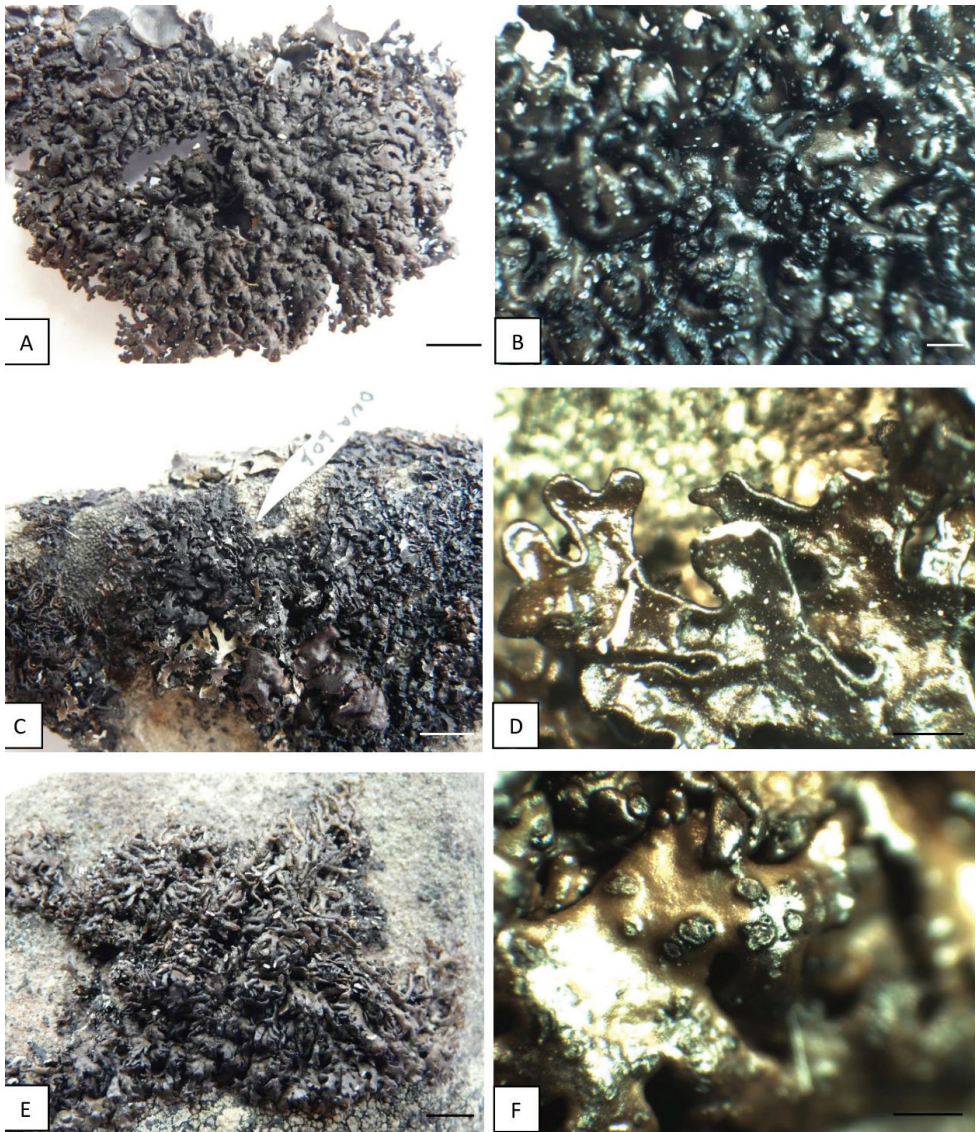
After phenotypic studies, we have concluded that all material is homogeneous and none of the analysed morphological and anatomical features coincides with geographically-distinct *M. stygia* populations (Fig. 9A–F). However, some differences may be observed in the secondary chemistry. In his paper, Esslinger (1977) recognised six chemical races within *M. stygia*. He stated that some of them are broadly distributed and others are more frequent in particular regions. All the currently-examined samples originating from Greenland and Central Europe belong to Race 1, containing fumaroprotocetraric and protocetraric acids. Specimens from Iceland represent Race 6, without secondary metabolites. Both races are known to occur in Japan, North America and Europe; however, there is a possibility that Race 6 is the only chemical Race occurring in Iceland. Production of some secondary metabolites may be induced by environmental factors (Culberson 1986; Leavitt et al. 2011) and does not always correspond with molecular data. Moreover, chemical differences can be observed within some recognised haplotype groups and even in the same haplotype (Matteucci et al. 2017). At the same time, chemical characters may be successfully used to support delimitation of lichen taxa, but in any case, they cannot be treated as an exclusive diagnostic trait (Elix et al. 2009; Spribille et al. 2011; Leavitt et al. 2013; Onut-Brännström et al. 2018; Mark et al. 2019.).

In conclusion, we can state that all of the potential species lineages within *Melanelia agnata* and *M. stygia* are cryptic, with very slight morphological, anatomical and chemical variation. We were unable to distinguish any distinctive feature that could be considered diagnostic and useful for the delimitation of new species, except molecular variation. The phenotypic differences mentioned above may reflect environmental or climate conditions, such as temperature, light, humidity or substrate and may not be connected with genetic differences. However, this study was limited to a small number of samples and one genetic marker, ITS; therefore, we refrain from describing new species because further study is pending. We suggest that an extended phylogeographic study is necessary and an increase in the number of herbarium specimens would probably give additional information. Even though our analyses complement the knowledge on lichens in Central Europe, many areas remain insufficiently explored. Additional sampling from Asia and Southern Europe may bring new data on the phylogenetic and phenotypic diversity of species from the brown *Parmeliae* group.





**Figure 8.** *Melanelia agnata* specimens treated **A** *Melanelia agnata* H-NYL 36086 (holotype) **B** *Melanelia agnata*, H-NYL 36086 (holotype) **C** *M. agnata*, AMNH 27562 (Iceland) **D** *M. agnata*, AMNH 30974 (Iceland) **E** *M. agnata*, C 19019 (Greenland) **F** *M. agnata*, C 19019 (Greenland) **G** *M. agnata*, Szczepańska 1050, WRSL (Poland) **H** *M. agnata*, Szczepańska 1050, WRSL (Poland). Scale bars: 0.5 cm (**A, C, E, G**); 0.5 mm (**B, D, F**); 1 mm (**H**).



**Figure 9.** *Melanelia stygia* specimens treated **A** *M. stygia*, AMNH 28243 (Iceland) **B** *M. stygia*, AMNH 16894 (Iceland) **C** *M. stygia*, C 19893 (Greenland) **D** *M. stygia*, C 19893 (Greenland) **E** *M. stygia*, Szczepańska 1160, WRSL (Poland) **F** *M. stygia*, Szczepańska 737, WRSL (Austria). Scale bars: 0.5 cm (**A**, **C**, **E**); 1 mm (**B**, **D**); 0.5 mm (**F**).

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## Supplementary material I

### Figure S1

Authors: Katarzyna Szczepańska, Beata Guzow-Krzemińska, Jacek Urbaniak

Data type: Images.

Explanation note: Phylogenetic relationships of brown Parmeliae, based on Bayesian analysis of the ITS rDNA dataset. Posterior probabilities and Maximum Likelihood bootstrap values are shown near the internal branches. Newly-generated sequences are described with isolate numbers preceding the species names and are marked in bold. GenBank accession numbers of sequences downloaded from GenBank are listed on the tree with species names.

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