Reindeer Diet in the Cairngorms:

Can fungal barcoding diagnose lichen species?

Image from: Ohio Moss and Lichen Association http://ohiomosslichen.org/lichen-cladonia-rangiferina/

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Abstract

Reindeer, *Rangifer tarandus* L., are known to consume lichens as part of their winter diet, however within the Cairngorm herd, little is known of their ecological impact. Therefore, this study forms preliminary data for a larger, long-term project to assess the diet of the Cairngorm reindeer. Using sequence data of the ITS region, this study sought to: (1) determine the efficacy of species discrimination within the genera *Cetraria* Ach., *Cladonia* P. Browne, *Ochrolechia* A. Massal. and *Umbilicaria* Hoffm. through DNA barcoding and (2) to determine if a specific sub-region, ITS1 or ITS2 could act as a stand-alone barcode within lichenised fungi, which are potentially important in reindeer diet. Barcode gaps were found using uncorrected (p) distances in 62.5% of studied lichens, with *Cladonia* only producing a barcode gap in 37.5% of species. Of the sub-regions, ITS1 out-performed ITS2 with 77.6% of species being accurately discriminated, compared to 62.4%, however both regions were out-performed by full length ITS (78.4%), as expected. ITS performs well as a DNA barcode despite notable incongruence seen between the phenotype and genotype within *Cladonia;* thus, the use of an additional barcode such as *cox1* or *rpb2* is recommended for species level identification, although not essential as genus or section identifications are sufficient for assessing diet. The origin of this incongruence should be explored further as *Cladonia* contribute significantly to the biomass of the lichens in the Cairngorms, with UK BAP priority species *Cladonia botrytes* (K.G. Hagen) Willd. being found there. Incomplete lineage sorting or introgression are hypothesised as potential origins.

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1.1.1 Lichen Diversity

With 98% of lichenised fungi belonging to the phylum Ascomycota, lichens represent a polyphyletic group that highlights convergent evolution across the fungi and demonstrates the evolutionary success of the lichen ecological strategy (Gargas *et al.*, 1995; Lutzoni, Pagel & Reeb, 2001; James *et al.*, 2006; Schoch *et al.*, 2009). Lichens are most simply characterised by the symbiotic relationship between a fungus and an alga but are not limited to two organisms and may often include a third or more, including bacteria and other fungi (Grube *et al.*, 2009; Lutzoni & Miadlikowska, 2009; Spribille, 2016). However, the stable symbiosis between the heterotrophic mycobiont and autotrophic photobiont provides an ecological strategy that is responsible for the success of both organisms and those that rely on them. In particular, the ecological strategy utilised by lichens have contributed to high levels of diversity in environments which are inhospitable for most vascular plants such as montane, desert and arctic habitats (Watkinson, 2015). As a result, lichens can exploit certain severe ecological niches within temperate and artic environments and may even outcompete vascular plants within these environments; for example, *Cladonia portentosa* (Dufour) Coem. has been shown to demonstrate nitrogen translocation and recycling, allowing the mat-forming species to thrive in nutrient poor environments and giving an advantage over vascular plants (Ellis *et al.*, 2005).

There are 19,000 known species of lichen; however, there are an estimated 28,000 lichen species on Earth (Lücking, Hodkinson & Leavitt, 2017). A significant portion of these unknown species are thought to be cryptic species. Cryptic diversity in lichens consists of two or more lichen forming fungi that show significant genotypic divergence but appear morphologically identical (Molina *et al.*, 2011; Grande & Leavitt, 2015; Leavitt *et al.*, 2016). High levels of cryptic diversity within lichens increases the challenges associated with conservation efforts, as population size and fitness may be difficult to judge (Bickford *et al.*, 2007; Crespo & Lumbsch, 2010). Furthermore, due to the difficulty of identifying cryptic species, the diversity may remain undiscovered and threatened to a point where conservation efforts are prohibitive in a cost/benefit analysis. With growing concern for the effects of climate change, many species may be at risk, particularly those with a preference for specific habitats, such as montane and tundra environments (Ellis *et al.*, 2007). It is therefore a necessity to consider a multidisciplinary approach to the conservation of lichens, one that

considers the hidden and cryptic diversity while simultaneously acting to conserve lichen biodiversity.

In a European context, Scotland shows high levels of lichen diversity with over 1,500 known species due to its climate, topography and its historically low pollution loads and relatively low intensity of land management (Ellis & Coppins, 2010). The climatic gradient across Scotland gives rise to ecological niches that are filled by highly specialised lichens (Ellis & Coppins, 2010); for example, the west of Scotland has a wetter, warmer climate supporting a temperate rainforest biome rich in epiphytes including lichens. In contrast, the east of Scotland is drier, with colder winters and continental, montane communities. Climatic conditions can further contribute towards lichen diversity through defining the ecological niche of the algal photobiont and the specificity of the lichen forming fungus (Casano *et al.*, 2011) . Lichen forming fungi have been shown to both be specific in their choice of photobiont or non-specific; as a result, lichen distributions may be influenced by not only the available photobionts, but also those that provide the most benefit in a site (Yahr, Vilgalys & Depriest, 2004, 2006; Casano *et al.*, 2011).

Lichen biodiversity flourishes within the Cairngorm plateau with over 700 of the approximately 1,500 Scottish lichen species being found there (Fryday, 1997). The Cairngorm plateau, due to its topography and climate, serves as a habitat for lichen species which thrive in arctic and montane conditions (Fryday, 2001). As a result, the Cairngorm plateau is a home to a wide variety of protected, near-threatened and endangered lichen species in the UK such as *Sporastatia testudinea* (Ach.) A. Massal. and *Alectoria ochroleuca* (Hoffm.) A. Massal. (Gilbert & Fox, 1985; Fryday, 1997). Furthermore, the Cairngorm plateau represents a habitat that is not found anywhere else in the British Isles; climate change is therefore a serious concern for specialised lichens that thrive in the Cairngorm tundra habitat (Ellis *et al.*, 2007). Many lichen species within the Cairngorms may be found growing sympatrically with related species, highlighting the lichen diversity within the mountain range, notably multiple species within the genera *Cetraria* Ach., *Cladonia* P. Browne, *Ochrolechia* A. Massal. and *Umbilicaria* Hoffm. This study is motivated by the need to be able to distinguish these species as potential components of an ungulate diet, and these groups are introduced below.

1.1.2 *Cetraria*

Cetraria (Parmeliaceae; Lecanorales) is a lichen genus typified by the species *Cetraria islandica* (L.) Ach. The genus is mostly found in boreal or arctic habitats with a symbiotic association with green algal photobionts (Fernández-Mendoza *et al.*, 2011). Lichenised-fungi which show specificity in their photobiont often share the same ecology as the alga (Yahr, Vilgalys & Depriest, 2004; Fernández-Mendoza *et al.*, 2011); in the case of *Cetraria* the association with green alga is representative of the lichens' preferred habitat as they are often found in drier colder climates (Fernández-Mendoza *et al.*, 2011).

Interspecific variation within the genus also provides insight into the evolutionary adaptations of lichen chemicals. Fumarprotocetraric acid is a chemical that may be found within certain *Cetraria* species; notably it is found in *C. islandica* while being absent in *Cetraria aculeata* (Schreb.) Fr. and *Cetraria muricata* (Ach.) Eckfeldt (Gudjónsdóttir & Ingólfsdóttir, 1997). An *ex situ* experiment was able to demonstrate an added resistance to SO_2 in *C. islandica* as a result of fumarprotocetraric acid within the thallus of the lichen, providing an added pollution tolerance compared to other species within the genus that lack fumarprotocetraric acid (Hauck, 2008).

Although the type species of *Cetraria, C. islandica*, is chemically and morphologically distinct, the genus is known to show a high degree of cryptic diversity. For example, species within the *C. aculeata* group including *C. aculeata, C. muricata* and *C. steppae* (Savicz) Kärnefelt, share common morphology across all taxa and may be difficult to identify based purely on morphological characters (Nadyeina *et al.*, 2013). Furthermore, the chemical composition of lichens within the group show little interspecific variation (Lutsak *et al.*, 2017). Norstictic acid, previously being regarded as an identifiable feature of *C. steppae,* may appear in taxa within the group but holds no weight in a phylogenetic clustering analysis and now only highlights chemotypes within taxa (Nadyeina *et al.*, 2013; Lutsak *et al.*, 2017). Molecular markers ITS and *rpb2* are useful in distinguishing taxa within this group, and previous attempts at barcoding *Cetraria* have been successful in discriminating *C. islandica* from *C. ericetorum* Opiz. In that complex, morphology and an analysis of the chemical composition proved difficult in discriminating species and chemotypes (Xu *et al.*, 2018). DNA barcoding may therefore be an important tool in the species identification of *Cetraria*.

1.1.3 *Cladonia*

Cladonia is a large genus of lichen forming fungi within the Cladoniaceae (Lecanorales) with approximately 500 known species (Pino-Bodas *et al.*, 2013). The genus is abundant within temperate and arctic environments, and many species within the genus are valued for antimicrobial properties (Ranković, Kosanić & Stanojković, 2011) or as food for reindeer. Although the phylogeny of *Cladonia* is unresolved, subgeneric taxonomic groups have been established through the use molecular, chemical and morphological data. Three currently recognised Subdivisions within *Cladonia* comprise I) a basal clade consisting of *Cladonia wainioi* Savicz, II) a *Cladonia* supergroup (corresponding to Section *Cladonia sensu* DePriest *et al.*, 2000), and III) the largest third group including the bulk of the genus and at least three supergroups, including the richly branching *Cladinae* (Stenroos *et al.*, 2002). Morphologically, species within the *Cladinae* group typically have a branching podetium with squamules and scyphi being absent, in contrast to many less-branching species among other supergroups, mostly with squamules and some with scyphi at the tips of the podetium (Stenroos *et al.*, 2002; Hodgetts, 1992; Athukorala *et al.*, 2016).

An easily distinguished section within the genus *Cladonia* Subdivision III is the *Cocciferae*. The *Cocciferae* are phylogenetically supported section characterised by red hymenial disks caused by the presence of rhodocladonic acid (Stenroos *et al.*, 2002). Species within the *Cocciferae* section show similar chemical compositions between species, and the presence of usnic, barbatic, squamatic and thamnolic acids in addition to zeorin having arisen multiple times within the section (Stenroos *et al.*, 2002; Steinová *et al.*, 2013). As a result, the chemical composition within the *Coccifera*e may to some extent mirror the evolutionary history of *Cladonia* and therefore help in identifying taxa to the species level using thin layer chromatography (TLC) (Orange *et al.*, 2001). TLC is has been useful in the identification of *Cladonia* section *Cocciferae*, a group which is difficult to identify even using DNA barcoding (Kelly *et al.*, 2011; Steinová *et al.*, 2013).

Although barcoding has been successful for clarifying species boundaries and taxonomic problems in some groups of *Cladonia* (e.g. *C. foliacea* (Huds.) Willd. (Pino-Bodas, *et al.*, 2010); *C. appalachensis* Yoshim. & Sharp ex Lendemer & R.C. Harris (Lendemer & Harris, 2013, 2014)), other groups within the genus are more problematic. For example, in the *C. gracilis* (L.) Willd. (Pino-Bodas *et al.*, 2013), *C. coccifera* (L.) Willd. (Steinová *et al.*, 2013) and *C. pyxidata* (L.) Hoffm. (Kotelko & Piercey-Normore, 2010) complexes, molecular phylogenies based in large part on the fungal barcoding marker are not

congruent with morphological species boundaries. Although the reasons why DNA barcoding is difficult with *Cladonia* are as of yet unknown, hybridisation and incomplete lineage sorting are hypothesised to play a role and should be explored further within the genus (Myllys *et al.*, 2003; Steinová *et al.*, 2013).

1.1.4 *Ochrolechia*

Ochrolechia is a genus of crustose lichen fungi characterised by large apothecia, and an areolate-cracked thallus within the family Pertusariaceae (Pertusariales) (Kukwa, 2009). *Ochrolechia* is typically found in arctic or boreal habitats with a worldwide distribution. Species within *Ochrolechia* utilise a diverse range of substrates including heathland plants, rock and soil (Zhang *et al.*, 2015); despite being a crustose genus, removing well-developed *Ochrolechia* specimens from their substrate is relatively easy compared to other crustose lichens, this has led to *Ochrolechia* spp. being a choice food resource for a variety of arctic/boreal fauna (Bergerud, 1972; Lawrey, 1980; Bokhorst *et al.*, 2007; Joo *et al.*, 2014).

Morphologically, identification of *Ochrolechia* may prove difficult as many of the key identifiable characters of taxa are shared amongst other species within the genus (Kukwa, 2009). As a result, secondary lichen metabolites and the chemical composition of the lichen are more valuable from a taxonomic perspective, particularly on the intrageneric level, such as the absence of gyrophoric and presence of variolaric acids characteristic of *Ochrolechia szatalensis* Vers., for example (Kukwa, 2009). Furthermore, the use of molecular methods have shown a high level of species discrimination when identifying *Ochrolechia* which may be a more reliable method of identifying *Ochrolechia* species (Kelly *et al.*, 2011; Joo *et al.*, 2014).

1.1.5 *Umbilicaria*

The genus *Umbilicaria* (Umbilicariaceae, Umbilicariales) is typically foliose with a central holdfast, known as the umbilicus, and is found across a wide geographical range that encompasses both the northern hemisphere and southern hemisphere (Ivanova *et al.*, 1999; Smith *et al.*, 2009). The group is largely found on rocks within arctic, boreal, temperate and montane habitats (Llano, 1956; Ivanova *et al.*, 1999).

Species within *Umbilicaria* show a high degree of extremophilic adaptations which have contributed to the success of the species in arctic habitats. Species such as *Umbilicaria cylindrica* (L.) Delise ex Duby have previously shown adaptations to harsh arctic environments through their photosynthetic activity which may operate in sub-zero temperatures, with full inhibition of photosynthetic activity only occurring at -30°C (Hájek *et al.*, 2016).

Taxonomically, *Umbilicaria* spp. can be difficult to distinguish from other species on an intrageneric level based on morphology alone; although some species within the genus are distinct in their morphology such as *Umbilicaria proboscidea* (L.) Schrad., species such as *U. polyphylla* (L.) Baumg. and *U. nylanderiana* (Zahlbr.) H. Magn. are almost indistinguishable through morphology (Posner, *et al.*, 1992). However, although the secondary product chemistry of the genus is not widely used to identify species due to the occurrence of gyrophoric acid seen in most species, it is an effective means of resolving taxonomic issues within the genus due to key identifiable chemicals such as umbilicaric, lecanoric and norstictic acids (Posner, *et al.*, 1992). Furthermore, the complete absence of secondary chemistry may be a useful identifiable character of species such as *Umbilicaria cylindrica*. Molecular analyses of *Umbilicaria* have had great success in resolving taxonomic issues within the genus while using the ITS locus (Ivanova *et al.*, 1999). Therefore, DNA barcoding using the ITS region may be an effective method in species discrimination of *Umbilicaria.*

1.2 Genetic Barcoding

1.2.1 What is Barcoding?

DNA barcoding is the process of sequencing a standardised locus of around 500 to 800 base pairs in an organism's DNA and utilising that sequence as a genetic marker to accurately identify conspecifics (Hebert, Cywinska, *et al.*, 2003). For effective barcoding to occur, comparison species and their associated barcode must be established within a reference library using a standardised gene region across all taxa (Kelly *et al.*, 2011). It is imperative for the species used in forming the reference library to be identified accurately and expertly with the voucher specimen from which the DNA was sourced being stored and accessible within a biological collection such as a herbarium with sequences and chromatograms being available online (Schoch *et al.*, 2012). Selected gene regions must show enough interspecific variation between taxa to discriminate between sequences and accurately identify species while

simultaneously showing little intraspecific variation to ensure all conspecifics are grouped together accurately (Hollingsworth *et al.*, 2009). When interspecific variation is greater than intraspecific variation a barcode gap is formed, therefore indicating a distinction between taxa enough to discriminate between species (Schoch *et al.*, 2012).

Due to the requirements for a small but specific gene region to act as an efficient barcode, there has been an effort to determine universal barcode regions within the three most species rich groups of eukaryotes, animals, plants and fungi. Initially the mitochondrial DNA region *cytochrome c oxidase 1* (*cox1*) region was established as the universal barcode for all living organisms due to the success of barcoding animals and protists using the *cox1* gene region (Hebert, Ratnasingham, *et al.*, 2003). Although the use of a universal barcode would be efficient, it is not practical due to differing rates of evolution between groups (Brirten, 1984; Wolfe *et al.*, 1987). The *cox1* region does not demonstrate suitable species discrimination within plants and is difficult to amplify while operating sub-optimally compared to other loci in fungi (Hollingsworth *et al.*, 2009; Schoch *et al.*, 2012). Thus, standard barcodes for the three main groups of eukaryotes have been established, *cox1* serving as the standard barcode for animals and protists, a concatenated sequence of plastid DNA regions *maturase K* (*matK*) and *RuBisCO large subunit* (*rbcL*) (Hollingsworth *et al.*, 2009) and the nuclear ribosomal DNA region the internal transcribed spacer (ITS) for fungi (Schoch *et al.*, 2012). Although these are accepted as standard barcode regions, there are still groups within animals, plants and fungi that are not discriminated using the them; for example, a better performance in species identification may be achieved when using ITS in combination with any plastid DNA region in land plants (Li *et al.*, 2011), whereas multilocus sequence typing has better discriminatory power among some fungi (Yahr, Schoch & Dentinger, 2016). Nevertheless, because of its high species discrimination power and relatively easy amplification, the ITS region shows high levels of success as a DNA barcode in a wide variety of groups within the fungi, including across lichens.

1.2.2 The Barcode Gap

The barcode gap utilises pairwise distances to determine the genetic differences between taxa and may be an effective tool in establishing the molecular species limit of a taxon (Meyer and Paulay, 2005). The barcode gap is established when intraspecific genetic

distance is less than the interspecific distances, thus delimiting between species whilst simultaneously accounting for intraspecific variation (Meyer & Paulay, 2005).

The extent of genetic variation between sequences may be measured through the pairwise distance which provides a means of determining whether two sequences are conspecific. There is a tendency within the literature to use the Kimura-2-parameter (K2P) (Kimura, 1980) to measure the pairwise distance between sequences, however the uncorrected (p) distance has been shown to have a higher yield of successful identifications within barcoding analyses, and K2P has been demonstrated as a suboptimal model in determining pairwise distances (Srivathsan & Meier, 2012). Generally, taxa in which the pairwise distance is ≤ 3% are accepted as conspecific (Hughes, *et al.*, 2009). For the barcoding of Fungi using ITS the threshold of ≤ 3% is mostly used throughout the literature (Begerow *et al.*, 2010). However, the 3% threshold was established when barcoding bacterial DNA from the SSU and may not be representative of the group in question, in some instances 3% may be too high as seen in *Aspergillus* Micheli (Nilsson *et al.*, 2008) or too low as seen in the genus *Cantharellus* Fr. where species such as *Cantharellus cibarius* Fr. show a high degree of intraspecific variation in the length of the ITS region (Feibelman, *et al.*, 1994). Thus 3% is not an effective universal threshold for genetic variation in fungal conspecifics (Bruns, Arnold & Hughes, 2008). Nonetheless, the 3% variation threshold is fairly consistent within Fungi, particularly when looking at the ITS region, and for the most part, is an accurate representation of intraspecific variation, even if taxa exist outside of the threshold (Nilsson *et al.*, 2008).

1.2.3 ITS as the Standard Fungal Barcode

The ITS is a gene region of predominantly non-coding nuclear ribosomal DNA, nestled in between the small subunit (SSU) and the large subunit (LSU), that may be partitioned into three sub-regions: ITS1, 5.8S and ITS2 (Figure 1.) (Schoch *et al.*, 2012;

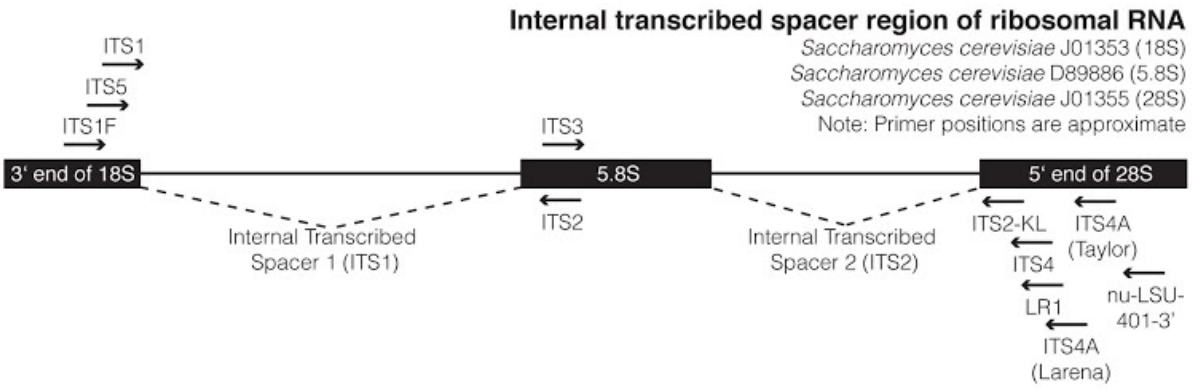


Figure 1. ITS gene regions and primer locations, obtained from Nelsen (2018).

Nelsen, 2018). The 5.8S locus is conserved and as a result does not produce adequate results in either phylogenetic or barcoding analyses due to a lack of variability and informative characters (Wang *et al.*, 2015). In contrast, both ITS1 and ITS2 as spacer regions show an enhanced rate of evolutionary change due to the region not providing a sequence-specific function, which provides both regions with enough informative characters for use in phylogenetic and barcoding analyses (Hillis & Dixon, 1991; Wang *et al.*, 2015). The ITS region has demonstrated success in a wide variety of eukaryotes including animals, plants and fungi (Kelly *et al.*, 2011; Wang *et al.*, 2015). ITS is outperformed by other loci in the majority of plant and animal taxa; however, the region can serve as an effective standard barcode within fungi, outperforming other gene regions such as the SSU, the LSU and *rpb1* (Schoch *et al.*, 2012). Although the ITS region is considered the standard, and it outperforms most loci in fungal barcode analyses, the region is not the most optimal within certain groups of fungi. For example, Ascomycotous yeasts and the Glomeromycota are more effectively barcoded through a two marker barcoding system utilising the LSU alongside ITS (Schoch *et al.*, 2012).

The ITS region of fungal genomes from complex (e.g. symbiotic) samples can be targeted by using specific primers (Gardes & Bruns, 1993). A primer is a short DNA strand that acts as a starting position for the synthesis of DNA; a specific primer will a consist of a nucleotide sequence that matches the sequence of the target group limiting the possibility of cross contamination (Gardes & Bruns, 1993). The use of the fungal specific primer ITS1F allows for novel applications when sequencing the DNA of fungi. Notably, the ITS1F primer can allow for the sequencing of DNA from mycorrhizal or epiphyllous fungi without sequencing DNA from the host plant (Gardes & Bruns, 1993) or allow for the sequencing of lichen forming fungi or fungi with associations with algae without contamination from the alga itself (Zuccaro *et al.*, 2008; Wornik & Grube, 2010).

1.2.4 Barcoding and its Role in Species Discrimination

Although barcoding may have its limitations when addressing certain groups of organisms, provided a standard gene region is used, barcoding may support novel applications of DNA identifications and play a significant role within taxonomic and conservation research. An example of this may be seen in the identification of hidden diversity through environmental samples and cryptic species, an application quite commonly used in fungi (O'Brien *et al.*, 2005; Balasundaram *et al.*, 2015) and may be seen in the genus *Inocybe*

(Ryberg *et al.*, 2008). This application may allow for the identification of new species and contribute to current understandings of fungal diversity, which is estimated to consist of 1.5-5 million species, with approximately 100,000 named (Yahr, Schoch & Dentinger, 2016).

An important criticism of the barcoding approach to identifying the species content of an environmental sample comes from the saturation of sequence databases such as GenBank with "dark taxa"; a taxon only recognised by its DNA sequence, with no voucher specimen nor with an acknowledged binomial associated with the taxon (Page, 2011; Yahr, Schoch & Dentinger, 2016). Furthermore, conflating genetic barcodes with taxonomy may allow species to be named based on the barcode without taking into account intraspecific variation or variation within the whole genome (Krishnamurthy & Francis, 2012).

Further novel applications of have been utilised in the conservation of species, notably in the application of monitoring wildlife and endangered species within illegal trade. DNA barcoding has previously been applied in identifying the illegal trade of shark fin soup sourced from endangered shark species (Fields *et al.*, 2015), identification of endangered plant species used in herbal remedies (Veldman *et al.*, 2014) and other applications such as the forensic identification of endangered *Taxus* spp. L. being used to make wooden products (Liu *et al.*, 2018). Another application of DNA barcoding in conservation includes dietary analysis, a technique that may be applied to conserve both the animal in question and food species (De Barba *et al.*, 2014).

1.2.5 Dietary Analysis

DNA metabarcoding may be applied in a dietary analysis to accurately identify what species an animal is eating and to generate an idea of trophic interactions within an ecosystem (De Barba *et al.*, 2014). Within a dietary analysis it is imperative for primers to be specific, particularly in a carnivorous or omnivorous species, to ensure that only the DNA of food species are sequenced (De Barba *et al.*, 2014; Su *et al.*, 2018). The analysis is cited as a non-invasive measure of determining an animal's diet through the sequencing of faecal DNA or gut contents of the animal and comparing the sequence against a reference barcode library of possible food species to identify exactly which species are used as a food source (Deagle *et al.*, 2010; Joo *et al.*, 2014). Therefore, a barcoding dietary analysis may provide information vital for the conservation of the animal species in question but may also provide an insight to the conservation of species used as a food source (Newmaster *et al.*, 2013; De Barba *et al.*,

2014; Srivathsan *et al.*, 2015). Furthermore, additional novel applications of dietary barcoding analyses are being utilised to understand conservation and disease transmission within species. Notably, metabarcoding dietary analyses have been utilised to determine the diet and any intestinal parasite infestations within the endangered species *Pygathrix nemaeus* L. (Srivathsan *et al.*, 2015). Additionally, dietary analyses have been used to determine the diet of the common vampire bat, *Desmodus rotundus* Geoffroy, a vector of the lethal rabies virus, information gathered from the dietary analysis may be applied in understanding the ecology and the risk of disease transmission associated with the species (Bohmann *et al.*, 2018). Furthermore, DNA barcoding has allowed an insight into ecosystem dynamics through dietary analyses particularly concerning niche partitioning and the resource exploitation of large herbivorous mammals that share a habitat. Indicating that different ecological niches were associated with different diets even amongst large herbivorous mammals in the same environment (Kartzinel *et al.*, 2015).

DNA barcoding is an effective method of discriminating the species composition of a faecal sample and remains a consistent and accurate method of identifying food species to the species or genus level (Newmaster *et al.*, 2013). Where most of the conservation concern from dietary analysis studies falls on the animal in question, a serious consideration should be taken for the food species within the trophic system. For example, overgrazing from large herbivorous species in habitats with an incomplete trophic system, i.e. an ecosystem missing an apex predator, can have serious implications for nutrient availability and for the population health of plant and fungal species (Morris & Letnic, 2017).

1.3 Reindeer Diet in the Cairngorms: Can Fungal Barcoding Diagnose Lichen Species?

Reindeer, *Rangifer tarandus tarandus* L. were historically part of Scotland's fauna, however due to climatic changes and hunting, the presence of *R. tarandus* in Scotland has dwindled since the last ice age and all current populations are a result of reintroduction from Scandinavian countries. Despite the changes in climate, the Cairngorm plateau provides a habitat in which populations of *R. tarandus* may thrive and since 1952 reindeer have been integrated into the Cairngorm ecosystem and economy, drawing in an average of 20,000 visitors per year. Although the Cairngorm reindeer are monitored by herders, they are allowed to roam the Cairngorm plateau freely, yet very little is known about the ecological impacts of the established reindeer herd. The diet of the herd while free roaming raises concern for the

conservation of endangered and protected lichen species within the Cairngorm plateau that are already under pressure from climate change (Ellis *et al.*, 2007).

Lichenised fungi are known to contribute towards the diet of *R. tarandus*, particularly in the winter months where lichens form a staple of the reindeer diet (Newmaster *et al.*, 2013; Joo *et al.*, 2014; Bergerud, 1972). *Rangifer tarandus* are one of the few animal species in which the enzyme lichenase may occur in the gut; the enzyme will break down lichenin, a starch found in many common lichen species (Kochan, 2006). With over 150 reindeer in the Cairngorms a significant amount of lichen biomass would be required to sustain the herd during the winter months. Furthermore, 700 of the 1,500 known lichen species of Scotland are found in the Cairngorm plateau, a variety of which are endangered or, within the UK, are only found there (Gilbert & Fox, 1985; Gordon *et al.*, 1998; Fryday, 2001).

Metabarcoding dietary analyses of *Rangifer tarandus* have previously shown to be effective at identifying the diet of *R. tarandus* with high levels of resolution and food species identification (Newmaster *et al.*, 2013; Joo *et al.*, 2014). A dietary analysis of the Cairngorm reindeer herd would give insight into which lichen species are being targeted as a food source and whether there is a conservation concern for those lichen species. In preparation for the dietary analysis, a reference barcode library using voucher specimens and sequences sourced from the Cairngorm plateau is required to ensure local haplotypes are included within the reference library. Furthermore, before the dietary analysis a consideration for the potential food species is required. Certain sections within the genus *Cladonia*, namely section *Cocciferae,* are notoriously difficult to barcode and a barcoding analysis may only provide an identification to the group (Pino-Bodas *et al.*, 2013; Steinová *et al.*, 2013; Kanz *et al.*, 2015).

Therefore, using the ITS region as a barcode, four lichen genera *Cetraria, Cladonia*, *Ochrolechia* and *Umbilicaria* will be tested for accurate species discrimination using DNA barcoding. The aim of the study will be to determine if species within the target genera are accurately discriminated from each other through the presence or absence of a barcode gap, by using the uncorrected (p) distances between taxa to determine genetic divergence between species and conspecifics, if the largest intraspecific distance is less than the smallest interspecific distance a barcode gap will be present, which is what we expect from biological species that are evolving independently. Furthermore, the study shall aim to compare the accuracy of species discrimination when using only ITS1, only ITS2 and finally using the full ITS region as a barcode to determine if one specific sub region is more effective in

discriminating problematic species and whether an ITS sub region may act as a barcode for lichenised fungi. A local BLAST database will be created from the whole ITS dataset; by dividing the ITS sequences into sub regions a BLAST search will be utilised testing the full ITS region and the ITS1 and ITS2 sub-regions as queries. If the top hit result once the query sequence has been removed is a conspecific the identification will be deemed as successful. Correct species identification will therefore determine the efficacy of species discrimination in ITS and the sub-regions for the target genera. The data from this thesis will be used as a cornerstone for a reference database as part of a larger, longer-term project that shall utilise video recording equipment alongside DNA barcoding to understand reindeer diet from free-ranging animals.

2. Materials and Methods

2.1 Taxon Sampling

All taxa were sampled from the Cairngorm mountain range in Scotland (ordinance survey grid reference NH and NO); taxa exempt of this prerequisite, notably *Cladonia stellaris* (Opiz) Pouzar & Vězda, *C. stygia* (Fr.) Ruoss and one sample of *C. rangiferina (L.)* Weber ex F. H. Wigg., did not originate from the Cairngorms and were instead sourced from a commercial reindeer feed of unknown origin, but presumed to be from Finland. The final dataset consisted of 75 separate collections of macrolichens from the area in which the Cairngorm reindeer herd roams, with significant diversity within the genera *Cetraria, Cladonia, Umbilicaria,* and the large crusts from the genus *Ochrolechia,* alongside sequences from elsewhere in Scotland from Kelly (*et al.,* 2011) and sequences from Scotland, Iceland and mainland Europe from Steinová (*et al.,* 2013).

Lichens which were abundant, foliose, fruticose or showed signs of grazing were initially targeted, crustose lichens were generally avoided due to the low likelihood of reindeer grazing on these species, however an exception was made for crustose species in the genus *Ochrolechia,* as they may easily be removed from the substrate and are known as important parts of reindeer diets in other regions (Joo *et al.,* 2014).

Only the genera in which multiple species with multiple samples were taken forward to test for lichen barcoding success. To represent the intraspecific variation of lichens within the reindeer's range at least three specimens from each species were collected if available (Kelly *et al.,* 2011). In the absence of three specimens per species, sequences sourced from the Cairngorms were taken from extant datasets (Kelly *et al.,* 2011; Steinová *et al.,* 2013) to ensure intraspecific variation was considered in all target species (Mark *et al.,* 2016).

All voucher information including EDNA number and GenBank accession number for all taxa used in the molecular analysis may be found in Appendix 2.

2.2 Identification

2.2.1 Initial Identification

Initial identification of the lichen occurred in the field at the time of collection based on prior knowledge and experience. Identifications were often made to the species level but in many instances only to genus. Identification was then confirmed, corrected or identified

further to species for all samples through the use of keys from the British Lichen Society (Smith *et al.*, 2009) and through the lichen field guide by Dobson (2011).

2.2.2 Thin Layer Chromatography

Lichen identifications were then confirmed or corrected using thin layer chromatography (TLC) to isolate the chemical composition of the lichen using the protocol outlined by Orange (*et al.*, 2001) with minor adjustments. Samples from all lichen collections were taken and left in an Eppendorf tube where three drops of acetone were then added. The sample was then left in acetone for 5 minutes to allow for the chemicals within the lichen to be extracted. Care was taken to ensure that there were no mixtures of species and that all samples were dry prior to the chemical extraction. A control sample was created from *Cladonia subcervicornis* (Vain.) Kernst. and *Pertusaria pseudocorallina* (Lilj.) Arnold which provided fumarprotocetraric acid, atranorin (*C. subcervicornis*) and norstictic acid (*P. pseudocorallina*) (Orange *et al.*, 2001). All chemical samples were run on glass TLC plates in solvent systems A (toluene/1, 4-dioxane/ acetic acid, 90:30:4) and G (toluene/ ethylacetate/ formic acid, 69.5:41.5:4).

The plates were examined and had all colours and position of pigments noted under daylight, short wave UV and long wave UV. New spots were carefully marked as they appeared using the parameters and marking system from Orange (*et al.*, 2001), including for hydrophobic compounds in water and after application of acid and charring. All plates were scanned and monitored for any colour changes that may occur while using the scan for comparison.

Identifying the substance isolated after TLC required calculation of R_f of the substance in both plates. The R_f is representative of the distance travelled by the substance relative to the solvent. Using the control substances and their known R_f values (Table 1.), the R_f values of the other spots were determined through the following equation and were used to identify the compound (Orange *et al.*, 2001).

$$\text{Relative } R_f \text{ of spot} = \frac{\text{Distance of spot from the baseline}}{\text{Distance of chosen standard from the baseline}} \times \text{Standard } R_f \text{ of chosen control}$$

The lichens were then identified to the species level using keys detailing lichen chemistry (Smith *et al.*, 2009; Dobson, 2011) and known chemistry information for *Umbilicaria* (Posner, Feige & Huneck, 1992) and *Ochrolechia* (Kukwa, 2009).

Table 1. Standard R_f values of control compounds from Orange *et al.* (2001).

Compound	R_f in solvent system A	R_f in solvent system G
Atranorin	75	90
Norstictic acid	40	57
Fumarprotocetraric acid	1	36

2.2.3 Molecular Identification

After the morphological and chemical assessment of all taxa had occurred, specimen identifications were confirmed through an NCBI BLAST search. In the event of a false identification, the chemical and morphological data was reviewed and examined in conjunction with the BLAST result to determine an accurate identification.

2.3 Molecular Techniques

2.3.1 DNA extraction

All samples for DNA extraction were assigned a unique accession number in the Edinburgh DNA (EDNA) database. The Qiagen Plant DNeasy kit (Qiagen, Germantown, MD, USA) and the protocol recommended by Qiagen were used for all DNA extractions with minor adjustments being made to accommodate fungal tissue (QIAGEN, 2012). Fresh lichen samples were prepared for extraction by removing a small fragment, not exceeding 20 mg in dry weight, and adding it to an Eppendorf tube alongside a pinch of acid-washed sand. Tweezers were cleaned with 70% ethanol and held in a flame to reduce the risk of contamination in between samples. Lichen material was homogenised using a mini-pestle and 200 µl of Buffer AP1 which had been heated to 65°C in a water bath. After the lichen material had sufficiently been ground with no visible material remaining, 4 µl of RNase A stock solution and 200 µl of Buffer AP1 were then added, the mixture was then incubated for 1 hour at 65°C in a Thermomixer set at 800rpm to lyse the cells.

For elution, 75 µl of Buffer AE was applied directly onto the membrane of the DNeasy Mini spin column and incubated at room temperature (15-25°C) for 5 minutes. The column was then centrifuged for 1 minute at 8,000rpm to elute the DNA from the membrane. The flow-through was then collected and added back onto the DNeasy Mini spin column and centrifuged again for 1 minute at 8,000rpm to increase the DNA concentration from the eluate.

Once the DNA had been extracted, it was stored at -20°C until required for use in the polymerase chain reaction (PCR). All samples were put in the Edinburgh DNA bank following sequencing.

2.3.2 Polymerase Chain Reaction (PCR)

DNA amplification reactions of 12.5 µl total volume included utilising 4.77 µl of ddH_2O, 0.2mM of dNTPs, 1x buffer (NH_4), 2.4M of $MgCl_2$, 0.4 µM of both the forward and reverse primer, 2.5 µl of 5x TBT-PAR, 0.05 U/µl of Taq and 1 µl of genomic DNA template per sample. A fungal specific primer was used to prevent the sequencing of any algal DNA from the lichen photobiont (Gardes & Bruns, 1993). Information regarding the primers utilised in this study may be found in Table 2.

The PCR initiated with DNA template denaturation at 95°C for 4 minutes before 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 90 seconds and extension at 72°C for 90 seconds before a final extension at 72°C for 10 minutes. PCR success was then checked using electrophoresis on 1% agarose gel stained with SYBR Safe (Invitrogen/Life Technologies, Carlsbad, CA, USA) and visualised through a Syngene G: BOX F3 Fluorescence Imaging System (Invitrogen/Life Technologies).

Table 2. Information on the primers used in this study. Fungal specific primers are indicated with an asterisk.

DNA region	Primer	Direction	Primer Sequence (5'-3')	Reference
ITS	ITS1F*	Forward	CTTGGTCATTTAGAGGAAGTAA	(Gardes & Bruns, 1993)
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	(White *et al.*, 1990)

2.3.3 Sequencing PCR

Following the PCR, 5 µl of PCR product was purified using 2 µl of ExoSAP IT (GE Healthcare) before being incubated at 37°C for 15 minutes then heated at 80°C for 15 minutes to inactivate the ExoSAP IT enzymes. After purification, the sample was sequenced using 6.68 µl of ddH_2O, 2 µl of 5x buffer, 0.32 µl of primer (10 µM), 0.5 µl of BigDye and 0.5 µl of purified PCR product per sample for both forward and reverse primers. PCR programmes consisted of 25 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 20 seconds and extension at 60°C for 4 minutes before being incubated at 4°C. Sequenced PCR product was then sent to Edinburgh Genomics.

2.4 Sequence Editing and Alignment

Using Sequencher v5.4.6 (Gene Codes Corporation) new sequences were trimmed of poor quality sequence data before forward and reverse sequences were aligned and manually checked and edited. Sequence data from Scottish *Cetraria*, *Cladonia*, *Ochrolechia* and *Umbilicaria* samples from Kelly *et al.* (2011) and from Northern European, Icelandic and Scottish *Cladonia* samples from (Steinová *et al.*, 2013) were added to the lichen dataset. The genera were grouped together independently before each genus dataset was aligned using MAFFT v7.402 (Katoh, Rozewicki & Yamada, 2017) to ensure species were aligned with only closely related species. Alignments were then manually checked, edited and the sub-region 18S, ITS1, 5.8S, ITS2 and 28S were annotated using Mesquite v3.51 (Maddison & Maddison, 2018).

2.5 Species Determination Through Barcoding

2.5.1 BLAST Based Identification of ITS and the sub-region ITS1 and ITS2

To determine whether a sub region could act as an effective barcode a local BLAST database was created using Geneious v 11.1.4, the database consisted of the full ITS sequence data from the dataset. The ITS region and sub-region were individually compared to the local BLAST database providing an identification for the whole ITS region, ITS1 and ITS2 for each sample. Identifications were considered successful if the top result was from a conspecific while excluding hits from the query sequence. Top results were filtered to include only those with the largest percent of query cover and percentage of pairwise identity.

2.5.2 Barcoding Analyses

Barcode gap analysis was performed using the pairwise distance between sequences gathered from the uncorrected (p) distances between all taxa (Srivathsan & Meier, 2012). The largest intraspecific distance was plotted against the smallest interspecific distance over the mean interspecific distance *sensu* Meier, *et al.*, (2008). A barcode gap was determined to be present if the largest intraspecific distance was less than the smallest interspecific distance. The frequency of intraspecific and interspecific genetic distance values were also noted and compared in each genus to determine the range of genetic divergence within conspecifics and to further investigate the barcode gap within the four genera (Pino-Bodas *et al.*, 2013). Sufficient sequences of European subspecies and varieties were not available form sampled taxa and from GenBank; therefore, taxa were grouped together based on their species level identification, regardless if the specimen had been identified further to subspecies or variety.

3. Results

3.1 Thin Layer Chromatography and Lichen Chemistry

All lichen compounds isolated through TLC may be seen in Table. 3. Isolated chemical compounds were mostly accurate when compared to the lichen compounds associated with each species in Smith *et al.* (2009), Posner, *et al.* (1992) and Kukwa (2009) with minor inconsistencies from sampled taxa and across keys. Notably, chemical composition of the sampled *Umbilicaria* spp. were not as accurate when compared with Smith *et al.*, (2009) but were more accurate with Posner, *et al.* (1992) who acknowledged umbilicaric acid in *U. polyphylla* and gyrophoric acid in *U. torrefacta* (Lightf.) Schrad. Further discrepancies include the absence of lichesterinic and protolichesterinic acids in *Cetraria aculeata* as noted in Smith *et al.* (2009) although the species is noted as having a similar composition to *C. muricata* in Kukwa (2009) which is consistent with the result.

With the *Cladonia* chemistry the only results inconsistent with Smith *et al.* (2009) were from the *C. gracilis* (Collection no. 056) and *C. ciliata* Stirt. (021) samples. The *C. gracilis* sample showed usnic acid and did not show fumarprotocetraric acid, whereas the *C. ciliata* sample initially failed to produce any compounds. However, other taxa on plates E619 and E620 that were expected to contain fumarprotocetraric acid failed to do so and thus were repeated on plate E625, where the *C. ciliata* sample produced fumarprotocetraric acid and unexpectantly atranorin. Due to this unexpected result, all *Cladinae* specimens were exposed to a para-phenylenediamine test and colour changes were noted to determine the presence of fumarprotocetraric acid. The identification for the *C. gracilis* and *C. ciliata* samples were therefore based on molecular data in a BLAST search alongside morphological and spot test data. Samples of *C. ciliata* appear variable as the varieties *C. ciliata var. ciliata* Stirt. and *C. ciliata var. tenuis* (Flörke) Ahti show distinct chemotypes, with *C. ciliata var. ciliata* containing fumarprotocetraric acid and *C. ciliata var. tenuis* containing usnic acid, a varied chemical composition was therefore expected within the species (Smith *et al.*, 2009).

Table 3. Chemical composition of the lichen species gathered for this study. Chemical presence is indicated with "+" or "+/-" if sometimes present.

Genus	Species	Number of Specimens	Substances Atranorin	Norstictic acid	Usnic acid	Squamatic acid	Zeorin	Porphyrilic Acid	Barbatic Acid	Perlatolic acid	Fumar-protocetraric acid	Gyrophoric acid	Umbilicaric acid
Cladonia	*C. arbuscula*	7			+								
	C. bellidiflora	3			+	+							
	C. ciliata var. ciliata	1	+								+		
	C. ciliata var. tenuis	1			+								
	C. coccifera	1			+		+	+					
	C. diversa	1			+		+	+					
	C. floerkeana	3							+				
	C. gracilis	1			+								
	C. portentosa	3			+					+			
	C. rangiferina	4	+								+		
	C. squamosa	1				+							
	C. stellaris	3			+								
	C. stygia	2	+								+		
	C. uncialis	3			+	+							
Cetraria	*C. aculeata*	5											
	C. islandica	4									+		
	C. muricata	5											
Ochrolechia	*O. androgyna*	1										+	
	O. frigida	4										+	
Umbilicaria	*U. cylindrica*	1											
	U. polyphylla	1										+	+
	U. proboscidea	1		+								+	
	U. torrefacta	3										+	

3.2 DNA Sequencing and Amplification

Of the 75 specimens collected only 11.4% of fresh samples failed to amplify using the ITS1F and ITS4 primers. All DNA sequences that were successfully amplified were of high quality. Notably, five specimens failed to amplify, *Cladonia stellaris* collection numbers 001 & 005, *C. stygia* 002 & 073, and *C. rangiferina* 074, were from the Finnish commercial reindeer feed with an unknown collection date. These samples likely failed due to the conditions they were stored in as they were stored in damp bags for extended periods of time and were not fresh. Other samples which proved difficult to amplify include *Cladonia squamosa* (Scop.) Hoffm. 027 & 032, amplification for these specimens either failed or were of poor quality despite coming from fresh material and were thus excluded from the dataset.

3.3 Species Discrimination

3.3.1 BLAST Based Identification

When using the local BLAST database 78.4% of species were correctly identified while using the full ITS region (Table 4). Notably, a large portion of species which were identified incorrectly using the local BLAST database were species within the *Cladonia coccifera* aggregate or section *Cocciferae*, including samples of *C. borealis* Stenroos, *C. coccifera*, *C. diversa* Asperges ex Stenroos and *C. floerkeana* (Fr.) Flörke. Of all *Cladonia* species only 70.2% were accurately identified to the species level, however all were identified to the genus level and those within the *Cocciferae* section were identified to another species within the group. Other genera showed greater success using the full ITS region with identifications of *Cetraria* at 100%, *Ochrolechia* at 92.9% and *Umbilicaria* at 92.3%.

Table 4. Local BLAST based identification success of the ITS region and sub-region in identifying species.

	ITS	**ITS1**	**ITS2**
Total Percentage of Correct Identification (%)	78.4	77.6	62.4
Cladonia Identification (%)	70.2	66.7	48.8
Cetraria Identification (%)	100	100	78.6
Ochrolechia Identification (%)	92.9	100	92.9
Umbilicaria Identification (%)	92.3	100	100

Using only the ITS1 region the identification rate against the local BLAST database was 77.6%. *Cladonia* had less identification success while using ITS1 with only 66.7% of identifications resulting in a hit with a conspecific; ITS1 was still capable of identifying *Cladonia* to the genus and similarly to the full ITS region most misidentifications were of the *C. coccifera* aggregate. The other genera demonstrated a greater percentage of identifications with the percentage of correct identifications of *Cetraria, Ochrolechia* and *Umbilicaria* species reaching 100%.

ITS2 provided the poorest percentage of successful identifications with only 62.4% of species being accurately identified. Furthermore, *Cladonia* and *Cetraria* showed a poorer percentage of correct identification than they had with ITS1 and ITS, with *Cladonia* at 48.8% and *Cetraria* at 78.5%. Notably, while using ITS2 some sequences were misidentified to genus such as *Cetraria sepincola* (Ehrh.) Hale (FR799152.1) with the top result being *Cladonia uncialis* (L.) Weber ex F. H. Wigg. (EDNA18-0051623). However, *Ochrolechia* and *Umbilicaria* showed a high degree of identification success using ITS2 with *Ochrolechia* at 92.9% and *Umbilicaria* at 100%.

3.3.2 Barcoding Gap Analysis

Of all the species utilised in the dataset only 62.5% demonstrated a barcode gap with a comparison between the maximum intraspecific uncorrected (p) distance and minimum interspecific distance across all specimens. However, on an intrageneric level 75% of *Cetraria* spp., 37.5% of *Cladonia* spp., 75% of *Ochrolechia* spp. and 100% of *Umbilicaria* spp. (Figure 2.) demonstrated a barcode gap while using the uncorrected (p) distances. Notably, *Cetraria aculeata, Cladonia arbuscula* (Wallr.) Rabenh., *C. borealis, C. ciliata, C. coccifera, C. diversa, C. floerkeana, C. squamosa* and *Ochrolechia androgyna* (Hoffm.) Arnold failed to demonstrate a barcode gap.

Furthermore, the frequency of genetic distances was measured to further analyse the presence of a barcode gap (Figure 3.). A distinct overlap may be seen in both intraspecific and interspecific distance frequencies in *Cladonia* further acknowledging the lack of a barcode gap seen within most of the sampled *Cladonia* species. Notably, on multiple occasions sequences belonging to species of the *Cladonia coccifera* aggregate such as *C. coccifera* and *C. diversa,* demonstrated interspecific distances of 0% indicating that the samples shared the same haplotype (Figures 2 & 3). Intraspecific variation within *Cetraria* appears to range from

0-3% genetic divergence and a barcode gap is formed despite interspecific genetic divergences starting as low as 3%. Both *Ochrolechia* and *Umbilicaria* demonstrate intraspecific divergence in a 0-3% range with few outliers; with the interspecific divergences being as high as 12% in *Ochrolechia* and from 5.2% in *Umbilicaria*.

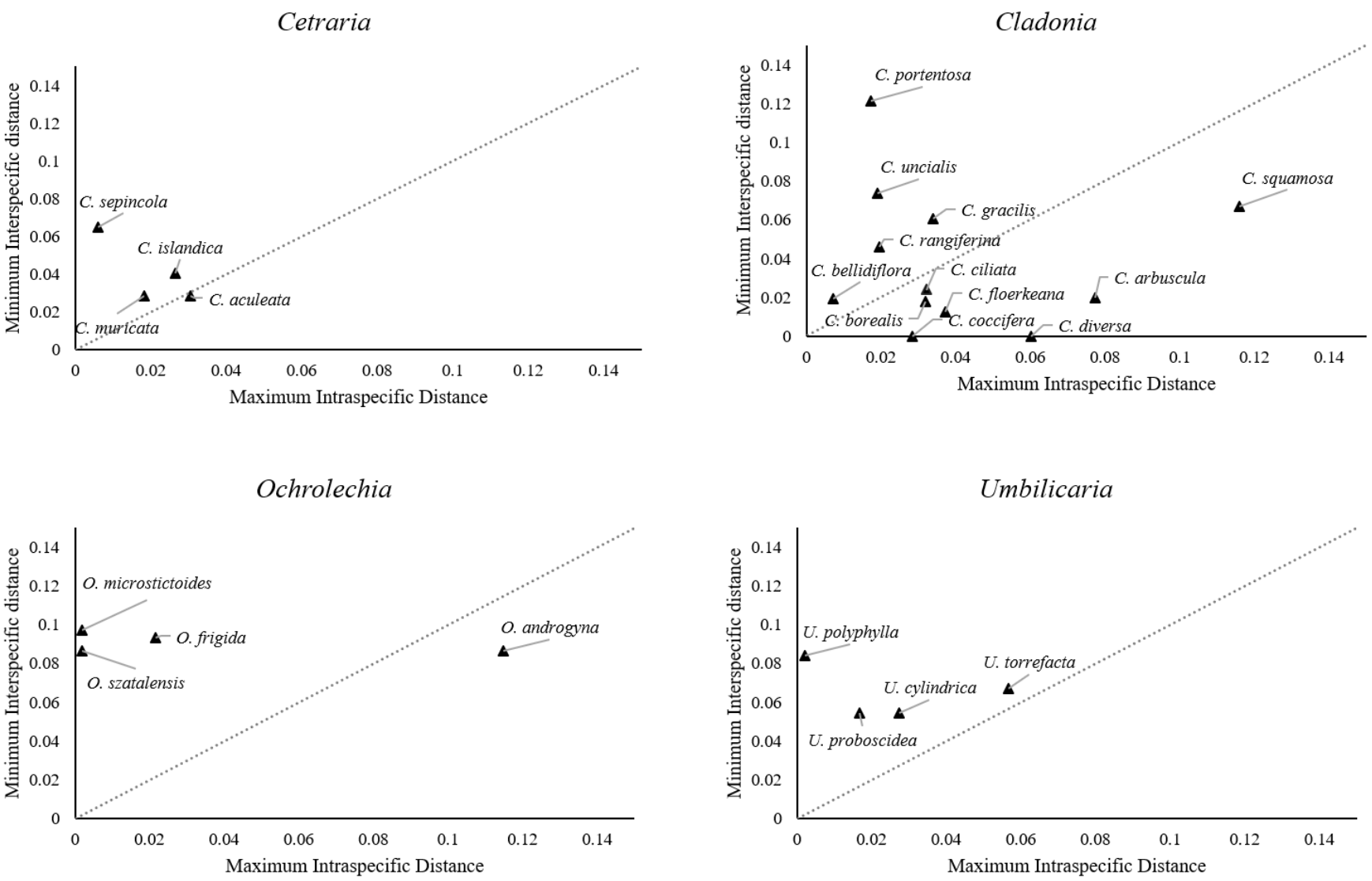


Figure 2. Barcode gap analysis of all genera using uncorrected (p) distance. Species which fall above the 1:1 line demonstrate a barcode gap as the maximum intraspecific genetic distance is less than the minimum interspecific distance, whereas species which fall below the line failed to do so.

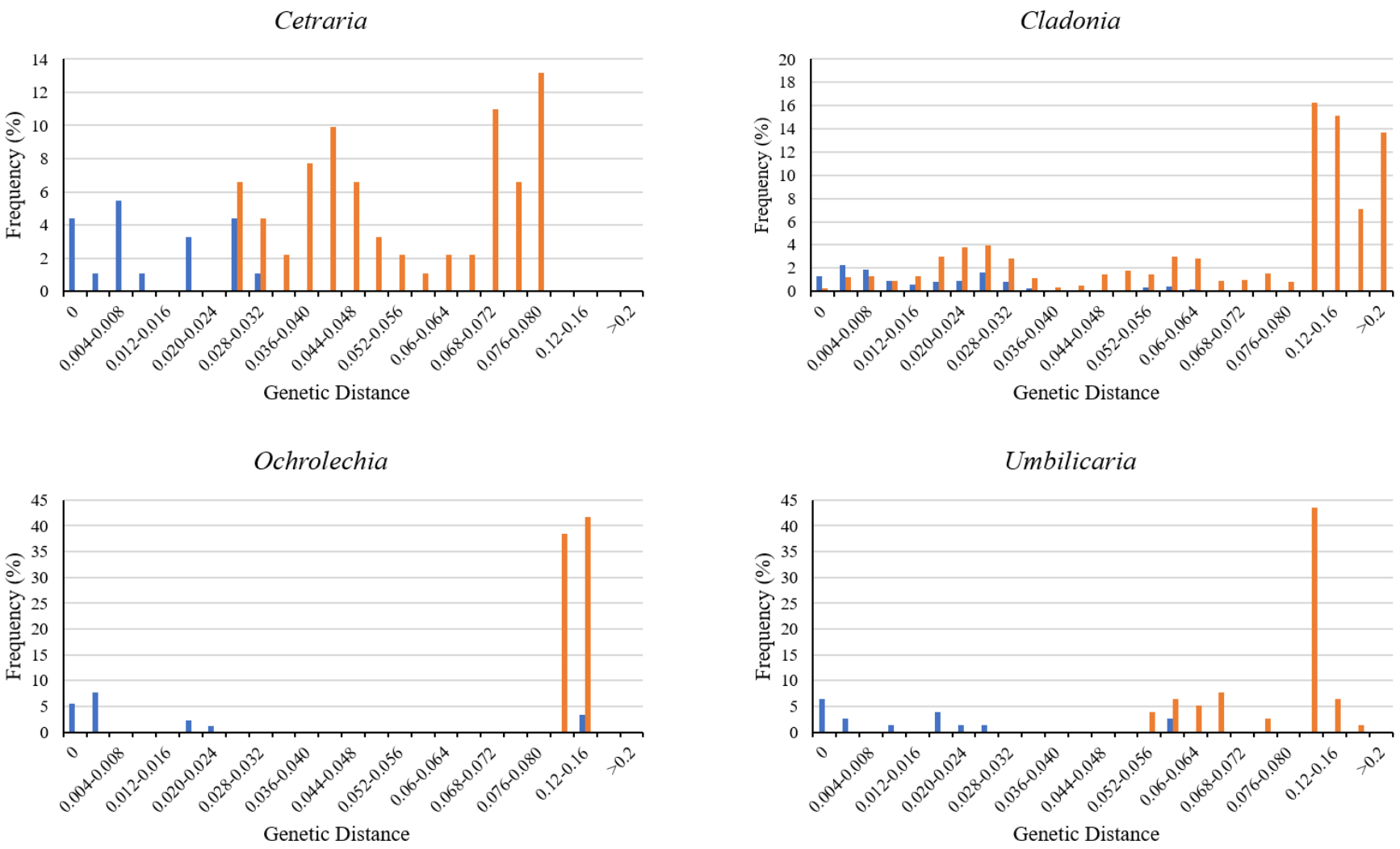


Figure 3. The frequency of genetic distances within each genus using ITS. Blue bars represent the frequency of genetic distances of conspecifics, orange bars represent the frequency of interspecific distances.

4. Discussion

4.1 Accuracy of Species Identifications

Species identifications were consistent based on morphology, chemistry and molecular data with the occasional problematic specimens. Notably, specimens from the *Cladinae* group in *Cladonia*, mostly *C. arbuscula, C. ciliata* and *C. rangiferina* proved difficult as diagnostic morphological characters as indicated by Smith *et al.* (2009) did not necessarily align with molecular data. A study of many accessions from a separate species in this group showed a high degree of both morphological and molecular variation, and it seems likely that the taxonomy in several of these species may require further work (Yahr *et al.*, 2006; Yahr, RBGE, pers. comm.). Further issues within this group include contradictory morphological and chemical characters; a sample of *Cladonia ciliata var. tenuis* (EDNA18-0051393) was identified based on morphology and molecular data but displayed atranorin a diagnostic chemical in *C. rangiferina* (Smith *et al.*, 2009) although this sample was positioned beside a *C. rangiferina* sample on plate E625 and may have experienced contamination. Due to time constraints this plate was not repeated. Despite this problematic group, morphological and chemical identifications for all species were considered accurate and for the most part were reflective of the molecular data.

Identifications through a NCBI BLAST search were mostly to the expected species and coincided with morphological and chemical identifications. Instances of contradiction mainly concerned species within the *Cladonia coccifera* aggregate. Notably, European sequences provided by Steinová *et al.* (2013) were unable to have their identity confirmed through morphological or chemical data as the voucher specimens were not stored within the herbarium at the Royal Botanic Garden Edinburgh, although identifications were checked by Ahti. However, a similar incongruence was noted by Steinová *et al.* (2013) where the traditional species circumscription based on morphology in the zeorin-containing members of the *C. coccifera* group were not supported by molecular data, with species such as *C. coccifera, C. deformis* (L.) Hoffm., and *C. pleurota* (Flörke) Schaer. appearing polyphyletic in both gene topographies. Despite this, the results of this study concerning *Cladonia* are consistent with the literature on the genetic divergence seen in *Cladonia* while looking at the ITS region (Myllys *et al.*, 2003; Pino-Bodas *et al.*, 2013; Steinová *et al.*, 2013) .

An additional problematic specimen was of *Ochrolechia androgyna* (EDNA18-0051717) which due to morphological and chemical characters was identified as *O.*

androgyna, molecular evidence did not group this sample with any other haplotype within the dataset, however the top NCBI BLAST result was to a specimen of *O. androgyna*. The NCBI BLAST database is limited on *Ochrolechia* sequences, with only 51 sequences available several are identified only to genus with very few representatives of species such as *O. androgyna* and *O. tartarea* (L.) A. Massal.

Sequence databases such as GenBank provide a collaborative resource which may act as a reference to aid in identification. However, publicly available databases such as GenBank are not regulated for sequence quality and sequences may be the subject of misidentification, contamination and sequencing errors that can hinder species identification (Shen, Chen & Murphy, 2013; Neaves *et al.*, 2018). An attempt was made to include sequences from *Ochrolechia tartarea* within the dataset however due to a limited number of European sequences, some of which may potentially be incorrect as they do not BLAST to any other sequence of *Ochrolechia tartarea*, the species was not included in the dataset. Further difficulties with GenBank include sequences that do not recognise voucher specimens, making the accuracy of identifications even more difficult to confirm and leading to non-repeatable scientific research (Culley, 2013; Yahr, Schoch & Dentinger, 2016).

4.2 DNA Barcoding

4.2.1 Barcoding of Lichenised Fungi using ITS

DNA barcoding appears to be an efficient method of identifying lichenised fungi to the species level. A barcode gap was visible in 62.5% of species examined, notably the majority of species which failed to display a barcode gap were in the genus *Cladonia*. With only 37.5% of *Cladonia* species demonstrating a barcode gap, ITS may not be the most appropriate barcode marker for the genus, despite this ITS may efficiently be used to identify *Cladonia* to genus and to group such as *Cladinae* and *Cocciferae*. The ITS region may however be an effective DNA barcode with the other genera used in the dataset where most of the species demonstrated a barcode gap.

Ochrolechia androgyna failed to demonstrate a barcode gap, however of the four samples utilised in the dataset all showed minimal genetic distances except from one sample, EDNA18-0051717 which skewed the dataset (figure 3.). As previously mentioned, the sample was unusual in morphology and chemistry, despite the sample resulting in a top hit with *O. androgyna* on a NCBI BLAST search, the sample on GenBank (GenBank accession number:

JN943616) and in this dataset were most likely misidentified. The sequence when included in a phylogeny and grouped with a specimen of *O. tartarea* (GenBank accession number: JN943620) and of *O. androgyna* (GenBank accession number: JN943616) further indicating the potential for both specimens to have been misidentified.

A clear distinction may be seen in the frequency of different interspecific and intraspecific distances within *Cetraria, Ochrolechia* and *Umbilicaria.* Notably, interspecific distances did not fall below 8% in *Ochrolechia* and 5.2% in *Umbilicaria* indicating the generally accepted ≤ 3% threshold for conspecific genetic divergence as appropriate within these genera (Begerow *et al.*, 2010). In *Cetraria* interspecific distances showed a minor overlap with intraspecific distances, where both inter and intraspecific distances appeared in the 2.4% - 3.2% range. Despite this, the majority of *Cetraria* species also demonstrated a barcode gap with the bulk of intraspecific distances occurring below the 2%. The only *Cetraria* species which failed to produce a barcode gap was *C. aculeata* a species which is a member of the *C. aculeata* group alongside *C. muricata* (Fernández-Mendoza *et al.*, 2011; Lutsak *et al.*, 2017). Although, a barcode gap was produced for *C. muricata* genetic distances between *C. muricata* and *C. aculeata* were low, reflecting the closely related nature of the two species. Furthermore, the widely accepted ≤ 3% of genetic divergence between species may not be a suitable threshold for the *C. aculeata* group, rather a more conservative ≤ 2% may be more efficient in identifying conspecifics. Despite *C. aculeata* failing to produce a barcode gap the barcoding of *Cetraria* was considered a success as *C. aculeata* was always discriminated from species out with the *C. aculeata* group.

4.2.2 ITS1 and ITS2 as Stand-Alone Barcodes

Within all four genera the ITS1 sub region out performed ITS2 except from *Umbilicaria* where ITS2 performed equally as well as ITS1. Unexpectantly, in *Umbilicaria* ITS1 and ITS2 outperformed the full ITS, which included ITS1, *5.8S* and ITS2, in the local BLAST based analysis. ITS1 was significantly more effective in discriminating *Cladonia* species where ITS2 occasionally failed to identify to the correct genus. Therefore, ITS2 would not be recommended as a stand-alone barcode for lichenised fungi.

ITS1, however, showed great success in discriminating species, even outperforming the full ITS in *Ochrolechia* and *Umbilicaria*. Despite this, discrimination of *Cladonia* was not as successful as the full ITS region. This highlights the potential of ITS1 as a stand-alone

barcode of lichenised fungi, however ITS is more efficient in discriminating species in problematic taxonomic groups such as *Cladonia*. Despite this, within a dietary analysis identifications to species are not always essential and genus or section level identifications may be sufficient (Newmaster *et al.*, 2013; De Barba *et al.*, 2014; Joo *et al.*, 2014; Su *et al.*, 2018). This suggests that with a high-throughput, but short-sequence format such as Illumina Hi-Seq and Mi-Seq platforms, ITS1 may be a suitable candidate barcode for dietary analyses involving these lichen groups.

4.3 Genetic Divergence

4.3.1 Issues in Barcoding *Cladonia*

Similar to other molecular studies involving *Cladonia* (Kotelko & Piercey-Normore, 2010; Steinová *et al.*, 2013; Pino-Bodas *et al.*, 2013), the phenotypes and chemotypes, which originally circumscribed *Cladonia*, have demonstrated incongruence with the genotype. Despite this being a common issue, few within the literature have aimed to answer why this occurs in *Cladonia* and instances where phylogenetically defined species do not fit in with morphological and chemical species concepts have often lead to species names being conserved on account of morphological species concepts such as *C. diversa* (Steinová *et al.*, 2013) or with *C. pyxidata* and *C. pocillum* (Ach.) Grognot (Kotelko & Piercey-Normore, 2010). Reasons cited for conserving names based on morphological species concepts often stem from ecological implications, such as using the morphological species as a bioindicator, as seen in Kotelko and Piercey-Normore's (2010) decision to conserve the names *C. pyxidata* and *C. pocillum*. Although this approach is not always encouraged, as acknowledging a phylogenetic species may promote the identification of morphological characters required for species discrimination and may further contribute to understanding speciation (Grube & Kroken, 2016). Furthermore, ecophysiological variation may account for the distinct morphologies seen in *Cladonia* while reflecting the genotype (Kotelko & Piercey-Normore, 2010). This is supported in other lichen genera such as *Usnea* Dill. ex. Adans. and *Cetraria*, species such as *Usnea florida* (L.) Weber ex F.H.Wigg. and *Cetraria aculeata* have demonstrated intraspecific ecophysiological variation where genetically similar morphs are morphologically distinct based on the surrounding environment (Articus *et al.*, 2002; Pérez-Ortega *et al.*, 2012).

Interpretations of this incongruence may indicate that the large genus *Cladonia* is not as speciose as previously thought and actually consists of similar species which demonstrate a high degree of phenotypic variation which may be influenced by environmental conditions (Kotelko & Piercey-Normore, 2010), or most likely, due to the size of the genus and the tendency of *Cladonia* species to form sympatric mats, may consist of recently diverged, incipient or cryptic species (Crespo & Lumbsch, 2010; Steinová *et al.,* 2013; Yahr, Schoch & Dentinger, 2016).

Another potential source of the incongruence seen between the *Cladonia* phenotype and genotype is incomplete lineage sorting (Myllys *et al.*, 2003). Incomplete lineage sorting is a phenomenon that may occur during speciation; when a polymorphic ancestral species with multiple haplotypes diverges. Through speciation the new species lineages may inherit both haplotypes. Further divergence may not pass both of the original haplotypes onto the new lineages, thus morphologically distinct species may share a haplotype with evolutionarily distant species leading to discrepancies between the phylogeny and the evolutionary history of the species (Myllys *et al*., 2003; Maddison & Knowles, 2006; Steinová *et al.*, 2013; Percy *et al.*, 2014). In this instance, haplotypes shared by species due to incomplete lineage sorting may explain why genetic distances between species in the *Cladonia coccifera* aggregate were so low and why the frequencies of genetic distances completely overlapped within the *Cladonia* distance matrix. Moreover, shared haplotypes resulting from incomplete lineage sorting may explain why a significant number of *Cladonia* species, such as *C. arbuscula* and *C. ciliata* in the *Cladinae* group or *C. coccifera, C. diversa* and *C. floerkeana* of the *Coccifera* group, failed to show a barcode gap.

Although incomplete lineage sorting may explain the incongruence between the genotype and phenotype of *Cladonia*, the incongruence may alternatively be explained through hybridisation and introgression leading to horizontal gene transfer between *Cladonia* species. Therefore, to determine the true nature of genotypic and phenotypic incongruence further research is required which may be achieved through a statistical approach *sensu* Joly *et al.* (2009) to distinguish whether *Cladonia* is experiencing incomplete lineage sorting or hybridisation.

4.3.2 Hybridisation and Horizontal Gene Transfer

Hybridisation is often under reported within fungi, as a result many reported instances of hybridisation focus on rusts (Brasier, 2000). However, mechanical hybrids have been noted in lichenised fungi, forming from initially different thalli of multiple distinct species coming together through hyphal fusion (Bridge & Hawksworth, 1998). Furthermore, hyphal fusion has previously shown to form intrageneric hybrids within lichens, as seen with *Alectoria* Ach. and *Bryoria* Brodo & D. Hawksw. (Brodo, 1978).

Hybridisation is an effective method of introducing genetic variability within a species through horizontal gene transfer and introgression which can increase the genetic diversity within a species and is evident within a wide variety of taxonomic groups (Grant, 2003; Balasundaram *et al.*, 2015; Steinová *et al.*, 2013; Hollingsworth, *et al*., 2017; Neaves *et al.*, 2018). Moreover, genes acquired through introgression and horizontal gene transfer may propagate throughout a population if the main mode of reproduction is asexual, as seen in other fungal groups (Brasier, 2000). Horizontal gene transfer can be defined as the interspecific transfer of a gene, often associated with bacteria the process has been observed in eukaryotes (Keeling & Palmer, 2008). Whereas, introgression is the establishment of a gene from another species through hybridisation and the backcrossing of the F1 hybrid with a parent species, thus transferring the gene between species (Arnold, 2004). Both introgression and horizontal gene transfer may account for the incongruence between the phenotype and genotype of *Cladonia*. However, horizontal gene transfer is less likely as it would imply a gene transfer event to have occurred recently between eukaryotes, an event which is possible but unlikely (Andersson, 2005; Rot *et al.*, 2006; Keeling & Palmer, 2008). Furthermore, reported instances of horizontal gene transfer within Eukaryotes are less frequent within animals and fungi than they are within plants, although this may be a result from gaps within the literature (Won & Renner, 2003; Danchin, 2016). Thus, introgression through hybridisation is more likely to explain the incongruence between phenotype and genotype within the *Cladonia* specimens than a horizontal gene transfer event.

Cladonia species within the Cairngorm plateau demonstrate a sympatric distribution and range, many are mat-forming lichens that overlap and grow over closely-related congenerics; therefore, gene flow between species as a result of hybridisation does appear plausible. Hyphal fusion is hypothesised to be responsible for incongruence within morphology and chemistry within the sympatric species *Cladonia mitis* Sandst. and *C. arbuscula* leading to intermediates that have a combined chemistry of both species (Ruoss,

1987). Moreover, both *C. mitis* and *C. arbuscula* have demonstrated incongruence between morphology and the phylogeny possibly indicating hyphal fusion as the source of this incongruence (Myllys *et al.*, 2003).

Hybridisation may therefore explain the incongruences between morphology, chemistry and genotypes demonstrated within the *Cladinae* group, namely specimens of *C. arbuscula, C. ciliata* and *C. rangiferina*. Within this section morphological data was incongruent with both molecular data and chemical data, notably a morphologically distinct specimen of *Cladonia ciliata var. tenuis* (EDNA18-0051393) contained atranorin, a diagnostic feature of *Cladonia rangiferina*; while genetic distance data indicated the specimen was closer to *C. ciliata* than the other samples of *C. rangiferina.* However, the incongruence within this specimen may also be explained through incomplete lineage sorting, indicating the specimen as *C. rangiferina* that shares a haplotype with *C. ciliata*.

4.3.3 Determining the Origin of Incongruence within *Cladonia*

Further research must be done to ascertain why such a strong incongruence between the phenotype and genotype of *Cladonia* exists, as the incongruence resurfaces frequently in molecular studies of *Cladonia* making the phylogeny of the genus difficult to interpret (Stenroos *et al.*, 2002; Myllys *et al.*, 2003; Kotelko & Piercey-Normore, 2010; Steinová *et al.*, 2013; Athukorala *et al.*, 2016). Understanding the cause of the incongruence in *Cladonia* can help within the interpretation of molecular studies including phylogenetic and barcoding analyses. Primarily, changes within the methodology can be made to account for processes such as incomplete lineage sorting and introgression to better understand the evolutionary history of problematic taxa (Maddison & Knowles, 2006; Eaton & Ree, 2013).

Due to time constraints the incongruence between morphological, chemical and molecular data within the *Cladonia* dataset was not explored further. However, the incongruence seen within *Cladonia* should be addressed, including further consideration to all sections within the genus. Notably from the results of this study, incongruence is seen within the *Cladinae* group and *Cocciferae* group and may potentially have independent origins. Therefore, the method for distinguishing whether incomplete lineage sorting or hybridisation is causing incongruence within *Cladonia* should encapsulate both sections within the genus while possibly extending to other sections. A statistical approach *sensu* Joly *et al.* (2009) is recommended to distinguish whether the incongruence of *Cladonia* is a result of incomplete

lineage sorting or introgression from hybridisation, to help interpret results from future phylogenetic and barcoding analyses concerning the genus.

4.4 Implications for the Cairngorm Reindeer Diet Project

The ITS region proved an effective DNA barcode in identifying the majority of tested lichen genera, although utilising ITS1 increased species discrimination in *Ochrolechia* and *Umbilicaria*, the overall success of the full ITS region in identifying a problematic genus such as *Cladonia* would highlight it as an appropriate DNA barcode to use within the reindeer diet project over both sub-regions. Of the sub-regions, ITS2 was not an effective barcode, which occasionally failed to identify to genus so would not be recommended for use in a dietary analysis. In contrast, ITS1 may be considered despite the poor discrimination seen in *Cladonia* as identifications were made to section.

However, *Cladonia* is a genus with approximately 500 species and congenerics make up a significant proportion of the Cairngorm lichen biomass. Knowing which species the reindeer are eating specifically is imperative for lichen conservation efforts. Many of the lichens on the UK biodiversity action plan (BAP) priority species list may be found within the Cairngorm plateau. Notably, within the genera of this study *Cladonia botrytes* (K.G. Hagen) Willd. is found within there.

Potentially, using another molecular marker alongside ITS could be more effective in discriminating *Cladonia* through barcoding (Kelly *et al.*, 2011). Notably *cox1* and *rpb2* have previously been cited as efficient barcodes alongside ITS in *Cladonia* (Pino-Bodas *et al.*, 2013). However, the most efficient secondary barcode between *cox1* and *rpb2* must still be researched to determine which one has higher rates of species discrimination alongside ITS. Although, the need for a species level identification may not be necessary, particularly when the species is morphologically and ecologically similar to other species within a genus or is not of conservation concern, a genus or section level identification may be sufficient in determining the ecological impacts of *Rangifer tarandus*. Notably, species within the *Cladinae* section share a mat-forming ecological habit and are not of conservation concern, therefore a genus or section level identification may suffice.

Of the other genera, species discrimination was effective using ITS or the ITS1 sub-region. Therefore, DNA barcoding should allow for higher success in species identification and discrimination of morphologically similar species such as *Cetraria muricata* and *C.*

aculeata which may not be readily identified through video recording equipment, although a species level identification may not be necessary. We were unable to sample the Schedule 8 BAP species *Alectoria ochroleuca* for this study, but it would be another species to specifically seek in a dietary study of the reindeer herd, as its largest populations are within reach of normal movements of the animals.

4.5 Conclusions

In summary, the ITS region has demonstrated its role as the standard fungal barcode, being able to discriminate most of the species tested. Of the ITS sub-regions, ITS1 demonstrated higher identification and discrimination success within the lichen genera *Cetraria, Cladonia, Ochrolechia* and *Umbilicaria* than the sub region ITS2, thus indicating the potential of ITS1 as a stand-alone barcode in fungi. Due to the difficulties surrounding the incongruence of *Cladonia,* ITS alone may not be sufficient as a DNA barcode and the identification of *Cladonia* may be more successful with a second barcode, possibly *cox1* or *rpb2*, further research is required to determine which region would act as a better barcode alongside ITS. Ensuring species discrimination is possible within *Cladonia* would benefit the long-term project, primarily due to the biomass of *Cladonia* within the Cairngorm plateau and the presence of a UK BAP priority species, *Cladonia botrytes*. However, for the reindeer diet project section level identification within *Cladonia* will suffice, due to the shared ecological niches of species within the sections. Thus, ITS1 could act as a stand-alone barcode for the dietary analysis despite the incongruence within *Cladonia*. Further research is also required to determine whether the origin of the incongruence seen within the phenotype and genotype of *Cladonia* as originating from incomplete lineage sorting, introgression from hybridisation, or from other unexplored possibilities such as our lack of understanding of morphological plasticity.

To conclude, ITS is an effective barcode for use in the reindeer diet project; it revealed a barcode gap within most of the species tested, although it may not be sufficient as a barcode for species discrimination for the genus *Cladonia.* The sub-region ITS1 has demonstrated potential as a stand-alone barcode, unlike ITS2 which was significantly worse in species discrimination. Although ITS1 demonstrated potential as a stand-alone barcode, it was not as efficient in species discrimination as ITS. However, species level identifications may not be necessary to understand the ecological impacts of the reindeer within the Cairngorms and

identifications to the genus or section level may suffice in determining the diet of the Cairngorm reindeer.

References

Andersson, J.O. (2005) Lateral gene transfer in eukaryotes. *Cellular and Molecular Life Sciences*. 62 (11), 1182–1197.

Arnold, M.L. (2004) Natural hybridization and the evolution of domesticated, pest and disease organisms. *Molecular Ecology*. 13 (5), 997–1007.

Articus, K., Mattsson, J.E., Tibell, L., Grube, M., *et al.* (2002) Ribosomal DNA and β-tubulin data do not support the separation of the lichens *Usnea florida* and *U. subfloridana* as distinct species. *Mycological Research*. 106, 412–418.

Athukorala, S.N.P., Pino-Bodas, R., Stenroos, S., Ahti, T., *et al.* (2016) Phylogenetic relationships among reindeer lichens of North America. *Lichenologist*. 48 (3), 209–227.

Balasundaram, S. V., Engh, I.B., Skrede, I. & Kauserud, H. (2015) How many DNA markers are needed to reveal cryptic fungal species? *Fungal Biology*. 119 (10), 940–945.

De Barba, M., Miquel, C., Boyer, F., Mercier, C., *et al.* (2014) DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Molecular Ecology Resources*. 14 (2), 306–323.

Begerow, D., Nilsson, H., Unterseher, M. & Maier, W. (2010) Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Applied Microbiology and Biotechnology*. 87 (1), 99–108.

Bergerud, A.T. (1972) Food Habits of Newfoundland Caribou. *The Journal of Wildlife Management*. 38 (3), 435–446.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., *et al.* (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*. 22 (3), 148–155.

Bohmann, K., Gopalakrishnan, S., Nielsen, M., Nielsen, L. dos S.B., *et al.* (2018) Using DNA metabarcoding for simultaneous inference of common vampire bat diet and population structure. *Molecular Ecology Resources*. (March), 1–14.

Bokhorst, S., Huiskes, A., Convey, P. & Aerts, R. (2007) External nutrient inputs into terrestrial ecosystems of the Falkland Islands and the Maritime Antarctic region. *Polar Biology*. 30 (10), 1315–1321.

Brasier, C. (2000) The rise of the hybrid fungi. *Nature*. 405 (6783), 134–135. doi:10.1038/35012193.

Bridge, P.D. & Hawksworth, D.L. (1998) What molecular biology has to tell us at the species level in lichenized fungi. *Lichenologist*. 30 (4–5), 307–320.

Brirten, R.J. (1984) Rates of DNA Sequence Evolution Differ Between Taxonomic Groups. *Science*. 39, 1393–1398.

Brodo, I.M. (1978) Changing Concepts Regarding Chemical Diversity in Lichens. *The Lichenologist*. 10, 1–11.

Bruns, T.D., Arnold, A.E. & Hughes, K.W. (2008) Fungal networks made of humans: UNITE, FESIN, and frontiers in fungal ecology. *New Phytologist*. 177 (3), 586–588.

Casano, L.M., Del Campo, E.M., García-Breijo, F.J., Reig-Armiñana, J., *et al.* (2011) Two *Trebouxia* algae with different physiological performances are ever-present in lichen thalli of *Ramalina farinacea*. Coexistence versus Competition? *Environmental Microbiology*. 13 (3), 806–818.

Crespo, A. & Lumbsch, H.T. (2010) Cryptic species in lichen-forming fungi. *IMA Fungus*. 1 (2), 167–170. doi:10.5598/imafungus.2010.01.02.09.

Culley, T.M. (2013) Why Vouchers Matter in Botanical Research. *Applications in Plant Sciences*. 1 (11), 1300076. doi:10.3732/apps.1300076.

Danchin, E.G.J. (2016) Lateral gene transfer in eukaryotes: Tip of the iceberg or of the ice cube. *BMC Biology*. 14 (1), 1–3.

Deagle, B.E., Chiaradia, A., McInnes, J. & Jarman, S.N. (2010) Pyrosequencing faecal DNA to determine diet of little penguins: Is what goes in what comes out? *Conservation Genetics*. 11 (5), 2039–2048.

DePriest, P.R., Piercey-Normore, M.D., Sikaroodi, M., Kärkkäinen, K., *et al.* (2000) Phylogenetic relationships among sections of *Cladonia* and *Cladina*. *Progress and Problems in Lichenology at the turn of the Millennium: The Fourth IAL* Symposium. Barcelona. 14.

Dobson, F. (2011) *Lichens: an Illustrated Guide to the British and Irish Species (6th edition)*.

Eaton, D.A.R. & Ree, R.H. (2013) Inferring Phylogeny and Introgression using RADseq Data: An Example from Flowering Plants (*Pedicularis*: Orobanchaceae). *Systematic Biology*. 62 (5), 689–706..

Ellis, C.J. & Coppins, B.J. (2010) Partitioning the role of climate, pollution and old-growth woodland in the composition and richness of lichen epiphytes in Scotland. *Lichenologist*. 42 (5), 601–614..

Ellis, C.J., Coppins, B.J., Dawson, T.P. & Seaward, M.R.D. (2007) Response of British lichens to climate change scenarios: Trends and uncertainties in the projected impact for contrasting biogeographic groups. *Biological Conservation*. 140 (3–4), 217–235.

Ellis, C.J., Crittenden, P.D., Scrimgeour, C.M. & Ashcroft, C.J. (2005) Translocation of ^{15}N indicates nitrogen recycling in the mat-forming lichen *Cladonia portentosa*. *New Phytologist*. 168 (2), 423–434.

Feibelman, T., Bayman, P. & Cibula, W.G. (1994) Length variation in the internal transcribed spacer of ribosomal DNA in chanterelles. *Mycological Research*. 98 (6), 614–618.

Fernández-Mendoza, F., Domaschke, S., García, M.A., Jordan, P., *et al.* (2011) Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. *Molecular Ecology*. 20 (6), 1208–1232.

Fields, A.T., Abercrombie, D.L., Eng, R., Feldheim, K., *et al.* (2015) A novel mini-DNA barcoding assay to identify processed fins from internationally protected shark species. *PLoS ONE*. 10 (2), 1–10.

Fryday, A. (1997) Montane lichens in Scotland. *Botanical Journal of Scotland*. 49 (1997), 367–374.

Fryday, A.M. (2001) Phytosociology of terricolous lichen vegetation in the Cairngorm Mountains, Scotland. *Lichenologist*. 33 (4), 331–351.

Gardes, M. & Bruns, T. (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology*. 2 (2), 113–118.

Gargas, A., DePriest, P.T., Grube, M. & Tehler, A. (1995) Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science*. 268 (5216), 1492–1495.

Gilbert, O.L. & Fox, B.W. (1985) Lichens Of High Ground In The Cairngorm Mountains, Scotland. *The Lichenologist*. 17 (1), 51–66.

Gordon, J.E., Macdonald, R., Thompson, D.B.A., Haynes, V.M., *et al.* (1998) Environmental sensitivity and conservation management in the Cairngorm Mountains, Scotland. *Ambio*. 27 (4), 335–344.

Grande, F.D. & Leavitt, S.D. (2015) Coalescent-Based Species Delimitation Approach Uncovers High Cryptic Diversity in the Cosmopolitan Lichen-Forming Fungal Genus *Protoparmelia* (Lecanorales , Ascomycota). *PLoS ONE*. 1–20.

Grant, B.R. (2003) Evolution in Darwin's Finches: a review of a study on Isla Daphne Major in the Galápagos Archipelago. *Zoology*. 106 (4), 255–259.

Grube, M., Cardinale, M., de Castro, J.V., Müller, H., *et al.* (2009) Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *The ISME Journal*. 3 (9), 1105–1115.

Grube, M. & Kroken, S. (2016) Molecular approaches and the concept of species and species complexes in lichenized fungi. *Mycological Research*. 104 1284–1294.

Gudjónsdóttir, G.A. & Ingólfsdóttir, K. (1997) Quantitative determination of protolichesterinic- and fumarprotocetraric acids in *Cetraria islandica* by high-performance liquid chromatography. *Journal of Chromatography A*. 757 (1–2), 303–306.

Hájek, J., Barták, M., Hazdrová, J. & Forbelská, M. (2016) Sensitivity of photosynthetic processes to freezing temperature in extremophilic lichens evaluated by linear cooling and chlorophyll fluorescence. *Cryobiology*. 73 (3), 329–334..

Hauck, M. (2008) Susceptibility to acidic precipitation contributes to the decline of the terricolous lichens *Cetraria aculeata* and *Cetraria islandica* in central Europe. *Environmental Pollution*. 152 (3), 731–735.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & DeWaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*. 270 (1512), 313–321.

Hebert, P.D.N., Ratnasingham, S. & de Waard, J.R. (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society B: Biological Sciences*. 270, S96–S99.

Hillis, D.M. & Dixon, M.T. (1991) Ribosomal DNA : Molecular Evolution and Phylogenetic Inference. *The Quarterly Review of Biology*. 66 (4), 411–453.

Hodgetts, N.G. (1992) *Cladonia* : a field guide. 1–42.

Hollingsworth, P.M., Forrest, L.L., Spouge, J.L., Hajibabaei, M., *et al.* (2009) A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the United States of America*. 106 (31), 12794–12797.

Hollingsworth, P.M., Neaves, L.E. & Twyford, A.D. (2017) Using DNA sequence data to enhance understanding and conservation of plant diversity at the species level. *Plant Conservation Science and Practice: The Role of Botanic Gardens*.

Hughes, K.W., Petersen, R.H. & Lickey, E.B. (2009) Using heterozygosity to estimate a percentage DNA sequence similarity for environmental species' delimitation across basidiomycete fungi. *New Phytologist*. 182 (4), 783–785.

Ivanova, N. V., Depriest, P.T., Bobrova, V.K. & Troitsky, A. V. (1999) Phylogenetic analysis of the lichen family Umbilicariaceae based on nuclear ITS1 and ITS2 rDNA sequences. *Lichenologist*. 31 (5), 477–489.

James, T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., *et al.* (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature*. 443 (7113), 818–822.

Joly, S., McLenachan, P.A. & Lockhart, P.J. (2009) A Statistical Approach for Distinguishing Hybridization and Incomplete Lineage Sorting. *The American Naturalist*. 174 (2), E54–E70.

Joo, S., Han, D., Lee, E.J. & Park, S. (2014) Use of length heterogeneity polymerase chain reaction (LH-PCR) as non-invasive approach for dietary analysis of Svalbard reindeer, *Rangifer tarandus platyrhynchus*. *PLoS ONE*. 9 (3). doi:10.1371/journal.pone.0091552.

Kanz, B., Brackel, W. von, Cezanne, R., Eichler, M., *et al.* (2015) DNA Barcodes for the Distinction of Reindeer Lichens: A Case Study Using *Cladonia rangiferina* and *C. stygia*. *Herzogia*. 28 (2), 445–464.

Kartzinel, T.R., Chen, P.A., Coverdale, T.C., Erickson, D.L., *et al.* (2015) DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. *Proceedings of the National Academy of Sciences*. 112 (26), 8019–8024.

Katoh, K., Rozewicki, J. & Yamada, K.D. (2017) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*. 1–7.

Keeling, P.J. & Palmer, J.D. (2008) Horizontal gene transfer in eukaryotic evolution. *Nature Reviews Genetics*. 9 (8), 605–618.

Kelly, L.J., Hollingsworth, P.M., Coppins, B.J., Ellis, C.J., *et al.* (2011) DNA barcoding of lichenized fungi demonstrates high identification success in a floristic context. *New Phytologist*. 191 (1), 288–300.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*. 16 (2), 111–120.

Kochan, T.I. (2006) Content of carbohydrates in enteral medium of various parts of digestive tract of the reindeer *Rangifer tarandus* during winter. *Journal of Evolutionary Biochemistry and Physiology*. 42 (1), 30–37.

Kotelko, R. & Piercey-Normore, M.D. (2010) *Cladonia pyxidata* and *C. pocillum*; genetic evidence to regard them as conspecific. *Mycologia*. 102 (3), 534–545.

Krishnamurthy, K.P. & Francis, R.A. (2012) A critical review on the utility of DNA barcoding in biodiversity conservation. *Biodiversity and Conservation*. 21 (8), 1901–1919.

Kukwa, M. (2009) The lichen genus *Ochrolechia* in the Baltic countries. *Folia Cryptogamica Estonia*. 46, 67–74.

Lawrey, J.D.. (1980) Correlations between Lichen Secondary Chemistry and Grazing Activity by *Pallifera varia*. *The Bryologist*. 83 (3), 328–334.

Leavitt, S.D., Kraichak, E., Vondrak, J., Nelsen, M.P., *et al.* (2016) Cryptic diversity and symbiont interactions in rock-posy lichens. *Molecular Phylogenetics and Evolution*. 99, 261–274.

Lendemer, J.C. & Harris, R.C. (2013) *Cladonia appalachensis*, belated description of a southern Appalachian lichen endemic from the Great Smoky Mountains. *Opuscula Philolichenum*. 12, 17–22.

Lendemer, J.C. & Harris, R.C. (2014) Studies in lichens and lichenicolous fungi No. 17 – Notes on lichens from the Coastal Plain of Southeastern North America. *Opuscula Philolichenum*. 13 (19), 8–19.

Li, D.-Z., Gao, L.-M., Li, H.-T., Wang, H., *et al.* (2011) Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proceedings of the National Academy of Sciences*. 108 (49), 19641–19646.

Liu, J., Milne, R.I., Möller, M., Zhu, G.-F., *et al.* (2018) Integrating a comprehensive DNA barcode reference library with a global map of yews (*Taxus* L.) for forensic identification. *Molecular Ecology Resources*. 0–3.

Llano, G.A. (1956) New Umbilicariaceae from the Western Hemisphere, with a key to the genera. *Journal of the Washington Academy of Sciences*. 46 (6), 183–185.

Lücking, R., Hodkinson, B.P. & Leavitt, S.D. (2017) The 2016 classification of lichenized fungi in the Ascomycota and Basidiomycota. *The Bryologist*. 120 (1), 58–69.

Lutsak, T., Fernández-Mendoza, F., Nadyeina, O., Şenkardeşler, A., *et al.* (2017) Testing the correlation between norstictic acid content and species evolution in the *Cetraria aculeata* group in Europe. *Lichenologist*. 49 (1), 39–56.

Lutzoni, F. & Miadlikowska, J. (2009) Lichens. *Current Biology*. 19 (13), R502–R503.

Lutzoni, F., Pagel, M. & Reeb, V. (2001) Major fungal lineagues are derived from lichen ancestors. *Nature*. 411 (June), 937–940.

Maddison, W.P. & Knowles, L.L. (2006) Inferring phylogengy despite incomplete lineage sorting. *Systematic Biology*. 55 (l), 21–30.

Maddison, W.P. & Maddison, D.R. (2018) *Mesquite: a modular system for evolutionary analysis. Version 3.51*. [Online]. Available from: http://www.mesquiteproject.org.

Mark, K., Cornejo, C., Keller, C., Flück, D., *et al.* (2016) Barcoding lichen-forming fungi using 454 pyrosequencing is challenged by artifactual and biological sequence variation 1. *Genome*. 59, 685–704.

Meier, R., Zhang, G. & Ali, F. (2008) The use of mean instead of smallest interspecific distances exaggerates the size of the 'barcoding gap' and leads to misidentification. *Systematic Biology*. 57 (5), 809–813.

Meyer, C.P. & Paulay, G. (2005) DNA barcoding: Error rates based on comprehensive sampling. *PLoS Biology*. 3 (12), 1–10.

Molina, M.C., Del-Prado, R., Divakar, P.K., S??nchez-Mata, D., *et al.* (2011) Another example of cryptic diversity in lichen-forming fungi: The new species Parmelia mayi (Ascomycota: Parmeliaceae). *Organisms Diversity and Evolution*. 11 (5), 331–342.

Morris, T. & Letnic, M. (2017) Removal of an apex predator initiates a trophic cascade that extends from herbivores to vegetation and the soil nutrient pool. *Proceedings of the Royal Society B: Biological Sciences*. 284 (1854).

Myllys, L., Stenroos, S., Thell, A. & Ahti, T. (2003) Phylogeny of bipolar Cladonia arbuscula and Cladonia mitis (Lecanorales, Euascomycetes). *Molecular Phylogenetics and Evolution*. 27 (1), 58–69.

Nadyeina, O., Lutsak, T., Blum, O., Grakhov, V., *et al.* (2013) Cetraria steppae Savicz is conspecific with *Cetraria aculeata* (Schreb.) Fr. according to morphology, secondary chemistry and ecology. *Lichenologist*. 45 (6), 841–856.

Neaves, L.E., Danks, M., Lott, M.J., Dennison, S., *et al.* (2018) Unmasking the complexity of species identification in Australasian flying-foxes. *PLoS ONE*. 13 (4), 1–19.

Nelsen, M.P. (2018) *Primer Maps*. 2018.

Newmaster, S.G., Thompson, I.D., Steeves, R.A.D., Rodgers, A.R., *et al.* (2013) Examination of two new technologies to assess the diet of woodland caribou: video recorders attached to collars and DNA barcoding. *Canadian Journal of Forest Research*. 43 (3), 897–900.

Nilsson, R.H., Kristiansson, E., Ryberg, M., Hallenberg, N., *et al.* (2008) Intraspecific ITS variability in the Kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics*. 2008 (4), 193–201.

O'Brien, H.E., Parrent, J.L., Jackson, J.A., Moncalvo, J., *et al.* (2005) Fungal Community Analysis by Large-Scale Sequencing of Environmental Samples. *Applied and Environmental Microbiology*. 71 (9), 5544–5550.

Orange, A., James, P.W., White, F.J. & British Lichen Society (2001) Thin Layer Chromatography. In: *Microchemical Methods for the Identification of Lichens*. British Lichen Society. pp. 50–69.

Page, R.D.M. (2011) *Dark taxa: GenBank in a post-taxonomic world*. . 2011.[Online] Available from: http://iphylo.blogspot.com/2011/04/dark-taxa-genbank-in-post-taxonomic.html

Percy, D.M., Argus, G.W., Cronk, Q.C., Fazekas, A.J., *et al.* (2014) Understanding the spectacular failure of DNA barcoding in willows (*Salix*): Does this result from a trans-specific selective sweep? *Molecular Ecology*. 23 (19), 4737–4756.

Pérez-Ortega, S., Fernández-Mendoza, F., Raggio, J., Vivas, M., *et al.* (2012) Extreme phenotypic variation in *Cetraria aculeata* (lichenized Ascomycota): Adaptation or incidental modification? *Annals of Botany*. 109 (6), 1133–1148.

Pino-Bodas, R., Martín, M.P. & Burgaz, A.R. (2010) Insight into the *Cladonia convoluta-C. foliacea* (Cladoniaceae, Ascomycota) complex and related species, revealed through morphological, biochemical and phylogenetic analyses. *Systematics and Biodiversity*. 8 (4), 575–586.

Pino-Bodas, R., Martín, M.P., Burgaz, A.R. & Lumbsch, H.T. (2013) Species delimitation in *Cladonia* (Ascomycota): A challenge to the DNA barcoding philosophy. *Molecular Ecology Resources*. 13 (6), 1058–1068.

Posner, B., Feige, G.B. & Huneck, S. (1992) Studies on the Chemistry of the Lichen Genus *Umbilicaria*-Hoffm. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences*. 47, 1–9.

QIAGEN (2012) *DNeasy Plant Handbook*. [Online]. Available from: https://ch1prd0102.outlook.com/owa/?hm=1&owa=1&wa=wsignin1.0%5Cnpapers2://publication/uuid/DB53BA74-A8C0-494F-8F7B-A83609B1F26D.

Ranković, B.R., Kosanić, M.M. & Stanojković, T.P. (2011) Antioxidant, antimicrobial and anticancer activity of the lichens *Cladonia furcata, Lecanora atra* and *Lecanora muralis*. *BMC Complementary and Alternative Medicine*. 11.

Rot, C., Goldfarb, I., Ilan, M. & Huchon, D. (2006) Putative cross-kingdom horizontal gene transfer in sponge (Porifera) mitochondria. *BMC Evolutionary Biology*. 6, 1–11.

Ruoss, E. (1987) Species differentiation in a group of reindeer lichens (*Cladonia* subg. *Cladina*). *Bibliotheca Lichenologica*. 25, Bibl. Lichenol.

Ryberg, M., Nilsson, R.H., Kristiansson, E., Töpel, M., *et al.* (2008) Mining metadata from unidentified ITS sequences in GenBank: A case study in *Inocybe* (Basidiomycota). *BMC Evolutionary Biology*. 8 (1), 1–14.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., *et al.* (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*. 109 (16), 6241–6246.

Schoch, C.L., Sung, G.H., López-Giráldez, F., Townsend, J.P., *et al.* (2009) The ascomycota tree of life: A phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Systematic Biology*. 58 (2), 224–239.

Shen, Y.-Y., Chen, X. & Murphy, R.W. (2013) Assessing DNA Barcoding as a Tool for Species Identification and Data Quality Control. *PLoS ONE*. 8 (2), e57125.

Smith, C.W., Aptroot, A., Coppins, B.J., Fletcher, A., *et al.* (2009) *The Lichens of Great Britain and Ireland*.

Spribille, T. (2016) Basidiomycetes yeast in the cortex of ascomycete macrolichens. *Science*. 8287, 1–11.

Srivathsan, A. & Meier, R. (2012) On the inappropriate use of Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. *Cladistics*. 28 (2), 190–194.

Srivathsan, A., Sha, J.C.M., Vogler, A.P. & Meier, R. (2015) Comparing the effectiveness of metagenomics and metabarcoding for diet analysis of a leaf-feeding monkey (*Pygathrix nemaeus*). *Molecular Ecology Resources*. 15 (2), 250–261.

Steinová, J., Stenroos, S., Grube, M. & Škaloud, P. (2013) Genetic diversity and species delimitation of the zeorin-containing red-fruited *Cladonia* species (lichenized Ascomycota) assessed with ITS rDNA and β-tubulin data. *Lichenologist*. 45 (5), 665–684.

Stenroos, S., Hyvönen, J., Myllys, L., Thell, A., *et al.* (2002) Phylogeny of the genus *Cladonia* s.lat. (Cladoniaceae, Ascomycetes) inferred from molecular, morphological, and chemical data. *Cladistics*. 18 (3), 237–278.

Su, M., Liu, H., Liang, X., Gui, L., *et al.* (2018) Dietary Analysis of Marine Fish Species: Enhancing the Detection of Prey-Specific DNA Sequences via High-Throughput Sequencing Using Blocking Primers. *Estuaries and Coasts*. 41 (2), 560–571.

Veldman, S., Otieno, J., Gravendeel, B., van Andel, T., *et al.* (2014) Conservation of Endangered Wild Harvested Medicinal Plants: Use of DNA Barcoding. In: *Novel Plant Bioresources: Applications in Food, Medicine and Cosmetics*. . pp. 81–88.

Wang, X.C., Liu, C., Huang, L., Bengtsson-Palme, J., *et al.* (2015) ITS1: A DNA barcode better than ITS2 in eukaryotes? *Molecular Ecology Resources*. 15 (3), 573–586.

Watkinson, S.C. (2015) *Mutualistic Symbiosis Between Fungi and Autotrophs*. Third Edit. . Elsevier Ltd. Available from: doi:10.1016/B978-0-12-382034-1.00007-4.

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics. *PCR Protocols*. 315–322.

Wolfe, K.H., Li, W.-H. & Sharp, P.M. (1987) Rates of Nucleotide Substitution Vary Greatly among Plant Mitochondrial, Chloroplast, and Nuclear DNAs. *Proceedings of the National Academy of Sciences of the United States of America*. 84 (24), 9054–9058.

Won, H. & Renner, S.S. (2003) Horizontal gene transfer from flowering plants to *Gnetum*. *Proceedings of the National Academy of Sciences of the United States of America*. 100 (19), 10824–10829.

Wornik, S. & Grube, M. (2010) Joint dispersal does not imply maintenance of partnerships in lichen symbioses. *Microbial Ecology*. 59 (1), 150–157.

Xu, M., Heidmarsson, S., Thorsteinsdottir, M., Kreuzer, M., *et al.* (2018) Authentication of Iceland Moss (*Cetraria islandica*) by UPLC-QToF-MS chemical profiling and DNA barcoding. *Food Chemistry*. 245, 989–996.

Yahr, R., Schoch, C.L. & Dentinger, B.T.M. (2016) Scaling up discovery of hidden diversity in fungi: Impacts of barcoding approaches. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 371 (1702).

Yahr, R., Vilgalys, R. & Depriest, P.T. (2006) Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. *New Phytologist*. 171 (4), 847–860.

Yahr, R., Vilgalys, R. & Depriest, P.T. (2004) Strong fungal specificity and selectivity for algal symbionts in Florida scrub *Cladonia* lichens. *Molecular Ecology*. 13 (11), 3367–3378.

Zhang, T., Wei, X.L., Zhang, Y.Q., Liu, H.Y., *et al.* (2015) Diversity and distribution of lichen-associated fungi in the Ny-Ålesund Region (Svalbard, High Arctic) as revealed by 454 pyrosequencing. *Scientific Reports*. 5 (August), 1–10.

Zuccaro, A., Schoch, C.L., Spatafora, J.W., Kohlmeyer, J., *et al.* (2008) Detection and identification of fungi intimately associated with the brown seaweed *Fucus serratus*. *Applied and Environmental Microbiology*. 74 (4), 931–941.

Appendices

Appendix 1. List of Field Collections

All field collections. Collections marked with an asterisk failed to sequence. [1] Represents samples sourced from commercial reindeer feed imported from Finland.

EDNA number	Species	Authority	Collector	Coll. No.	Locality
EDNA18-0051282	*Cladonia stellaris**[1]	(Opiz) Pouzar & Vězda	J. Howieson	001	Finland
EDNA18-0051283	*Cladonia stygia**[1]	(Fr.) Ruoss	J. Howieson	002	Finland
EDNA18-0051284	*Cetraria islandica*	(L.) Ach.	J. Howieson	003	Cairngorms, Scotland
EDNA18-0051285	*Cladonia arbuscula*	(Wallr.) Rabenh.	J. Howieson	004	Cairngorms, Scotland
EDNA18-0051286	*Cladonia bellidiflora*	(Ach.) Schaer.	J. Howieson	005	Cairngorms, Scotland
EDNA18-0051287	*Cladonia rangiferina*	(L.) Weber ex F. H. Wigg	J. Howieson	006	Cairngorms, Scotland
EDNA18-0051288	*Cladonia diversa*	Asperges ex Stenroos	J. Howieson	007	Cairngorms, Scotland
EDNA18-0051289	*Cladonia floerkeana*	(Fr.) Flörke	J. Howieson	008	Cairngorms, Scotland
EDNA18-0051381	*Flavocetraria nivalis*	(L.) Kärnefelt & Thell	J. Howieson	009	Cairngorms, Scotland
EDNA18-0051382	*Umbilicaria torrefacta*	(Lightf.) Schrad.	J. Howieson	010	Cairngorms, Scotland
EDNA18-0051383	*Umbilicaria proboscidea*	(L.) Schrad.	J. Howieson	011	Cairngorms, Scotland
EDNA18-0051384	*Cetraria aculeata*	(Schreb.) Fr.	J. Howieson	012	Cairngorms, Scotland
EDNA18-0051385	*Ochrolechia frigida*	(Sw.) Lynge	J. Howieson	013	Cairngorms, Scotland
EDNA18-0051386	*Cladonia bellidiflora*	(Ach.) Schaer.	J. Howieson	014	Cairngorms, Scotland
EDNA18-0051387	*Cladonia floerkeana*	(Fr.) Flörke	J. Howieson	015	Cairngorms, Scotland
EDNA18-0051388	*Thamnolia vermicularis**	(Sw.) Ach. ex Schaer.	J. Howieson	016	Cairngorms, Scotland
EDNA18-0051389	*Platismatia glauca*	(L.) Culb. & C. Culb.	J. Howieson	017	Cairngorms, Scotland
EDNA18-0051390	*Pseudephebe pubescens*	(L.) Choisy	J. Howieson	018	Cairngorms, Scotland
EDNA18-0051391	*Cladonia arbuscula*	(Wallr.) Rabenh.	J. Howieson	019	Cairngorms, Scotland
EDNA18-0051392	*Cladonia bellidiflora*	(Ach.) Schaer.	J. Howieson	020	Cairngorms, Scotland
EDNA18-0051393	*Cladonia ciliata var. ciliata*	Stirt.	J. Howieson	021	Cairngorms, Scotland
EDNA18-0051394	*Umbilicaria torrefacta*	(Lightf.) Schrad.	J. Howieson	022	Cairngorms, Scotland
EDNA18-0051395	*Pseudevernia* sp.	Zopf.	J. Howieson	023	Cairngorms, Scotland
EDNA18-0051396	*Melanelia stygia*	(L.) Essl	J. Howieson	024	Cairngorms, Scotland
EDNA18-0051532	*Cladonia portentosa*	(Dufour) Coem.	J. Howieson	025	Cairngorms, Scotland
EDNA18-0051533	*Umbilicaria cylindrica*	(L.) Delise ex Duby	J. Howieson	026	Cairngorms, Scotland
EDNA18-0051534	*Cladonia squamosa**	(Scop.) Hoffm.	J. Howieson	027	Cairngorms, Scotland
EDNA18-0051535	*Cornicularia normaerica**	(Gunn.) Du Rietz	J. Howieson	028	Cairngorms, Scotland
EDNA18-0051536	*Cladonia portentosa*	(Dufour) Coem.	J. Howieson	029	Cairngorms, Scotland
EDNA18-0051537	*Cetraria muricata**	(Ach.) Eckfeldt	R. Yahr	030	Kindrogan, Scotland

EDNA18-0051538	*Bryoria* sp.	Brodo & D.Hawksw.	J. Howieson	031	Cairngorms, Scotland
EDNA18-0051539	*Cladonia squamosa**	(Scop.) Hoffm.	J. Howieson	032	Cairngorms, Scotland
EDNA18-0051602	*Pseudephebe pubescens*	(L.) Choisy	J. Howieson	033	Cairngorms, Scotland
EDNA18-0051603	*Hypogymnia physodes**	(L.) Nyl.	J. Howieson	034	Cairngorms, Scotland
EDNA18-0051604	*Cladonia arbuscula*	(Wallr.) Rabenh.	J. Howieson	035	Cairngorms, Scotland
EDNA18-0051605	*Cladonia uncialis*	(L.) Weber ex F. H. Wigg.	R. Yahr	036	Kindrogan, Scotland
EDNA18-0051606	*Cetraria muricata*	(Ach.) Eckfeldt	R. Yahr	037	Kindrogan, Scotland
EDNA18-0051607	*Sphaerophorus* sp.*	Pers.	J. Howieson	038	Cairngorms, Scotland
EDNA18-0051608	*Cladonia rangiferina*	(L.) Weber ex F. H. Wigg	R. Yahr	039	Kindrogan, Scotland
EDNA18-0051609	*Cladonia portentosa*	(Dufour) Coem.	R. Yahr	040	Kindrogan, Scotland
EDNA18-0051610	*Cladonia arbuscula*	(Wallr.) Rabenh.	J. Howieson	041	Cairngorms, Scotland
EDNA18-0051611	*Cladonia ciliata var. tenuis*	(Flörke) Ahti	R. Yahr	042	Kindrogan, Scotland
EDNA18-0051612	*Cladonia arbuscula**	(Wallr.) Rabenh.	R. Yahr	043	Kindrogan, Scotland
EDNA18-0051613	*Umbilicaria polyphylla*	(L.) Baumg.	J. Howieson	044	Cairngorms, Scotland
EDNA18-0051614	*Alectoria negricans*	(Ach.) Nyl.	J. Howieson	045	Cairngorms, Scotland
EDNA18-0051615	*Cladonia squamosa*	(Scop.) Hoffm.	J. Howieson	046	Cairngorms, Scotland
EDNA18-0051616	*Bryoria* sp.	Brodo & D.Hawksw.	R. Yahr	047	Kindrogan, Scotland
EDNA18-0051617	*Cladonia rangiferina*	(L.) Weber ex F. H. Wigg	R. Yahr	048	Kindrogan, Scotland
EDNA18-0051618	*Cladonia stellaris*	(Opiz) Pouzar & Vězda	J. Howieson	049	Cairngorms, Scotland
EDNA18-0051619	*Cladonia uncialis*	(L.) Weber ex F. H. Wigg.	J. Howieson	050	Cairngorms, Scotland
EDNA18-0051620	*Platismatia glauca*	(L.) Culb. & C. Culb.	J. Howieson	051	Cairngorms, Scotland
EDNA18-0051621	*Cladonia* sp.	P. Browne	R. Yahr	052	Kindrogan, Scotland
EDNA18-0051622	*Cladonia arbuscula*	(Wallr.) Rabenh.	J. Howieson	053	Cairngorms, Scotland
EDNA18-0051623	*Cladonia uncialis*	(L.) Weber ex F. H. Wigg.	J. Howieson	054	Cairngorms, Scotland
EDNA18-0051624	*Sphaerophorus* sp.	Pers.	J. Howieson	055	Kindrogan, Scotland
EDNA18-0051625	*Cladonia gracilis*	(L.) Willd	R. Yahr	056	Kindrogan, Scotland
EDNA18-0051626	*Cladonia sulphurina*	(Michaux) Fr.	R. Yahr	057	Kindrogan, Scotland
EDNA18-0051627	*Cladonia arbuscula**	(Wallr.) Rabenh.	R. Yahr	058	Kindrogan, Scotland
EDNA18-0051710	*Cladonia coccifera*	(L.) Willd.	J. Howieson	059	Cairngorms, Scotland
EDNA18-0051711	*Cetraria islandica*	(L.) Ach.	J. Howieson	060	Cairngorms, Scotland
EDNA18-0051712	*Ochrolechia frigida*	(Sw.) Lynge	J. Howieson	061	Cairngorms, Scotland
EDNA18-0051713	*Cetraria aculeata*	(Schreb.) Fr.	J. Howieson	062	Cairngorms, Scotland
EDNA18-0051714	*Ochrolechia frigida*	(Sw.) Lynge	J. Howieson	063	Cairngorms, Scotland
EDNA18-0051715	*Ochrolechia frigida*	(Sw.) Lynge	J. Howieson	064	Cairngorms, Scotland
EDNA18-0051716	*Cetraria islandica*	(L.) Ach.	J. Howieson	065	Cairngorms, Scotland
EDNA18-0051717	*Ochrolechia androgyna*	(Hoffm.) Arnold	J. Howieson	066	Cairngorms, Scotland
EDNA18-0051718	*Umbilicaria torrefacta*	(Lightf.) Schrad.	J. Howieson	067	Cairngorms, Scotland
EDNA18-0051719	*Cladonia floerkeana*	(Fr.) Flörke	J. Howieson	068	Cairngorms, Scotland

EDNA18-0051720	*Cetraria aculeata*	(Schreb.) Fr.	J. Howieson	069	Cairngorms, Scotland
EDNA18-0051721	*Cetraria aculeata*	(Schreb.) Fr.	J. Howieson	070	Cairngorms, Scotland
EDNA18-0051722	*Cetraria aculeata*	(Schreb.) Fr.	J. Howieson	071	Cairngorms, Scotland
EDNA18-0051723	*Cetraria muricata*	(Ach.) Eckfeldt	J. Howieson	072	Cairngorms, Scotland
EDNA18-0051724	*Cladonia stygia**[1]	(Fr.) Ruoss	J. Howieson	073	Finland
EDNA18-0051725	*Cladonia rangiferina**[1]	(L.) Weber ex F. H. Wigg	J. Howieson	074	Finland
EDNA18-0051726	*Cladonia stellaris**[1]	(Opiz) Pouzar & Vězda	J. Howieson	075	Finland

Appendix 2, Sequences and Voucher Specimens used in the Barcoding Dataset

List of all voucher specimens that were used in the barcode study. Vouchers marked with an asterisk denote samples kept within a private collection.

Family	Species	Authority	Locality, Collection and Herbarium	EDNA number	GenBank Accession Number
Parmeliaceae	*Cetraria aculeata*	(Schreb.) Fr.	Scotland, *Howieson* 012(E)	EDNA18-0051384	
	Cetraria aculeata		Scotland, *Howieson* 062(E)	EDNA18-0051713	
	Cetraria aculeata		Scotland, *Howieson* 069(E)	EDNA18-0051720	
	Cetraria aculeata		Scotland, *Howieson* 070(E)	EDNA18-0051721	
	Cetraria aculeata		Scotland, *Howieson* 071(E)	EDNA18-0051722	
	Cetraria islandica	(L.) Ach.	Scotland, *Howieson* 003(E)	EDNA18-0051284	
	Cetraria islandica		Scotland, *Howieson* 060(E)	EDNA18-0051711	
	Cetraria islandica		Scotland, *Howieson* 065(E)	EDNA18-0051716	
	Cetraria muricata	(Ach.) Eckfeldt	Scotland, *Howieson* 037(E)	EDNA18-0051606	
	Cetraria muricata		Scotland, *Howieson* 072(E)	EDNA18-0051723	
	Cetraria muricata		Iceland, *Thell* ISL-9722(TUR)		AF228302.1
	Cetraria sepincola	(Ehrh.) Hale	Scotland, *Ellis & Coppins*L633: 5(E)	EDNA09-01467	FR799152.1
	Cetraria sepincola		Scotland, *Ellis & Coppins* L632: 4(E)	EDNA09-01570	FR799151.1
	Cetraria sepincola		Scotland, *Ellis & Coppins* L604: 225(E)	EDNA09-01580	FR799153.1
Cladoniaceae	*Cladonia arbuscula*	(Wallr.) Rabenh.	Scotland, *Howieson* 004(E)	EDNA18-0051285	
	Cladonia arbuscula		Scotland, *Howieson* 019(E)	EDNA18-0051391	
	Cladonia arbuscula		Scotland, *Howieson* 035(E)	EDNA18-0051604	

Cladonia arbuscula		Scotland, *Howieson* 043(E)	EDNA18-0051612	
Cladonia arbuscula		Scotland, *Howieson* 053(E)	EDNA18-0051622	
Cladonia arbuscula		Finland, *Myllys* 215(TUR)		AY170789.1
Cladonia bellidiflora	(Ach.) Schaer.	Scotland, *Yahr & Kwella* 22(E)	EDNA11-02006	
Cladonia bellidiflora		Scotland, *Yahr & Kwella* 22(E)	EDNA11-02006b	
Cladonia bellidiflora		Scotland, *Howieson* 005(E)	EDNA18-0051286	
Cladonia bellidiflora		Scotland, *Howieson* 014(E)	EDNA18-0051386	
Cladonia bellidiflora		Scotland, *Howieson* 020(E)	EDNA18-0051392	
Cladonia bellidiflora		Finland, *Stenroos* 5152(TUR)		AF453700.1
Cladonia borealis	Stenroos	Scotland, *Yahr* 5221(E)	EDNA10_02718	
Cladonia borealis		Scotland, *Yahr* 5221(E)	EDNA11_00616	
Cladonia borealis		Scotland, *Yahr* 5211(E)	EDNA11_01998	
Cladonia borealis		Finland, *Stenroos* 5157(TUR)		AF454434.1
Cladonia borealis		Iceland, *Ahti* 54928(H)		AF454435.1
Cladonia ciliata var. ciliata	Stirt.	Scotland, *Howieson* 021(E)	EDNA18-0051393	
Cladonia ciliata var. ciliata		Ireland, *Rikkinen* 2000(TUR)		AF458310
Cladonia ciliata var. tenuis	(Flörke) Ahti	Scotland, *Howieson* 042(E)	EDNA18-0051611	
Cladonia ciliata var. tenuis		Portugal, *Aht & Burgaz* 55883(H)		AF458311
Cladonia coccifera	(L.) Willd.	Scotland, *Yahr* 5289(E)	EDNA10_03998	
Cladonia coccifera		Scotland, *Yahr* 5289(E)	EDNA10_03998b	
Cladonia coccifera		Scotland, *Yahr & Kwella* 8(E)	EDNA11_02002b	
Cladonia coccifera		Scotland, *Yahr & Kwella* 18(E)	EDNA11_02003a	
Cladonia coccifera		Scotland, *Yahr & Kwella* 18(E)	EDNA11_02003b	

Cladonia coccifera		Scotland, *Yahr & Kwella* 18(E)	EDNA11_02003c	
Cladonia coccifera		Scotland, *Howieson* 059(E)	EDNA18-0051710	
Cladonia coccifera		Finland, *Stenroos* 5155(TUR)		AF454436.1
Cladonia coccifera		Czech, *Peksa* 84(PRC)		HE611154
Cladonia coccifera		Austria, *Hafellner* 66608(GZU)		HE611155
Cladonia coccifera		Austria, *Hafellner* 66785(GZU)		HE611156
Cladonia coccifera		Austria, *Hafellner* 66214(GZU)		HE611157
Cladonia coccifera		Czech Republic, *Bouda*778*		HE611158
Cladonia coccifera		Czech, *Peksa* 359(PRC)		HE611159
Cladonia coccifera		Czech, Steinová 43(PRC)		HE611160
Cladonia coccifera		Czech Republic, *Steinová* 81(PRC)		HE611161
Cladonia coccifera		Spain, *Steinová* 401(PRC)		HE611162
Cladonia coccifera		Austria, *Steinová* 242(PRC)		HE611163
Cladonia coccifera		Norway, *Steinová* 332(PRC)		HE611171
Cladonia coccifera		Finland, *Steinová* 334(PRC)		HE611172
Cladonia diversa	Asperges ex. Stenroos	Scotland, *Ellis & Harrold* s.n.(E)	EDNA09_02362	
Cladonia diversa		Scotland, *Ellis & Harrold* s.n.(E)	EDNA09_02386	
Cladonia diversa		Scotland, *Ellis & Harrold* L696: 45(E)	EDNA09-02362	FR799158.1
Cladonia diversa		Scotland, *Ellis & Harrold* L697: 46(E)	EDNA09-02386	FR799159.1
Cladonia diversa		Scotland, *Ellis & Harrold* L698: 47(E)	EDNA09-02387a	
Cladonia diversa		Scotland, *Ellis & Harrold* L698: 47(E)	EDNA09-02387b	FR799160.1
Cladonia diversa		Scotland, *Yahr* 5195(PRC)	EDNA10_02715	
Cladonia diversa		Scotland, *Yahr* 5195(PRC)	EDNA10_02715b	

Cladonia diversa		Scotland, *Yahr* 5231(E)	EDNA10_03997	
Cladonia diversa		Scotland, *Yahr & Kwella* 13(E)	EDNA11_02005a	
Cladonia diversa		Scotland, *Yahr & Kwella* 13(E)	EDNA11_02005b	
Cladonia diversa		Scotland, *Yahr & Kwella* 13(E)	EDNA11_02005c	
Cladonia diversa		Scotland, *Howieson* 007(E)	EDNA18-0051288	
Cladonia diversa		Czech Republic, *Bouda*777*		HE611164
Cladonia diversa		Portugal, *Steinová* 400(PRC)		HE611165
Cladonia diversa		Denmark, *Vondrák* 6242(CBFS)		HE611166
Cladonia diversa		Belgium, *Steinová* 351(PRC)		HE611167
Cladonia diversa		Belgium, *Steinová* 352(PRC)		HE611168
Cladonia diversa		Netherlands, *Steinová* 353(PRC)		HE611169
Cladonia floerkeana	(Fr.) Flörke	Scotland, *Howieson* 008(E)	EDNA18-0051289	
Cladonia floerkeana		Scotland, *Howieson* 015(E)	EDNA18-0051387	
Cladonia floerkeana		Scotland, *Howieson* 068(E)	EDNA18-0051719	
Cladonia floerkeana		Finland, *Stenroos* 5582(TUR)		AF453697.1
Cladonia gracilis	(L.) Willd.	Scotland, *Ellis & Harrold* L700: 87	EDNA09-02363	FR799162
Cladonia gracilis		Scotland, *Ellis & Harrold* L701: 90	EDNA09-02398	FR799163
Cladonia gracilis		Scotland, *Ellis & Harrold* L702: 100	EDNA09-02404	FR799164
Cladonia gracilis		Scotland, *Howieson* 056(E)	EDNA18-0051625	
Cladonia portentosa	(Dufour) Coem.	Scotland, *Ellis & Davies* L673: 58(E)	EDNA09-02090	FR799167.1
Cladonia portentosa		Scotland, *Ellis & Harrold* L704: 48(E)	EDNA09-02388	FR799166.1
Cladonia portentosa		Scotland, *Ellis & Harrold* L705: 115(E)	EDNA09-02413	FR799168.1
Cladonia portentosa		Scotland, *Howieson* 025(E)	EDNA18-0051532	

	Cladonia portentosa		Scotland, *Howieson* 029(E)	EDNA18-0051536	
	Cladonia portentosa		Scotland, *Howieson* 040(E)	EDNA18-0051609	
	Cladonia rangiferina	(L.) Weber ex F. H. Wigg.	Scotland, *Howieson* 006(E)	EDNA18-0051287	
	Cladonia rangiferina		Scotland, *Howieson* 039(E)	EDNA18-0051608	
	Cladonia rangiferina		Luxembourg, *Cezanne & Eichler* 9402 (FR)		KT792792.1
	Cladonia squamosa	(Scop.) Hoffm.	Scotland, *Howieson* 046(E)	EDNA18-0051615	
	Cladonia squamosa subsp. *squamosa*	(Nyl. ex Leight.) Hoffm.	Scotland, *Ellis & Harrold* L709: 92(E)	EDNA09-02365	FR799171.1
	Cladonia squamosa subsp. *squamosa*		Scotland, *Ellis & Harrold* L710: 95(E)	EDNA09-02401	FR799172.1
	Cladonia uncialis	(L.) Weber ex F. H. Wigg.	Scotland, *Howieson* 036(E)	EDNA18-0051605	
	Cladonia uncialis		Scotland, *Howieson* 050(E)	EDNA18-0051619	
	Cladonia uncialis		Scotland, *Howieson* 054(E)	EDNA18-0051623	
Pertusariaceae	*Ochrolechia androgyna*	(Hoffm.) Arnold	Scotland, *Ellis & Coppins* L551: 222(E)	EDNA09-01528	FR799238.1
	Ochrolechia androgyna		Scotland, *Ellis & Coppins* L552: 258(E)	EDNA09-01547	FR799239.1
	Ochrolechia androgyna		Scotland, *Ellis & Coppins* L553:294(E)	EDNA09-01558	FR799240.1
	Ochrolechia androgyna		Scotland, *Howieson* 066(E)	EDNA18-0051717	
	Ochrolechia frigida	(Sw.) Lynge	Scotland, *Howieson* 013(E)	EDNA18-0051385	
	Ochrolechia frigida		Scotland, *Howieson* 061(E)	EDNA18-0051712	
	Ochrolechia frigida		Scotland, *Howieson* 063(E)	EDNA18-0051714	

	Ochrolechia frigida		Scotland, *Howieson* 064(E)	EDNA18-0051715	
	Ochrolechia microstictoides	Räsänen	Scotland, *Ellis & Coppins* L554: 259(E)	EDNA09-01548	FR799241.1
	Ochrolechia microstictoides		Scotland, *Ellis & Coppins* L555: 280(E)	EDNA09-01589	FR799242.1
	Ochrolechia microstictoides		Scotland, *Ellis & Coppins* L556: 284(E)	EDNA09-01590	FR799243.1
	Ochrolechia szatalaensis	Vers.	Scotland, *Ellis & Davies* L681: 61(E)	EDNA09-02104	FR799244.1
	Ochrolechia szatalaensis		Scotland, *Ellis & Harrold* L752: 151(E)	EDNA10-00041	FR799245.1
	Ochrolechia szatalaensis		Scotland, *Ellis & Harrold* L753: 154(E)	EDNA10-00042	FR799246.1
Umbilicariaceae	*Umbilicaria cylindrica*	(L.) Delise ex Duby	Scotland, *Ellis & Harrold* L723: 81b(E)	EDNA10-00738	FR799300.1
	Umbilicaria cylindrica		Scotland, *Ellis & Harrold* L730: 89a(E)	EDNA10-00743	FR799306.1
	Umbilicaria cylindrica		Scotland, *Howieson* 026(E)	EDNA18-0051533	
	Umbilicaria polyphylla	(L.) Baumg.	Scotland, *Ellis & Harrold* L724:82(E)	EDNA09-02392	FR799301.1
	Umbilicaria polyphylla		Scotland, *Ellis & Harrold* L725: 81a(E)	EDNA10-00737	FR799302.1
	Umbilicaria polyphylla		Scotland, *Howieson* 044(E)	EDNA18-0051613	
	Umbilicaria proboscidea	(L.) Schrad.	Scotland, *Ellis & Harrold* L726: 72(E)	EDNA09-02390	FR799303.1
	Umbilicaria proboscidea		Scotland, *Ellis & Harrold* L728:109(E)	EDNA09-02408	FR799304.1
	Umbilicaria proboscidea		Scotland, *Ellis & Harrold* L729: 81c(E)	EDNA10-00739	FR799305.1
	Umbilicaria proboscidea		Scotland, *Howieson* 011(E)	EDNA18-0051383	
	Umbilicaria torrefacta	(Lightf.) Schrad.	Scotland, *Howieson* 010(E)	EDNA18-0051382	
	Umbilicaria torrefacta		Scotland, *Howieson* 022(E)	EDNA18-0051394	
	Umbilicaria torrefacta		Scotland, *Howieson* 067(E)	EDNA18-0051718	

Appendix 3. Thin Layer Chromatography Data.

TLC information for the *Cladonia* plate E619. Species marked with an asterisk were not identified or due to poor DNA or sample quality were removed from the final dataset. Chemical presence is indicated with "+".

Plate	Lane	Species	Collection number	EDNA	Substance						
					Norstictic Acid	Atranorin	Squamatic acid	Usnic acid	Porphyrilic acid	Barbatic acid	Zeorin
E619	1	*C. bellidiflora*	005	EDNA18-0051286			+	+			
	2	*C. diversa*	007	EDNA18-0051288				+	+		+
	3	Control	N/A	N/A	+	+					
	4	*C. floerkeana*	008	EDNA18-0051289						+	
	5	*C. bellidiflora*	014	EDNA18-0051386			+	+			
	6	*C. floerkeana*	015	EDNA18-0051387						+	
	7	*C. bellidiflora*	020	EDNA18-0051392			+				
	8	*C. portentosa*	046	EDNA18-0051615							
	9	*C. rangiferina*	048	EDNA18-0051617		+					
	10	*C. stellaris*	049	EDNA18-0051618				+			
	11	*C. uncialis*	050	EDNA18-0051619			+	+			
	12	*Cladonia sp.* *	052	N/A							
	13	*C. arbuscula*	053	EDNA18-0051622				+			
	14	*C. uncialis*	054	EDNA18-0051623			+				
	15	*C. gracilis*	056	EDNA18-0051625				+			
	16	*C. sulphurina**	057	EDNA18-0051626			+	+			
	17	Control	N/A	N/A	+	+					
	18	*C. arbuscula**	058	N/A				+			
	19	N/A	N/A	N/A							

Appendix 3 (cont.)

TLC information from the *Cladonia* plate E620. Species marked with an asterisk were not identified or due to poor DNA or sample quality were removed from the final dataset. Chemical presence is indicated with "+".

Plate	Lane	Species	Collection number	EDNA	Substance Norstictic acid	Atranorin	Squamatic acid	Usnic acid	Perlatolic acid
E620	1	*C. stellaris**	001	N/A				+	
	2	*C. stygia**	002	N/A		+	+		
	3	Control	N/A	N/A	+	+		+	
	4	*C. arbuscula*	004	EDNA18-0051285				+	
	5	*C. arbuscula*	019	EDNA18-0051391				+	
	6	*C. rangiferina*	006	EDNA18-0051287					
	7	*C. ciliata var. ciliata*	021	EDNA18-0051393					
	8	*C. portentosa*	025	EDNA18-0051532				+	+
	9	*Cladonia squamosa**	027	N/A			+		
	10	*C. portentosa*	029	EDNA18-0051536				+	+
	11	*C. squamosa**	032	N/A			+		
	12	*C. arbuscula*	035	EDNA18-0051604				+	
	13	*C. uncialis*	036	EDNA18-0051605			+	+	
	14	*C. rangiferina*	039	EDNA18-0051608					
	15	*C. portentosa*	040	EDNA18-0051609				+	+
	16	*C. arbuscula*	041	EDNA18-0051610				+	
	17	Control	N/A	N/A	+	+			
	18	*C. ciliata var. tenuis*	042	EDNA18-0051611				+	
	19	*C. arbuscula**	043	EDNA18-0051612				+	

Appendix 3 (cont.)

TLC information for the *Cetraria* plate E621. Species marked with an asterisk were not identified or due to poor DNA or sample quality were removed from the final dataset. A known control for each species was used in the *Cetraria* plate alongside the chemical controls. Chemical presence is indicated with "+".

Plate	Lane	Species	Collection number	EDNA	Substance Norstictic Acid	Atranorin	Fumarprotocetraric acid
E621	1	N/A	N/A	N/A			
	2	N/A	N/A	N/A			
	3	Control	N/A	N/A	+	+	
	4	*C. aculeata*	012	EDNA18-0051384			
	5	*C. aculeata*	070	EDNA18-0051721			
	6	*C. aculeata*	071	EDNA18-0051722			
	7	*C. aculeata*	Control	N/A			
	8	*C. islandica*	003	EDNA18-0051284			+
	9	*C. islandica*	060	EDNA18-0051711			+
	10	*C. islandica*	065	EDNA18-0051716			+
	11	*C. islandica*	Control	N/A			+
	12	*C. muricata**	030	N/A			
	13	*C. muricata*	037	EDNA18-0051606			
	14	*C. muricata*	062	EDNA18-0051713			
	15	*C. aculeata*	069	EDNA18-0051720			
	16	*C. muricata*	072	EDNA18-0051723			
	17	Control	N/A	N/A	+	+	
	18	*C. muricata*	Control	N/A			
	19	N/A	N/A				

Appendix 3 (cont.)

TLC information for *Ochrolechia, Umbilicaria* and *Cladonia*, plate E622. Species marked with an asterisk were not identified or due to poor DNA or sample quality were removed from the final dataset. Chemical presence is indicated with "+".

					Substance								
Plate	**Lane**	**Species**	**Voucher**	**EDNA**	**Norstictic Acid**	**Atranorin**	**Gyrophoric acid**	**Umbilicaric acid**	**Usnic acid**	**Zeorin**	**Porphyrilic acid**	**Barbatic acid**	**Fumar-protocetraric acid**
E622	1	*O. androgyna*	066	EDNA18-0051717			+						
	2	*O. frigida*	013	EDNA18-0051385			+						
	3	Control	N/A	N/A	+	+							
	4	*O. frigida*	061	EDNA18-0051712			+						
	5	*O. frigida*	063	EDNA18-0051714			+						
	6	*O. frigida*	064	EDNA18-0051715			+						
	7	*U. cylindrica*	026	EDNA18-0051533									
	8	*U. polyphylla*	044	EDNA18-0051613			+	+					
	9	*U. proboscidea*	011	EDNA18-0051383	+		+						
	10	*U. torrefacta*	010	EDNA18-0051382			+						
	11	*U. torrefacta*	022	EDNA18-0051394			+						
	12	*U. torrefacta*	067	EDNA18-0051718			+						
	13	*C. coccifera*	059	EDNA18-0051710					+	+	+		
	14	*C. floerkeana*	068	EDNA18-0051719								+	
	15	*C. rangiferina**	074	N/A		+							+
	16	*C. stellaris**	075	N/A					+				
	17	Control	N/A	N/A	+	+							
	18	*C. stygia**	073	N/A		+							+
	19	N/A	N/A	N/A									

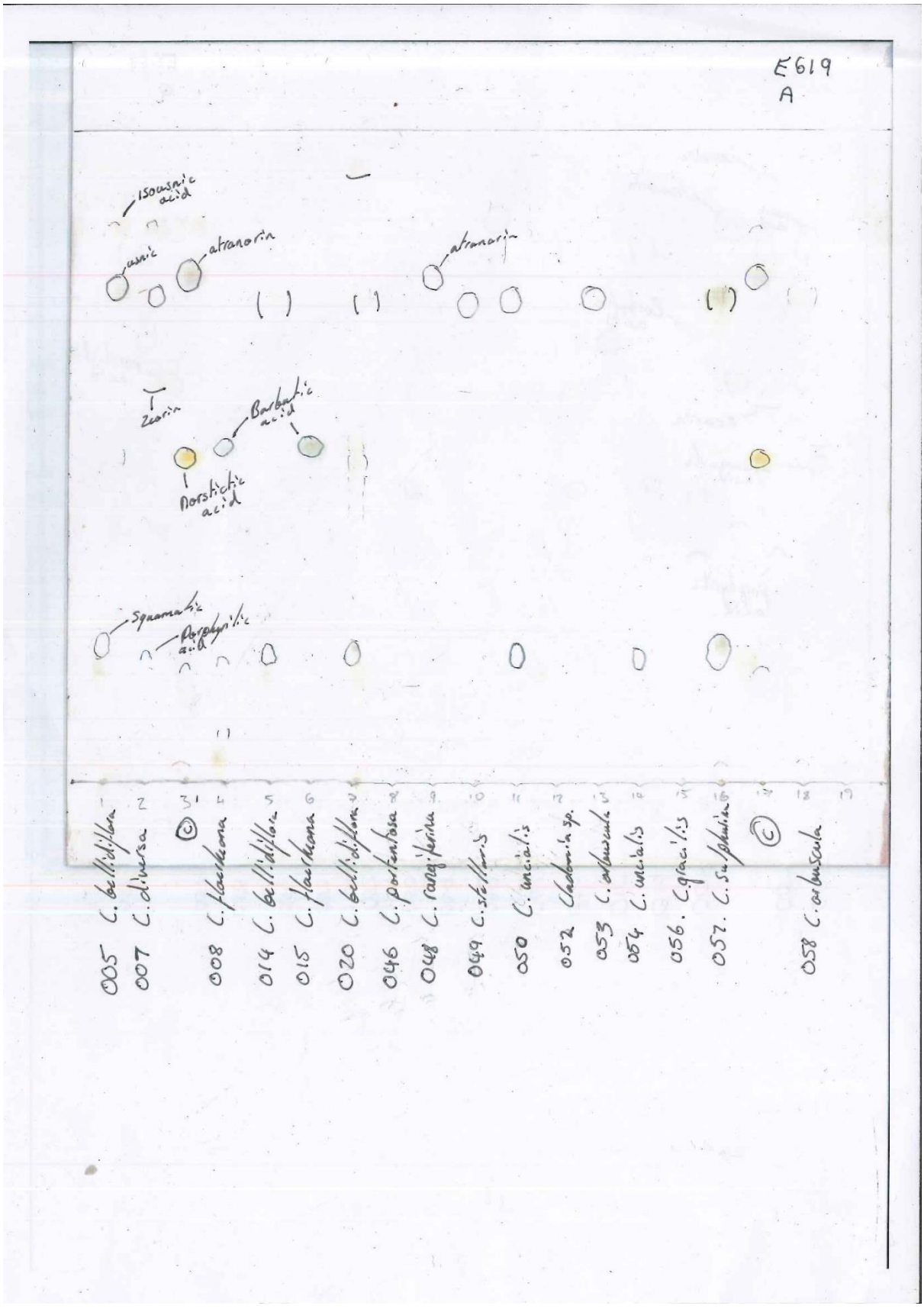
Appendix 3 (cont.)

TLC information for *Cladonia* spp. that failed initial TLC, plate E625. Chemical presence is indicated with "+".

Plate	Lane	Species	Collection number	EDNA	Substance	
					Fumarprotocetraric acid	Atranorin
E625	1	N/A	N/A	N/A		
	2	N/A	N/A	N/A		
	3	Control	N/A	N/A	+	
	4	*C. rangiferina*	048	EDNA18-0051617	+	+
	5	*C. ciliata var. ciliata*	021	EDNA18-0051393	+	+
	6	N/A	N/A	N/A		
	7	N/A	N/A	N/A		
	8	N/A	N/A	N/A		
	9	N/A	N/A	N/A		
	10	N/A	N/A	N/A		
	11	N/A	N/A	N/A		
	12	N/A	N/A	N/A		
	13	N/A	N/A	N/A		
	14	N/A	N/A	N/A		
	15	N/A	N/A	N/A		
	16	N/A	N/A	N/A		
	17	N/A	N/A	N/A		
	18	N/A	N/A	N/A		
	19	N/A	N/A	N/A		

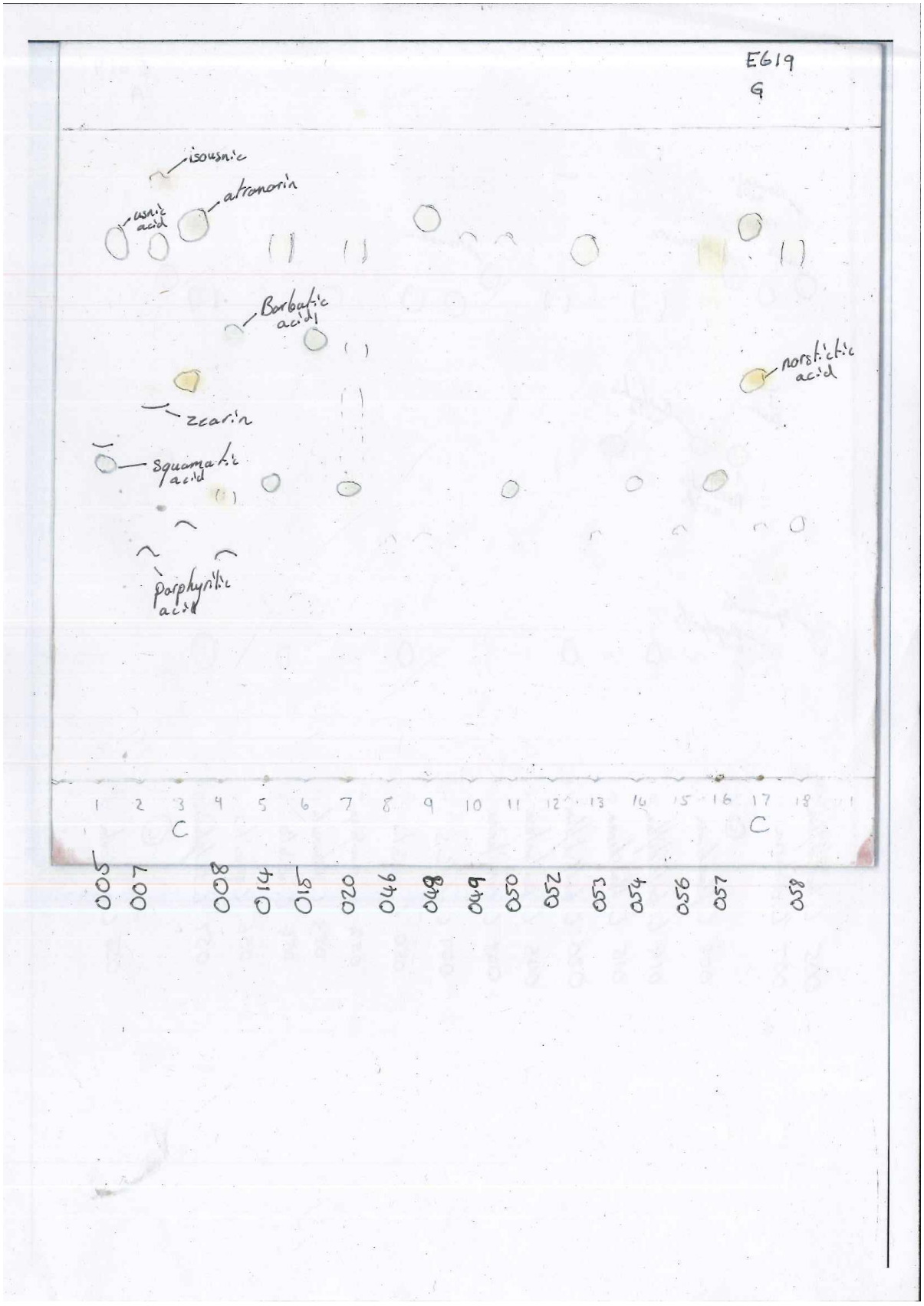
Appendix 4. Thin Layer Chromatography Plates

Plate E619, solvent system A.



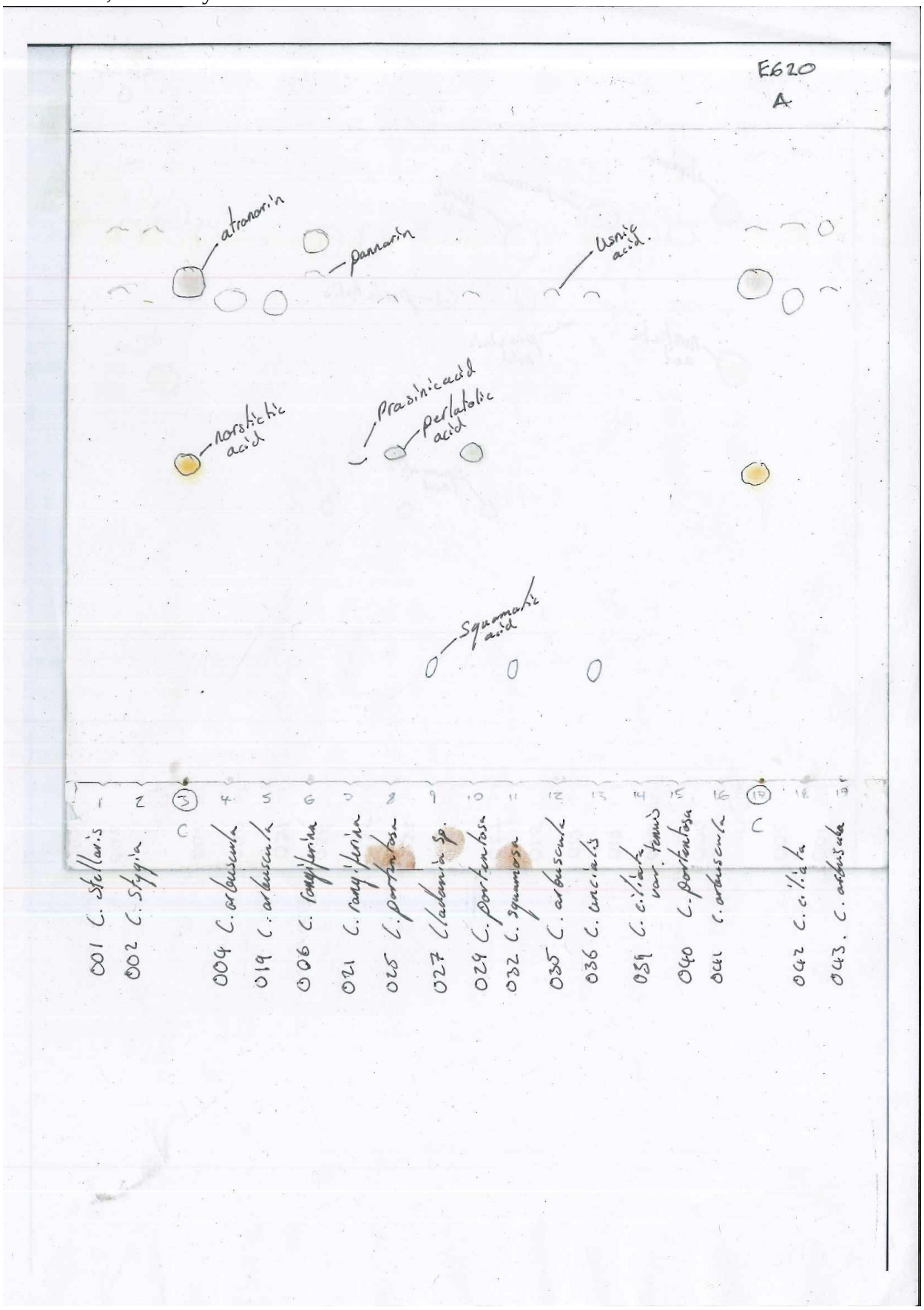
Appendix 4 (cont.)

Plate E619, solvent system G.



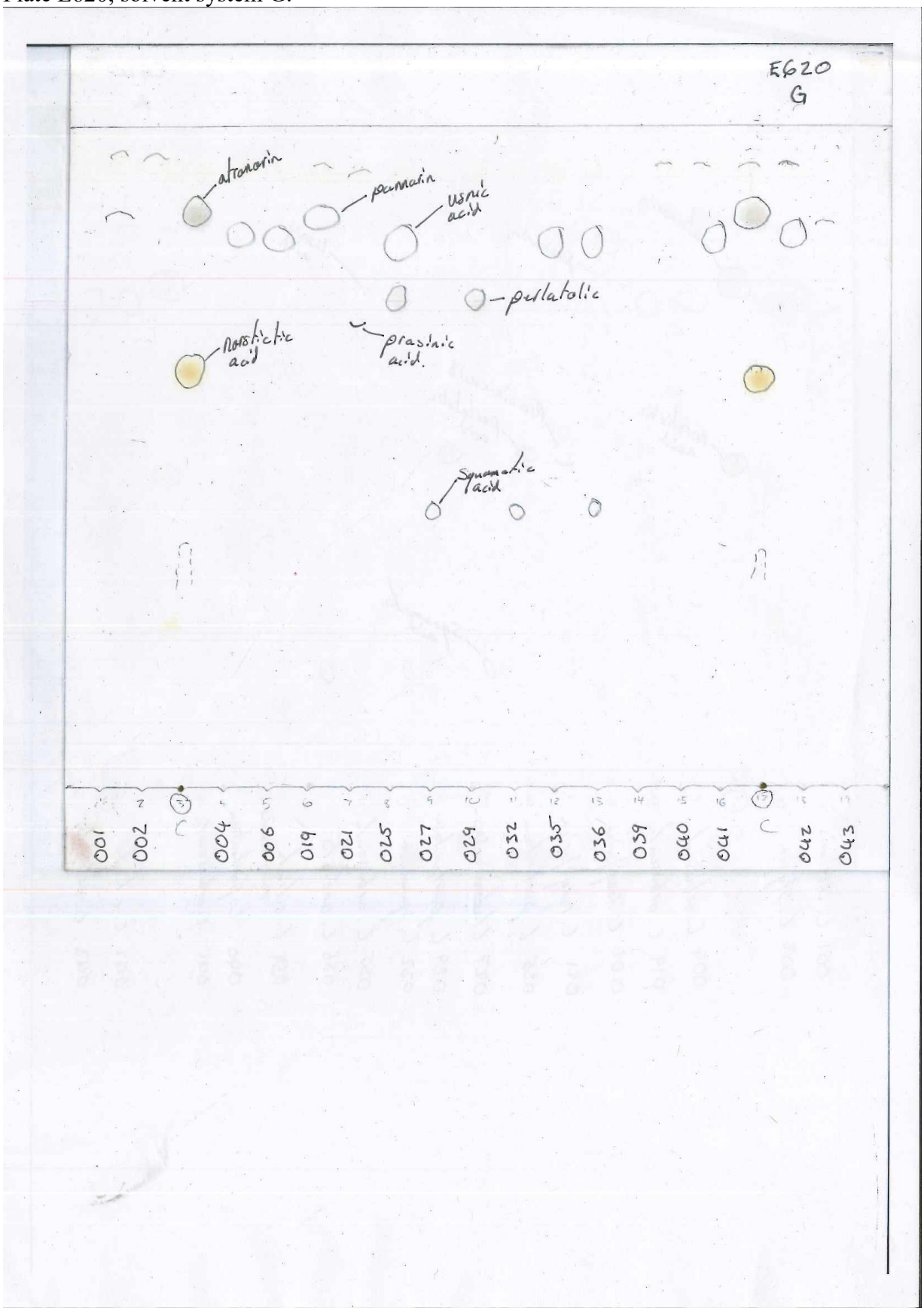
Appendix 4 (cont.)

Plate E620, solvent system A.



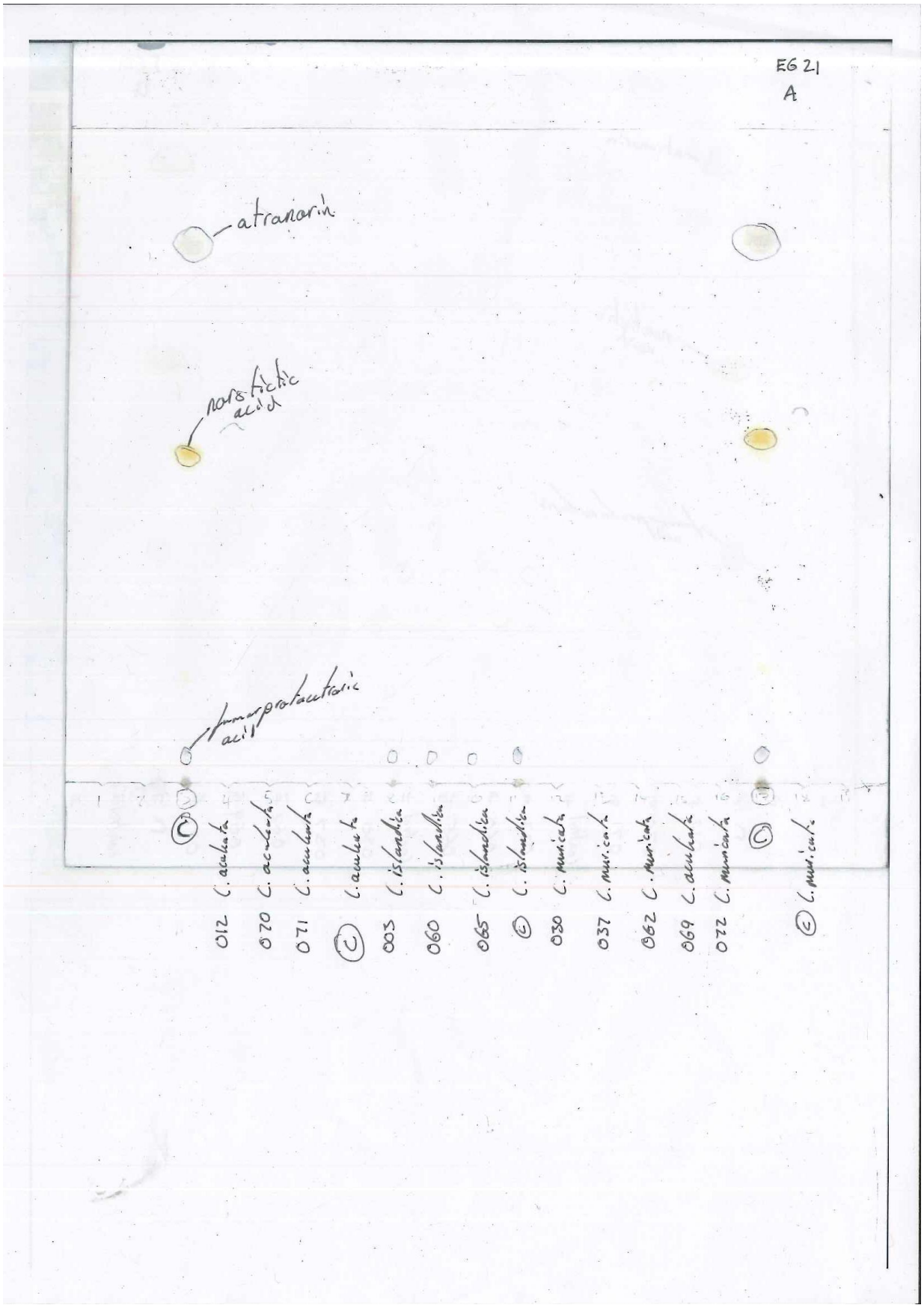
Appendix 4 (cont.)

Plate E620, solvent system G.



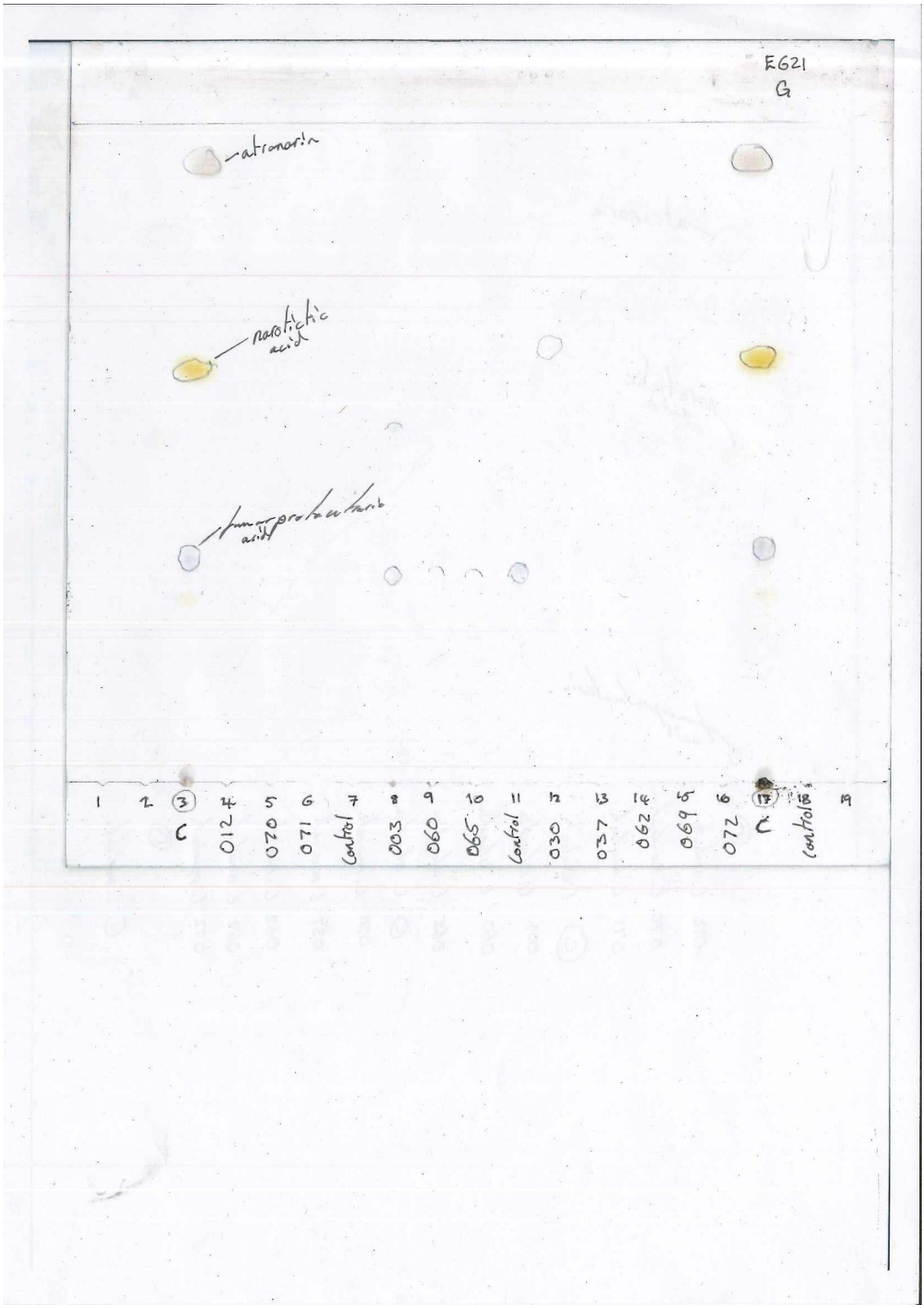
Appendix 4 (cont.)

Plate E621, solvent system A.



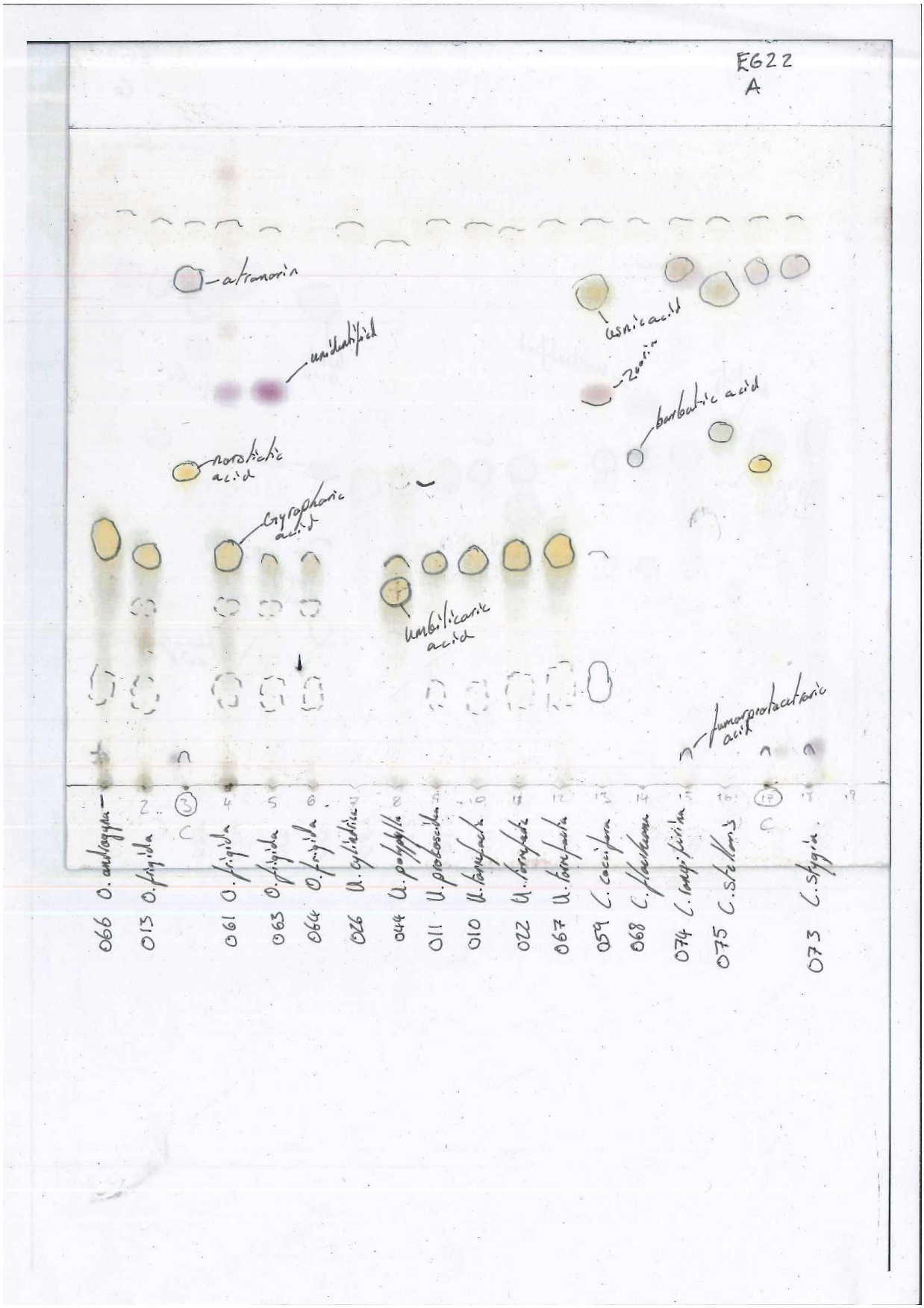
Appendix 4 (cont.)

Plate E621, solvent system G.



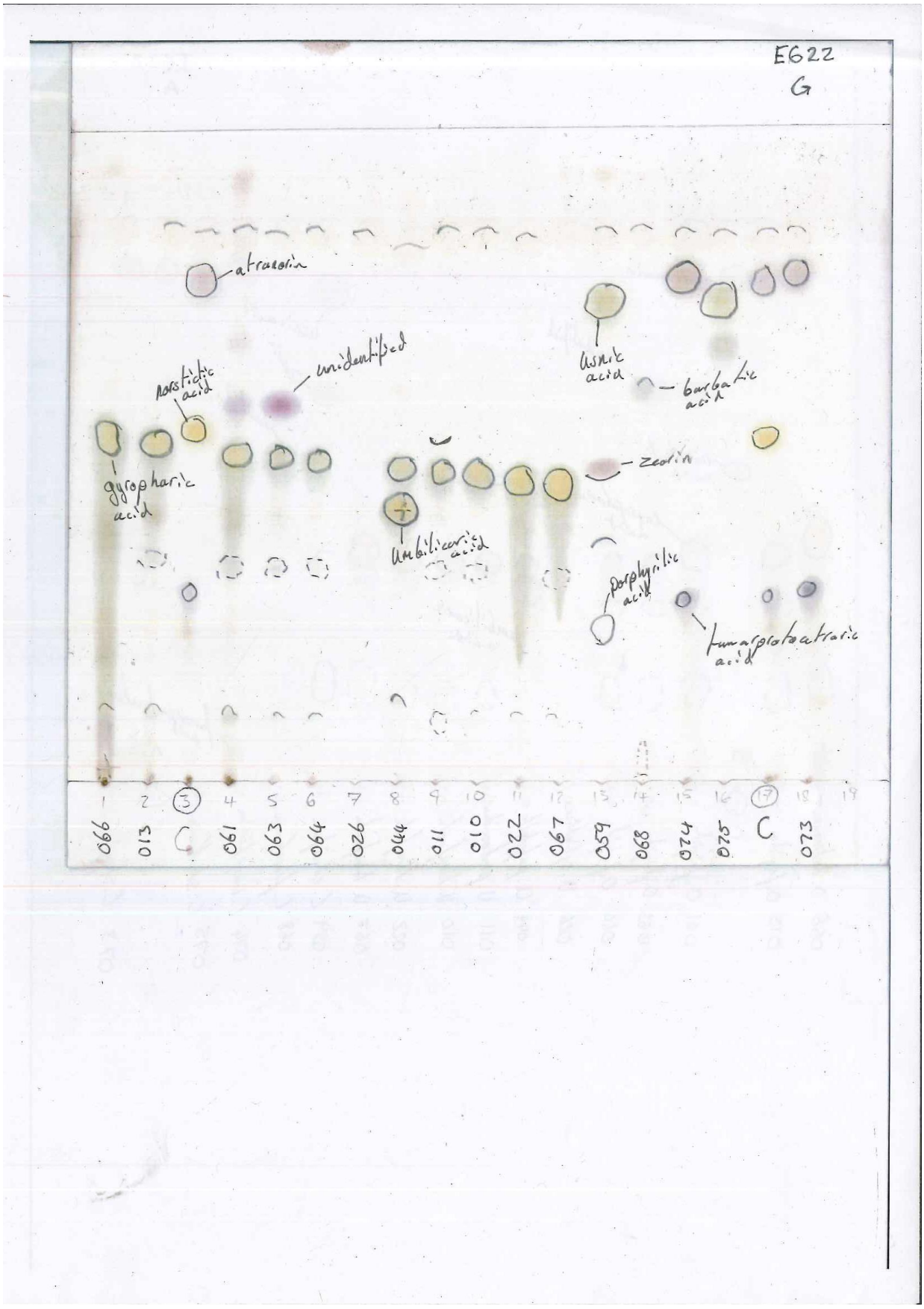
Appendix 4 (cont.)

Plate E622, solvent system A.



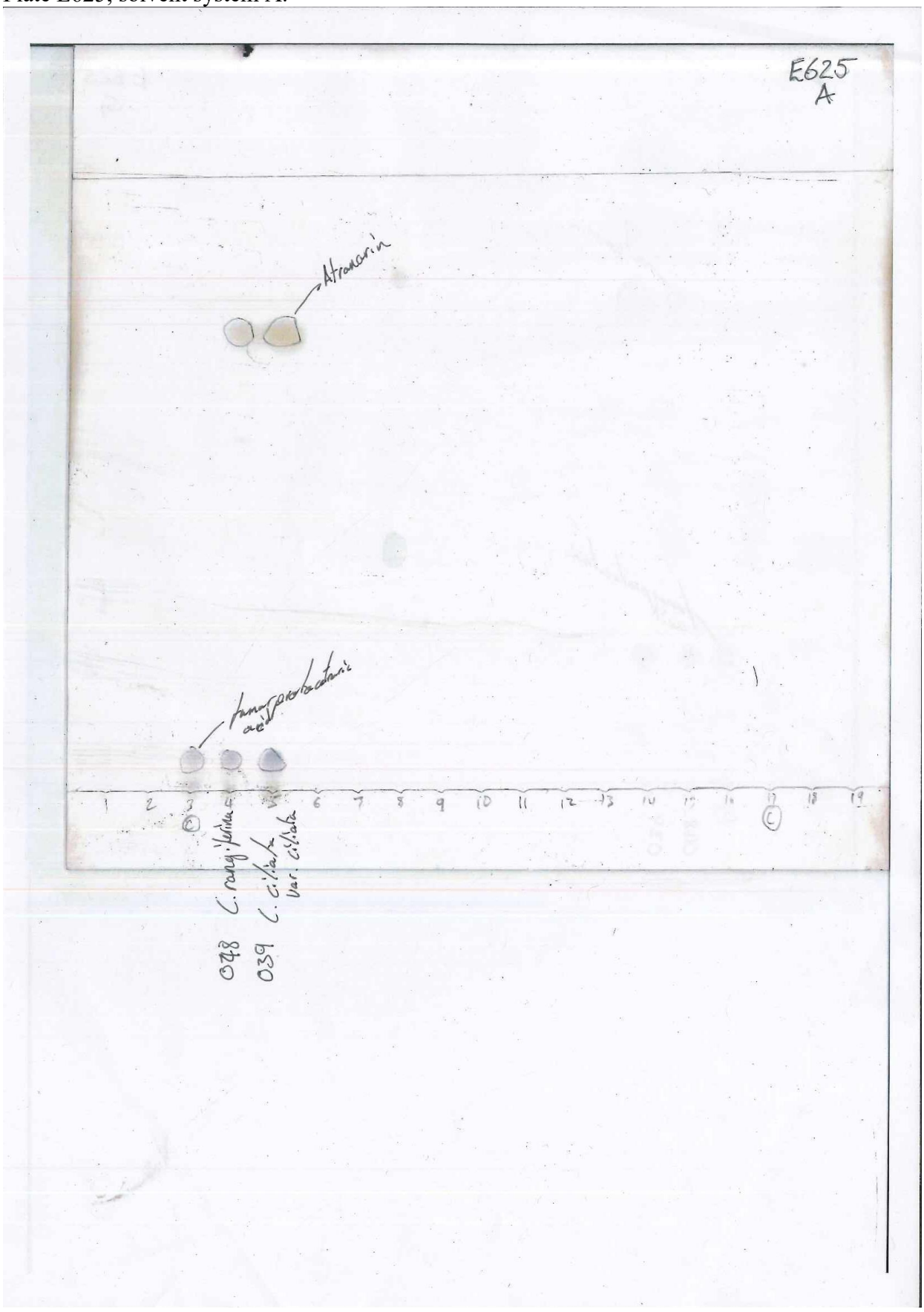
Appendix 4 (cont.)

Plate E622, solvent system G.



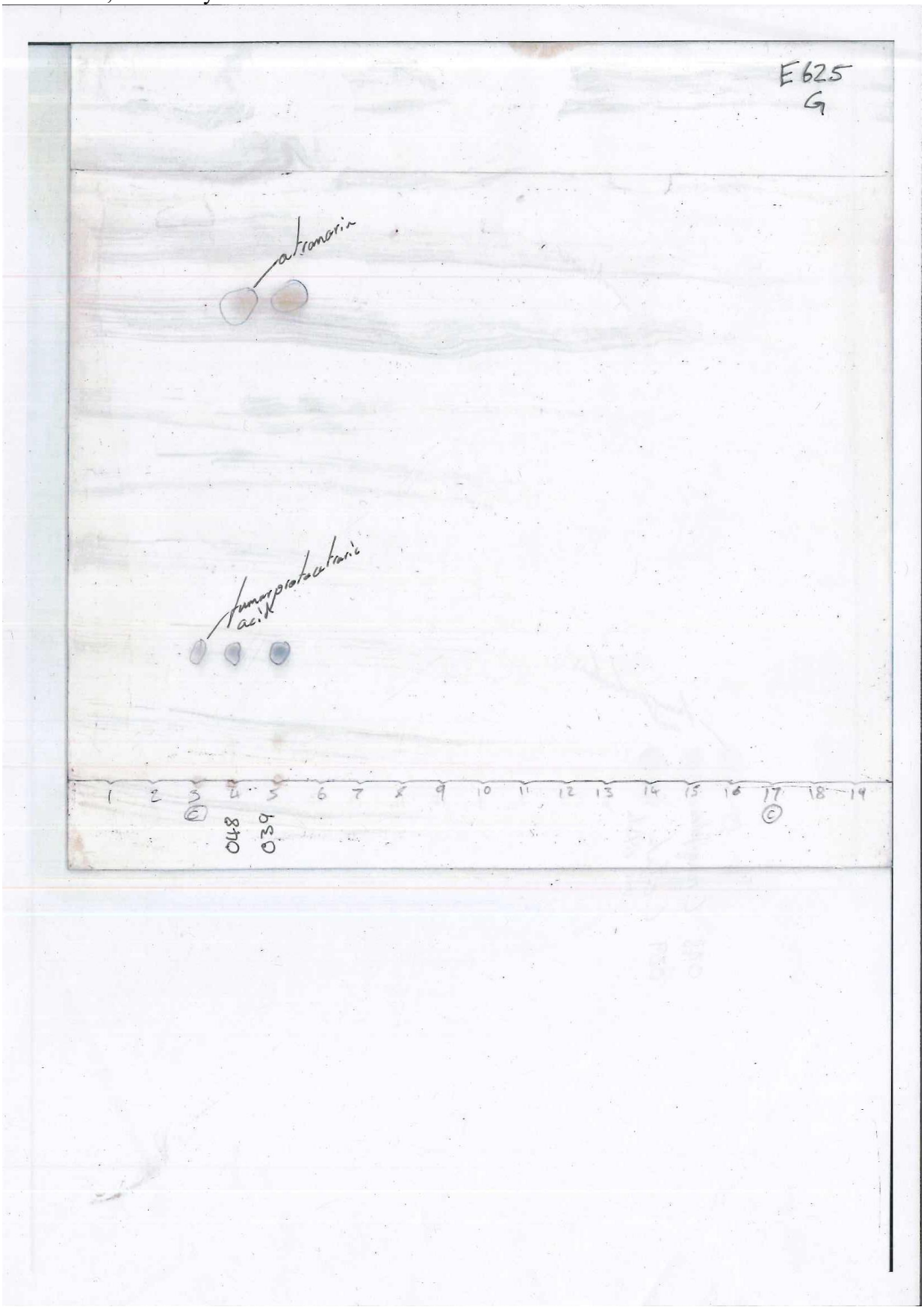
Appendix 4 (cont.)

Plate E625, solvent system A.



Appendix 4 (cont.)

Plate E625, solvent system G.



Appendix 5. Uncorrected (p) Distance Matrices

The uncorrected (p) distance matrix for all *Cetraria* sequences. Distances between conspecifics are highlighted with a black border.

	aculeata-EDNA18-0051721	aculeata-EDNA18-0051722	aculeata-EDNA18-0051720	aculeata-EDNA18-0051713	aculeata-EDNA18-0051384	muricata-EDNA18-0051606	muricata-EDNA18-0051723	muricata AF228302.1	islandica-EDNA18-0051284	islandica-EDNA18-0051711	islandica-EDNA18-0051716	sepincola-_FR799151.1_	sepincola-_FR799153.1	sepincola-_FR799152.1_
aculeata- EDNA18-0051721	0	0	0	0.006073	0.024292	0.046559	0.02834	0.02834	0.046559	0.040486	0.048583	0.076613	0.078629	0.078629
aculeata- EDNA18-0051722	0	0	0	0.006073	0.024292	0.046559	0.02834	0.02834	0.046559	0.040486	0.048583	0.076613	0.078629	0.078629
aculeata- EDNA18-0051720	0	0	0	0.006073	0.024292	0.046559	0.02834	0.02834	0.046559	0.040486	0.048583	0.076613	0.078629	0.078629
aculeata- EDNA18-0051713	0.006073	0.006073	0.006073	0	0.030364	0.052632	0.034413	0.034413	0.052632	0.046559	0.054656	0.082661	0.084677	0.084677
aculeata- EDNA18-0051384	0.024292	0.024292	0.024292	0.030364	0	0.048682	0.032454	0.032454	0.046653	0.040568	0.046653	0.080808	0.082828	0.082828
muricata- EDNA18-0051606	0.046559	0.046559	0.046559	0.052632	0.048682	0	0.018256	0.018256	0.056795	0.058824	0.055102	0.084848	0.086869	0.086869
muricata- EDNA18-0051723	0.02834	0.02834	0.02834	0.034413	0.032454	0.018256	0	0	0.03854	0.040568	0.048682	0.070707	0.072727	0.072727
muricata AF228302.1	0.02834	0.02834	0.02834	0.034413	0.032454	0.018256	0	0	0.03854	0.040568	0.048682	0.070707	0.072727	0.072727
islandica- EDNA18-0051284	0.046559	0.046559	0.046559	0.052632	0.046653	0.056795	0.03854	0.03854	0	0.018293	0.026423	0.062753	0.064777	0.064777
islandica- EDNA18-0051711	0.040486	0.040486	0.040486	0.046559	0.040568	0.058824	0.040568	0.040568	0.018293	0	0.00813	0.072874	0.074899	0.074899
islandica- EDNA18-0051716	0.048583	0.048583	0.048583	0.054656	0.046653	0.055102	0.048682	0.048682	0.026423	0.00813	0	0.080972	0.082996	0.082996
sepincola-_FR799151.1_	0.076613	0.076613	0.076613	0.082661	0.080808	0.084848	0.070707	0.070707	0.062753	0.072874	0.080972	0	0.004073	0.002037
sepincola-_FR799153.1	0.078629	0.078629	0.078629	0.084677	0.082828	0.086869	0.072727	0.072727	0.064777	0.074899	0.082996	0.004073	0	0.00611
sepincola-_FR799152.1_	0.078629	0.078629	0.078629	0.084677	0.082828	0.086869	0.072727	0.072727	0.064777	0.074899	0.082996	0.002037	0.00611	0

Appendix 5 (cont.)

The uncorrected (p) distance matrix between all *Cladonia* species may be found on the supplementary disk provided. All distances between conspecifics are highlighted by a black border.

Appendix 5 (cont.)

The uncorrected (p) distance matrix for all *Ochrolechia* sequences. Distances between conspecifics are highlighted with a black border.

	androgyna-_FR799238.1_	androgyna-_FR799239.1_	androgyna-_FR799240.1	androgyna-EDNA18-0051717	frigida-EDNA18-0051385	frigida-EDNA18-0051712	frigida-EDNA18-0051715	frigida-EDNA18-0051714	microstictoides-_FR799241.1_	microstictoides-_FR799242.1_	microstictoides-_FR799243.1_	szatalaensis-_FR799244.1_	szatalaensis-_FR799246.1_	szatalaensis-_FR799245.1
androgyna-_FR799238.1_	0	0	0	0.114786	0.116959	0.124031	0.125969	0.122093	0.135659	0.135659	0.133721	0.088409	0.088409	0.086444
androgyna-_FR799239.1_	0	0	0	0.114786	0.116959	0.124031	0.125969	0.122093	0.135659	0.135659	0.133721	0.088409	0.088409	0.086444
androgyna-_FR799240.1	0	0	0	0.114786	0.116959	0.124031	0.125969	0.122093	0.135659	0.135659	0.133721	0.088409	0.088409	0.086444
androgyna-EDNA18-0051717	0.114786	0.114786	0.114786	0	0.093204	0.093204	0.095146	0.091262	0.118217	0.118217	0.120155	0.112621	0.112621	0.11068
frigida-EDNA18-0051385	0.116959	0.116959	0.116959	0.093204	0	0.019531	0.021484	0.017578	0.128155	0.128155	0.130097	0.113281	0.113281	0.111328
frigida-EDNA18-0051712	0.124031	0.124031	0.124031	0.093204	0.019531	0	0.001953	0.001953	0.124272	0.124272	0.126214	0.12233	0.12233	0.120388
frigida-EDNA18-0051715	0.125969	0.125969	0.125969	0.095146	0.021484	0.001953	0	0.003906	0.126214	0.126214	0.128155	0.124272	0.124272	0.12233
frigida-EDNA18-0051714	0.122093	0.122093	0.122093	0.091262	0.017578	0.001953	0.003906	0	0.12233	0.12233	0.124272	0.120388	0.120388	0.118447
microstictoides-_FR799241.1_	0.135659	0.135659	0.135659	0.118217	0.128155	0.124272	0.126214	0.12233	0	0	0.001961	0.099222	0.099222	0.097276
microstictoides-_FR799242.1_	0.135659	0.135659	0.135659	0.118217	0.128155	0.124272	0.126214	0.12233	0	0	0.001961	0.099222	0.099222	0.097276
microstictoides-_FR799243.1_	0.133721	0.133721	0.133721	0.120155	0.130097	0.126214	0.128155	0.124272	0.001961	0.001961	0	0.101167	0.101167	0.099222
szatalaensis-_FR799244.1_	0.088409	0.088409	0.088409	0.112621	0.113281	0.12233	0.124272	0.120388	0.099222	0.099222	0.101167	0	0	0.00198
szatalaensis-_FR799246.1_	0.088409	0.088409	0.088409	0.112621	0.113281	0.12233	0.124272	0.120388	0.099222	0.099222	0.101167	0	0	0.00198
szatalaensis-_FR799245.1	0.086444	0.086444	0.086444	0.11068	0.111328	0.120388	0.12233	0.118447	0.097276	0.097276	0.099222	0.00198	0.00198	0

Appendix 5 (cont.)

The uncorrected (p) distance matrix for all *Umbilicaria* sequences. Distances between conspecifics are highlighted with a black border.

	cylindrica-_FR799300.1	cylindrica-_FR799306.1	cylindrica-EDNA18-0051533	proboscidea-_FR799303.1	proboscidea-_FR799305.1	proboscidea-_FR799304.1	proboscidea-EDNA18-0051383	torrefacta-EDNA18-0051394	torrefacta-EDNA18-0051718	torrefacta-EDNA18-0051382	polyphylla-_FR799301.1	polyphylla-EDNA18-0051613	polyphylla-_FR799302.1
cylindrica-_FR799300.1	0	0.008457	0.023158	0.0587	0.0587	0.0587	0.0587	0.100209	0.100209	0.146138	0.09434	0.09434	0.096436
cylindrica-_FR799306.1	0.008457	0	0.027368	0.054507	0.054507	0.0587	0.054507	0.096033	0.096033	0.141962	0.098532	0.098532	0.100629
cylindrica-EDNA18-0051533	0.023158	0.027368	0	0.060797	0.060797	0.060797	0.060797	0.102296	0.102296	0.148225	0.09434	0.09434	0.096436
proboscidea-_FR799303.1	0.0587	0.054507	0.060797	0	0	0.016842	0	0.067086	0.067086	0.113208	0.085954	0.085954	0.08805
proboscidea-_FR799305.1	0.0587	0.054507	0.060797	0	0	0.016842	0	0.067086	0.067086	0.113208	0.085954	0.085954	0.08805
proboscidea-_FR799304.1	0.0587	0.0587	0.060797	0.016842	0.016842	0	0.016842	0.073375	0.073375	0.119497	0.083857	0.083857	0.085954
proboscidea-EDNA18-0051383	0.0587	0.054507	0.060797	0	0	0.016842	0	0.067086	0.067086	0.113208	0.085954	0.085954	0.08805
torrefacta-EDNA18-0051394	0.100209	0.096033	0.102296	0.067086	0.067086	0.073375	0.067086	0	0	0.056723	0.112735	0.112735	0.114823
torrefacta-EDNA18-0051718	0.100209	0.096033	0.102296	0.067086	0.067086	0.073375	0.067086	0	0	0.056723	0.112735	0.112735	0.114823
torrefacta-EDNA18-0051382	0.146138	0.141962	0.148225	0.113208	0.113208	0.119497	0.113208	0.056723	0.056723	0	0.158664	0.158664	0.160752
polyphylla-_FR799301.1	0.09434	0.098532	0.09434	0.085954	0.085954	0.083857	0.085954	0.112735	0.112735	0.158664	0	0	0.002101
polyphylla-EDNA18-0051613	0.09434	0.098532	0.09434	0.085954	0.085954	0.083857	0.085954	0.112735	0.112735	0.158664	0	0	0.002101
polyphylla-_FR799302.1	0.096436	0.100629	0.096436	0.08805	0.08805	0.085954	0.08805	0.114823	0.114823	0.160752	0.002101	0.002101	0