EpiAir Final Report

Twig epiphyte communities as indicators of environmental quality in the border region of Ireland.

Maria L. Cullen & Howard F. Fox

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Executive Report

Summary

In 2011 and 2012, over 2500 vegetation samples were collected from stream-sides as part of the Tellus Border geochemistry survey. This survey work also gathered samples of stream water and stream sediment from the same localities. The vegetation samples took the form of twigs from trees or bushes. Vegetation samples were between 4g to 51g in mass (average 26g with a SD 7.9g) and were dated using ring counts from 478 samples were between 1-14 years in age (average 6 years and SD 2.25 years).

The material was sorted, dried and stored in their original paper packets at the Geological Survey of Ireland. The EpiAir project studied over 700 twig sets for the lichens, algae, fungi, mosses and liverworts – epiphytes - growing on these twigs. Species from other biological groups were also identified, *e.g.* mussel scale insects, wherever possible.

Work was carried out using x10-x20 stereoscope and x100-x600 compound microscope. Host twigs were identified using botanical keys. Epiphytes were identified from experience, keys and microscopic features where necessary. A small number of samples were processed by genetic sequencing. A database was constructed of 5,103 biological records. These data were analysed using R statistics and maps were plotted using Quantum GIS.

Presentations have been made on the EpiAir project in Galway (Irish Plant Scientists' Annual Meeting, May 2013), at the Air Pollution Workshop in Portland, Oregon (June 2013) and at the Tellus Border Project meeting recently held in Monaghan (October 2013).

Irish legislation, air quality monitoring methods, modelling, reporting and regulation were examined regarding the Tellus Border area.

For example, there is only one EPA air quality monitoring station, at Kilkitt, Co. Monaghan, currently working in the entire Tellus Border region. This station measures Ozone, NOx and SOx levels but it does not monitor ammonia, a significant agricultural air pollutant and a cause of eutrophication.

We recommend that a reasonable level of monitoring for ammonia be established in this rural area to support model-based reporting.

It is welcome that under an Ambient Atmospheric Ammonia in Ireland project, shared between University College Dublin and Trent University Ontario, 6 sites have commenced ammonia monitoring in the Tellus Border region, albeit on a volunteer basis, following earlier work by de Kluizenaar and Farrell (2000).

Project Background and Objectives

The Tellus Border project is a geochemical and geophysical study of Counties Sligo, Donegal, Leitrim, Cavan, Monaghan and Louth in Ireland. Twigs from bushes and trees by streams and wet ditches were part of the vegetation sampled across the region for a trace-element geochemistry archive. These twigs support an epiphyte biodiversity 'by-catch'. This biodiversity is being analysed in the EpiAir project and the information content is being interpreted with regard to rural air pollution.

The epiphytic organisms include lichens, fungi, bryophytes (mosses and liverworts) and algae. The lichens, in particular, are sensitive to anthropogenic pollution of air and water (in mists and precipitation). Furthermore, certain algae can constitute thin 'blooms' on twig surfaces overgrowing epiphytic lichens and bryophytes. The methodology for this project is simple and robust. A sample size of 700 packets of twig material has been screened for their epiphyte species composition.

Our objective is to document impacts of eutrophication on epiphytic biodiversity in the Tellus Border region. We are exploring the influence of anthropogenic sources, almost exclusively agricultural, on air pollution. In particular, the aim is to detect and map biodiversity signals of excessive nitrogen inputs evident from analysis of the distribution of epiphytes in their capacity as biomonitors.

Basic Premises

That epiphyte species diversity is variable on twigs

That some epiphyte species are nitrophytes, neutrophytes or nitrogen tolerant and some are pollution-sensitive

That epiphyte recruitment and success of juveniles is reduced by air pollution and habitat structure factors such as the long history of forest management by clear-felling

That there should be several eutrophication signals in the epiphyte flora

That this eutrophication signal, and bioindication in general, is based on the amount of eutrophication (mainly due to ammonia) experienced in an area being sufficient to alter and to reduce that area's epiphyte biodiversity

That epiphyte species on twigs are pioneers

Air pollution limits recruitment and success of juvenile epiphytes

That environmental geochemistry data provides element and ionic tolerance ranges for species presence/recruitment success to be understood

That epiphytes can be used as a proxy for air quality affecting aspects of human health in the Tellus Border Region

That empirical definition of nitrophytic epiphytes can be developed *a postiori* based on combined analysis of species presence and geochemistry data

That tree host is important to epiphytes as different tree species may be an inhibitor or promoter of epiphyte diversity

That GIS can be used to analyse location effects and therefore reduce an urban pollution signal from the data

That biodiversity data with substrate ring counts and GIS of nitrophytic species spatial distributions can elucidate the influence of clear-felling and loss of epiphyte source material so that this diversity reducing signal can be reduced

Lichens and other epiphytes in the context of air pollution studies

Many lichen species are sensitive to different types of air pollution as they are to a large extent filter-feeders of air and moisture on tree branch and trunk surfaces. Species of lichens containing cyano-bacterial algal photobionts require high humidity in order to grow so they rely on Ireland's clean Atlantic air to survive and we have an important biomass of these lichens – the Lobarion community. Although only one Lusitanian lichen species is protected in Ireland, all species of lichen that are fruticose or bushy in growth form, with large surface areas, such as *Usnea* spp. are sensitive to air pollution.

Studies of ecological effects suggest that emissions reductions are needed to protect sensitive ecosystem components (Fenn et al. 2003)

Critical levels and loads

According to the CAFE Directive; "critical level" means a level fixed on the basis of scientific knowledge, above which direct adverse effects may occur on trees, other plants or natural ecosystems but not on humans. Enhanced N accumulation in lichen material and shifts in lichen community functional groups have been the most sensitive responders to atmospheric N deposition.

"Critical load" is defined as an amount that in the long-term will not cause adverse effects to the structure and functions of ecosystems (UNECE, 2011).

Critical level: is the concentration of a pollutant in the atmosphere, below which vegetation is unlikely to be damaged according to present knowledge. However, this concept is flawed as N–impacts include local extinction of oligotrophs, a biodiversity vegetation composition damage.

Critical load: the critical load is the amount of pollutant deposited below which significant harmful effects on specified elements of the environment do not occur, according to current knowledge.

The idea of a critical load was developed to provide a receptor-based system for measuring emission reduction targets for pollutants, in the areas of acidification and

nitrogen enrichment. Critical loads of N for nitrogen enrichment have been based on the results of field monitoring experiments, and links between N deposition and changes in vegetation. Critical loads of acidity, which include the contributions from N and S (sulphur), are generally set to limit changes in the chemistry of soil or soil solution so that the biological system is protected. The critical limits have been set to prevent changes in the species present, or changes in the ecosystem (Hornung et al., 2001.)

An empirical critical load of 3.1 kg N ha-1 yr-1 was calculated for enhanced lichen tissue N concentrations, which corresponded with the initiation of community change (Fenn et al., 2008). At a throughfall N deposition level of 5.2 kg N ha-1 yr-1 the lichen community being studied in California shifted from acidophyte dominance to neutrophyte dominance. Lichen species classified as acidophytes were extirpated at a critical load of 10.2 kg N ha-1 yr-1 (Fenn et al., 2008).

The Nitrogen Cascade and cyanobacterial epiphytes

The modern Nitrogen cycle is fundamentally different from the Nitrogen cycle that evolved naturally between the geophysical environment and the natural biosphere. (Galloway et al., 2003, Bebout et al., 2013).

Over the course of about two centuries, human involvement with Nitrogen has progressed from its discovery in the late-18th century by chemists, to absolute human dominance of the terrestrial biogeochemical cycle of Nitrogen in the late-20th century. Humans have had the ability to generate reactive Nitrogen, by fixing Nitrogen (N2) gas through the Haber-Bosch process to create ammonium and nitrate. Reactive Nitrogen (Nr) is defined as ammonium, ammonia, nitrite, nitrate, nitrogen oxides and water soluble organic nitrogen (Hastings et al., 2013).

The natural atmospheric Nitrogen fixation from electrical storms and lightning is no longer the limiting factor for growth of plants and food production in the environment. The production of food and energy, so critical to human survival and feeding the world's population, has resulted in substantial increases in the amount of Nr used in the environment. Reactive Nitrogen fixed by the chemical industry is now pervasive in fertilizers and unused waste reactive Nitrogen has permeated and been distributed through the environment via biogeochemical processes. The cascade of environmental impacts of reactive Nitrogen release by humans include Ozone release, Particulate Matter from fertilizer dust, forest degradation and

eutrophy, soil acidification, groundwater contamination, atmospheric, water and inshore coastal water eutrophication leading to mammal deaths, algal bloom formation, ocean acidification and harming the natural biological process of fixing Nitrogen by cyanobacterial species of forest ecosystems, rather than agro-ecosystems, which have evolutionarily inherited the genetically and enzymatically optimised ancestry for living and Nitrogen fixing in oligotrophic habitats.

Biologically fixed Nitrogen is a natural source of reactive Nitrogen. In nature in terrestrial ecosystems, cyanobacteria are the main nitrogen fixers, as in the anaerobic layer of soil of rice paddies, legume root nodules, Alder tree root nodules, and in symbioses with some epiphytic lichens (e.g. *Collema, Leptogium, Pannaria, Lobaria, Sticta*) containing a cyanobacterial photobiont.

This cascade of waste reactive Nitrogen through the environment has had a biodiversity impact by limiting the survival of forest epiphyte species which are adapted to oligotrophic conditions before the 18th Century with low background reactive Nitrogen inputs. These benign oligotrophic atmospheric conditions while common in forests in Europe in 19th Century and early 20th Century are now replaced in the eutrophic atmosphere of our forests of the late 20th century and early 21st Century.

Eutrophication and elevated Nitrogen critical loads occur over much of Europe, Eastern North America, India, China, Brazil and other regions with intensive agriculture.

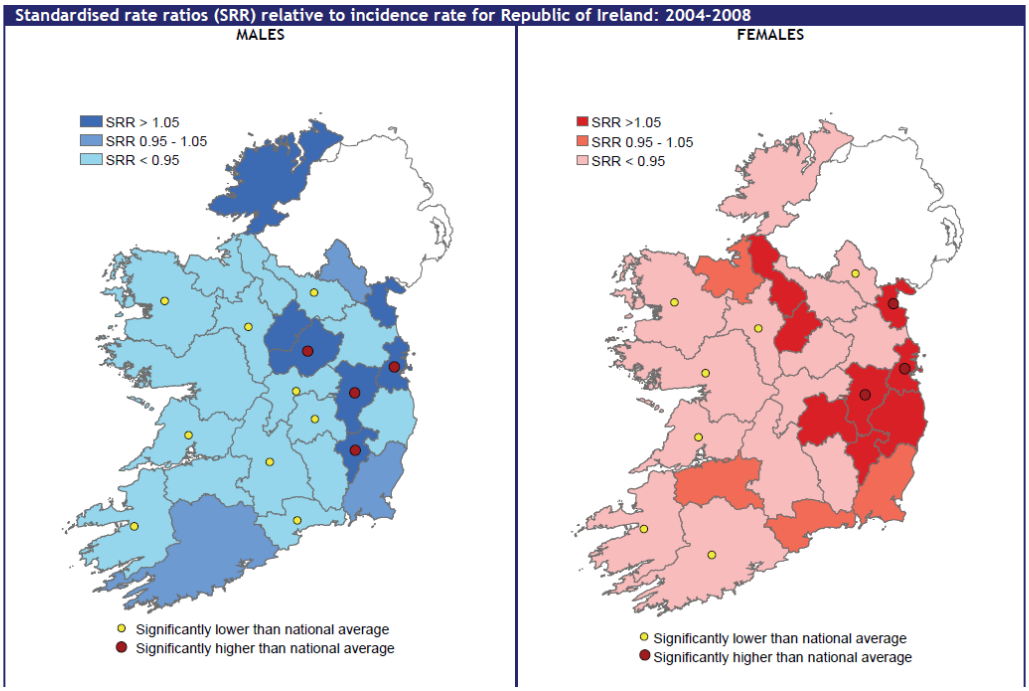
The decline in the regional extent and range of cyanobacterial based lichen epiphytes has been catastrophic due to the combined effects of eutrophication and adverse impacts of inappropriate forest management. The pervasiveness of the eutrophication signal among epiphytes in the rural Irish landscape is an uncomfortable reality of what we found from the EpiAir twig biodiversity species compositional analysis. Due to the rarity of oligotrophic lichens in the dataset, apart from the most successful epiphyte recruitment areas in the Lough Allen basin with very high twig epiphyte species biodiversity, the concept that air of pristine quality is prevalent over most of the rural Tellus Border region we can safely conclude is compromised.

Capital investment and incentivisation for waste reactive Nitrogen stream processing technologies including agro-water treatment and biogas recovery by the agricultural sector need to be developed at individual farm enterprise level is

required to deal with reactive Nitrogen pollution issues, and their contribution to climate change abatement measures.

Human Health in the Tellus Border Region

19% of children currently suffer from asthma and 10% of adults have the disease in Ireland. This makes Ireland the 4th highest in the world for incidences of asthma and has implications for employment, education and healthcare. For example, 8% of individuals with asthma in Co. Monaghan required hospital treatment in 2012. General comfort is also an issue, particularly at certain times of year, as between 60-80% of those with asthma in Ireland also suffer from hay fever (allergic rhinitis) (Asthma Society of Ireland statistics).



(Lung cancer numbers 2004-2008 from National Cancer Registry Statistics, 2011).

According to Prof. Tim McDonnell, Ireland has the highest rate of hospital admissions for chronic obstructive pulmonary disease (COPD) in the developed world. Once fine particles (e.g. PM2.5, PM10) are in the lungs, they can adversely affect the heart, blood vessels, and lungs.

Both indoor and outdoor air pollution are relevant to the sufferer of pulmonary or related cardiac disease. These authors welcome the formation of the Lung Health Alliance as an umbrella body for many organisations who represent those with different lung and related ailments in Ireland *e.g.* those with asthma, COPD or cystic fibrosis. In the USA, the Centre for Disease Control and Prevention (CDC) has a Tracking Network that uses methods developed by the U.S. Environmental

Protection Agency (EPA) and others to estimate how lowering air pollution levels can affect health. The EPA's Benefits Mapping and Analysis Program (BenMAP) is a geographic information system (GIS) based program that helps to calculate health impacts of air pollution. Using GIS data in the Tellus Border Region, air quality work can be instigated and monitored in co-operation with the EPA and umbrella bodies such as the Lung Health Alliance for the benefit of sufferers of asthma, lung cancer, COPD, Cystic Fibrosis along with other issues such as heart ailments that are knock-on problems. With our incidences of lung diseases among the highest in the world, a better understanding of the local causes, allergens and agrivants should be among the preventative measures we take to protect the Irish population from these health problems. A key question is whether threshold concentrations exist below which air pollution has no effect on population health (Brunekreef & Holgate 2002).

A recent OECD report states that by 2050, air pollution will become the biggest cause of premature death, killing an estimated 3.6 million people a year. Premature deaths from exposure to elevated concentrations of ground-level ozone are projected to more than double worldwide, from 385,000 to nearly 800,000.

Lung Health and Air Quality

The incidence of lung cancer in the Tellus Border region between 2004 and 2008 was calculated to be higher than average for both men and women in Co. Louth (using European Age Standardised Incidence Rates EASIR methodology). Men in Co. Donegal, particularly around Letterkenny and west Donegal, and Co. Monaghan were found to be at greater risk than average. So too were women in Cos. Leitrim and Co. Sligo although these data were based on county boundaries with variation in population density (National Cancer Registry 2011; www.ncri.ie).

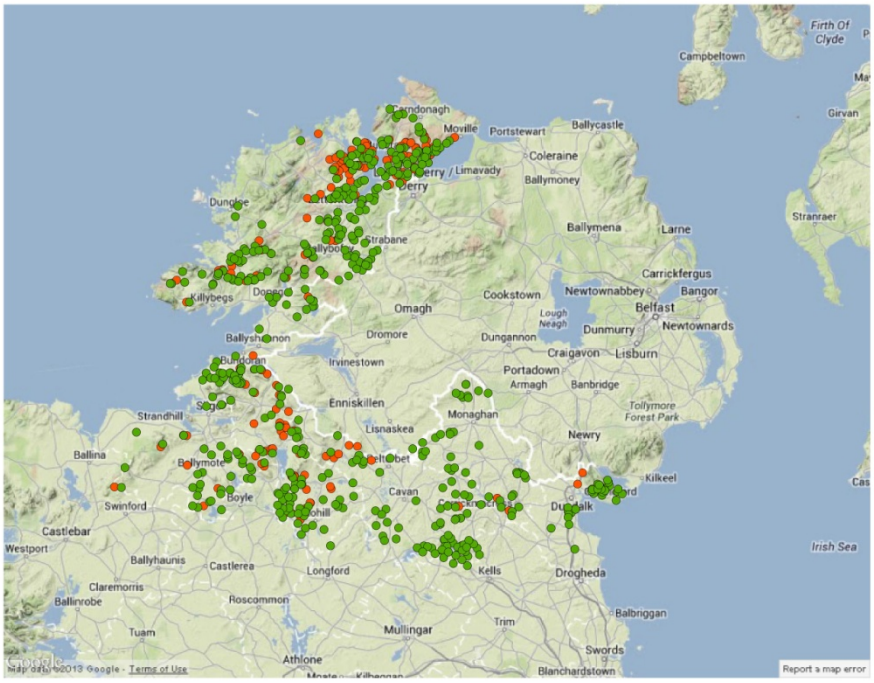
The cost of lung diseases – asthma, lung cancer, COPD (Chronic Obstructive Pulmonary Disease) and also directly related heart diseases to sufferers, their families and the country can be significant in terms of quality of life and finances.

Poor air quality in both indoors and outdoors is considered the most significant environmental factor in cases of reduced lung health.

Our Findings

Results from our work include the following observations:

540 twigs host green algae. *Desmococcus olivaceus* was found on 527 twigs or 75.2% of the overall number, constituting an aerial green algal bloom, which is a characteristic signal of eutrophication of our environment.



Map of *Desmococcus olivaceus* distribution (present green, absent red) on twigs studied as part of EpiAir project, a subset from Tellus Border vegetation sample set.

Eutrophic lichen species are far more prevalent in the Tellus Border region than pollution sensitive species.

The lichen *Evernia prunastri* has declined in range throughout the Tellus Border region in the last century.

The algal parasite *Psammina stipitata* was found New to Ireland.

The lichen *Caloplaca holocarpa* appears to be increasing in population in Ireland while *Fellhanera bouteillei* is far more common than previous recording would intimate. This is true to a lesser extent for the lichen *Lecania cyrtella*. These are small occasional species and obviously overlooked as they are specific to twig habitats.

A partial baseline of epiphytic bio-monitoring in areas of the Tellus Border region is useful in planning regulation where land use change is considered.

Generation of data useful in the ongoing bio-monitoring of critical level/load selection and to inform policy to combat eutrophication in Ireland.

Identification of *Epicoccum nigrum* Ehrenb. ex Schlecht. from a MegaBLAST (Basic Local Alignment Sequencing Tool) nucleotide sequence analysis of fungal DNA (from packet 586013V). This species can colonize nasal sinuses and cause allergic fungal sinusitis (AFS) (Noble *et al.* 1997).

Discussion

The Nitrogen Cascade and cyanobacterial epiphytes

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Nitrogen inputs. These benign oligotrophic atmospheric conditions while common in forests in Europe in 19th Century and early 20th Century are now replaced in the eutrophic atmosphere of our forests of the late 20th century and early 21st Century.

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Capital investment and incentivisation for waste reactive Nitrogen stream processing technologies including water treatment and biogas recovery by the agricultural sector need to be developed at individual farm enterprise level is required to deal with the reactive Nitrogen pollution issue, and its contribution to climate change abatement measures.

Recommendations for TELLUS research

Archive of Tellus Border vegetation samples in a low humidity storage environment to minimise contamination by potentially harmful xerophilic fungi (*Aspergillus glaucus*) which grow on improperly dried herbarium collections, for example in the Irish National Herbarium, DBN, housed in the National Botanic Gardens, which has a drying room, so long as unrestricted access to the collection is openly facilitated to all citizens.

We recommend that the vegetation sampling is continued in other counties with the additions of a few additional details to the protocol recording of environmental details. It would be helpful to have data on woody plant height, hedgerow or tree line landscape and management elements after general hedgerow survey criteria in use in Ireland (Folkes, Heritage Council endorsed survey methods), digital time, date, geo-code stamped photography from 5m to show plant habit to assist field identity, and to keep the woody plant twig selection from single 50 to 100cm lengths for division and packing, so that twig sampled includes surfaces from 3 to 8 years old.

In the vegetation protocol, laboratory processing could include twig weighings when dried for storage and twig countings, and maximum annual ring counts, and field notation to indicate whether live and/or dead attached twig is in the sample on the basis of live leaf, live bud or dead bud presences. Epiphyte growth continues after attached twig death, and this niche diversity is an important biodiversity signal for sampling.

In dealing with the quantification of the spatio-temporal fluxes in the Nitrogen cycle, we endorse that long term monitoring data be collected at many stations in Ireland for Ammonia and Ammonium. For example in the UK, there are 31 NAMN stations as part of a 313 station European network.

Technical Report

2. Methods

What gets measured gets managed.
Peter Druker

Identification of epiphyte species and host twigs with references

Twig samples from each packet were identified to woody species on the basis of bark morphology and branching and bud characteristics. The number of twig samples per packet were then counted and then weighed using a Salter 1029WHDRT weighing scales.

Microscopy

Tree rings of largest twigs in each packet were counted with the aid of a x10-x40 stereomicroscope. Each batch of woody material was then screened for epiphytes using a stereoscope. Dissections were made for interesting and critical species and examined under x100, x400 x600 magnification using a Nikon Eclipse 80i microscope. Photomicrographs made of microscopic slide features of note. Contamination by *Aspergillus glaucus* agg. was noted on some material.

Genetics

For PCR the following protocol was followed:
Samples of lichen were powdered using liquid Nitrogen to freeze them and then autoclaved micropestles to crush them. The samples were small in mass so a modified treatment was used to extract DNA.
An EZNA Fungal DNA Mini Kit was used as the basis for DNA extraction:
200ul of FG1 was added to each 2ml tube and samples were shaken to mix
Tubes were incubated in a water bath set at 65C for 10 minutes during which samples were inverted twice to mix
Tubes were then placed on ice for 5 minutes
They were centrifuged at 10,000g for 10 minutes
The supernatant was transferred to fresh microcentrifuge tubes
0.7% volume of Isopropanol (ice cold) was added to each tube
Tubes were inverted to precipitate DNA
The samples were centrifuged at 10,000g for 2 minutes

The supernatant was removed
Tubes were inverted on paper towels in a laminar flow cabinet
300ul of sterilised deionised water preheated to 65C was added to each tube to resuspend the DNA pellet
The Nanodrop checking of these samples showed that any DNA had been removed at the Supernatant stage for the majority of samples
While the supernatant was likely to contain the DNA of more than one fungus, it was decided to use purified supernatant samples where necessary
Thermoscientific GeneJet PCR Purification Kit was used for two samples 6009.1S and 6013.1S to assess if this measure would be adequate for DNA purification
For these two samples:
The samples were transferred to fresh Genejet columns and 700ul of Wash buffer was added
The samples centrifuged for 1 minute and the throughflow discarded
30ul of Elution buffer was added to the columns over fresh microcentrifuge tubes and the samples centrifuged for a further minute before the elute was collected
The following samples were selected for PCR:

1. 6009.1 Opegrapha vulgata
2. 6031.14 Scoliciosporum umbrinum*
3. 6031.1S Lecanora chlarotera
4. 6009.1SP Opegrapha vulgata (same as sample 1 but purified supernatant)*
5. 6013.1SP Lecanora chlarotera (supernatant, purified)*
6. 6031.6S Lecanora albella (supernatant sample)
7. 6031.13S Lecidella elaeochroma (supernatant sample)*
8. Water blank

Thermoscientific Phusion Blood Direct PCR Kit was used as extremely high resistance to inhibitors found in blood and these inhibitors are similar to those in wood and fungi.

2x Phusion Blood PCR Buffer	100ul
Primer ITS1F	5ul
Primer ITS4R	5ul
Phusion Blood Polymerase	4ul
DNA (1ul)	
H20 purified	85ul

The PCR took place under the following conditions in a Thermocycler:
1. 98C 5 minutes

2. 98C 1 second
3. 59C 5 seconds
4. 72C 30 seconds
5. 72C 1 minute
6. 10C resting Temperature
Steps 2-4 were repeated for 37 cycles
A 30ml agarose gel of 2% concentration with 1.5ul of added Ethidium bromide was prepared for electrophoresis and PCR products were allowed to run along this gel in a bath of TAEx1 at 40mV. A UV transilluminator with camera attached to computer running GelCapture software was used to image the gel.
2ul of 100 bp ladder was added to the first and last well to gauge the size of PCR amplification products.
5ul of PCR product was used in each well with 1ul of Bromophenol blue dye
Bands were seen between 500 and 600 base pairs for samples 6031.14C, 6009.1SC, 6013.1SC and 6031.13SC although samples 6009.1SC and 6013.1SC displayed double bands. With more time these might have been removed by re-PCR.

Four samples were selected after Gel electrophoresis for purification and sequencing:
6031.14 Scolisiosporum umbrinum
6009.1SP Opegrapha vulgata (Double bands)
6013.1SP Lecanora chlarotera (Double bands)
6031.13S Lecidella elaeochroma

Thermoscientific GeneJet PCR Purification Kit was used.
1. 1:1 volume of Binding Buffer was mixed thoroughly with each sample.
2. The solution was added to labelled Genejet purification columns that were centrifuged for 60 seconds. The throughflow was discarded.
3. 700ul of Wash buffer was added to each purification column and these were centrifuged for 60 seconds. Again the throughflow was discarded.
4. The empty purification columns were centrifuged for a further 60 seconds.
5. The four purification columns were transfered to fresh microcentrifuge tubes and 50ul of Elution buffer added to each; these were centrifuged for 60 seconds.
6. The purified DNA was stored at -20C.
After Nanodrop measurement of the DNA concentration, 15ul of each sample (at c. 7ng/ul) was sent for sequencing against a 1:30ul solution of ITS4R.

Statistics

R was used

GIS

Quantum GIS® was used primarily in the portrayal of EpiAir and Tellus Border data in graphic and map forms. Spatial variation of data was helpful in examining differences in epiphyte biodiversity and species distributions as well as the ability to these results with compare stream water or soil element concentrations.

3. Results

The first condition of understanding a foreign country is to smell it.
Rudyard Kipling

Metadata about the file EpiAirData.ods

This spreadsheet is composed of two sheets with data originated by Maria Cullen, Sean O'Donovan and Howard Fox in the pilot study in summer 2012 and the EpiAir project in 2013.

The epiphyte floristic results are presented in a sheet named "EpiAirfloradata" with 5104 rows where the 4 column headers (aaDisplay name, Organism, VegPlot & count) are in row 1.

aaDisplay name – This is the standardized epiphyte species identification. This is a corrected, censored and derived field from the input identification data in the original EpiAir data entry spreadsheet. The data entry spreadsheet was compiled by Maria Cullen, Sean O'Donovan and Howard Fox from twig epiphyte identifications made in the pilot study in summer 2012 and from those made during the EpiAir project from April 2013-to August 2013. The data cleaning process corrects for typos, multiple identifications reported in one cell, omits notes and other biological materials (hairs, feathers, scale insects, mites, etc.) found on the twigs.

Organism – this is a category group for grouping organisms from a list of alga, fungus, lichen, liverwort, moss, no flora and no lichen. The no flora group was required to admit VegPlot numbers for twigs where the diversity was assessed and found to be bereft of epiphytes. The no lichen group was required to acknowledge the lack of lichen species in the VegPlot twig sample studied. This data was used to help populate an epiphyte species trait dataset with taxonomic data on biological and reproductive properties of the identified species.

Vegplot – This is the packet number written on the sample examined during the EpiAir project.

Count – this is a presence count field of either 1 or 0. The coding is 1 for an accepted records of a species identified as being in a vegetation plot and 0 for dealing with cells where no flora, no lichen, bereft of epiphytes are reported. and storage spoilage fungus. It is used for deriving a diversity statistic with the number of plots in which each of 180 or so species are present.

The plot attribute data are included in a spreadsheet named "701plotsall". This spreadsheet "701plotsall" has 17 columns and 702 rows and the column headers are in row 1. This spreadsheet was designed to hold our substrate identification data and some analytical results on the organism group species compositional diversity counts.

The column headers are VegPlot Coll1 Coll2 Twig final grams Ring max Num Twigs Floristic data Data Match VegPlot Alga tot fungus tot hepatic tot lichen tot moss tot epiph tot

VegPlot signifyies the VegPlot number on the packet studied. Coll1 and Coll 2 is for the two field collector's initials written on the packet. Twig final is the EpiAir team's determination of the substrate identity during the screening process. Grams relates to the weight of the twigs measured from May 2013 to August 2013. Twigs studied in the pilot were not weighed retrospectively. Ring max is the tree rings present on the cut end of the twig sample. It is an age proxy for the twig bark, but of course ring increments for counting do not accrue on twigs on death.

We did not database some plot attributes - for example – we omitted vegplot packet numbers for samples that were opened and were rejected by the EpiAir team for screening due to an unhealthy excess of biodeterioration and spoilage fungi (Aspergillus glaucus). The biodeterioration fungi had developed on the epiphyte material after field collection in 2011 and 2012 and before being noted during the EpiAir project in 2013.

The vegplot packet collector acronyms are written on vegplot packets made by the field team in 2011 and 2012. This was a quality control field to help identify plot number data entry errors and digit inversions in the 58XXXX digit string

COMPOSITION OF EPIPHYTE BIOTA

Our identifications include almost 180 epiphyte taxa. The identifications are presented in order of decreasing frequency.

Epiphyte	**Organism**	**Total**
Desmococcus olivaceus	alga	527
Lecidella elaeochroma	lichen	316
Lecanora chlarotera	lichen	271
Opegrapha atra	lichen	239
Physcia tenella	lichen	238
Arthonia radiata	lichen	199
Arthopyrenia punctiformis	lichen	141
Frullania dilitata	hepatic	128
Xanthoria parietina	lichen	127

Aspergillus glaucus	fungus	121
Ulota crispa	moss	119
Anisomeridium biforme	lichen	117
Lecania cyrtella	lichen	110
Vouauxiella lichenicola	fungus	109
Fuscidea lightfootii	lichen	107
Fellhanera bouteillei	lichen	106
Phaeographis smithii	lichen	103
Ramalina farinacea	lichen	94
Porina aenea	lichen	93
Arthonia punctiformis	lichen	86
Bacidia phacodes	lichen	82
Graphis scripta	lichen	76
Metzgeria furcata	hepatic	74
Melanelia subaurifera	lichen	69
Opegrapha vulgata	lichen	62
Opegrapha herbarum	lichen	59
Parmotrema perlatum	lichen	59
Lepraria incana	lichen	51
Metzgeria fruticulosa	hepatic	48
Anisomeridium polypori	lichen	46
Pertusaria leioplaca	lichen	46
Physcia aipolia	lichen	45
Graphis elegans	lichen	41
Parmelia sulcata	lichen	41
Opegrapha varia	lichen	38
Trentepohlia	alga	33
Rinodina sophodes	lichen	33
Caloplaca ferruginea	lichen	29
Japewiella tavaresiana	lichen	29
Arthonia cinnabarina	lichen	28
Scoliciosporum umbrinum	lichen	28
Lecanora albella	lichen	26
Hypotrachyna revoluta	lichen	24
Microlejeunea ulicina	hepatic	23
Micarea prasina	lichen	23
Lecanora argentata	lichen	22

Usnea subfloridana	lichen	22
Candelariella reflexa	lichen	21
Normandina pulchella	lichen	20
Hypnum cupressiforme	moss	17
Lecanora symmicta	lichen	16
Arthonia ilicina	lichen	15
Caloplaca cerinella	lichen	15
Lecanora expallens	lichen	15
Arthopyrenia	lichen	14
Zygogonium	alga	13
Hymenochaete corrugata	fungus	13
Scoliciosporum chlorococcum	lichen	13
Orthotrichum	moss	13
Bacidia arceutina	lichen	12
Stenocybe pullatula	lichen	12
Dimerella lutea	lichen	11
Eopyrenula	lichen	11
Lecanora carpinea	lichen	11
Lecanora pallida	lichen	11
Dimerella pineti	lichen	10
Tomasellia gelatinosa	lichen	10
Caloplaca holocarpa	lichen	9
Physcia adscendens	lichen	9
Hypogymnia physodes	lichen	8
Micarea nitschkeana	lichen	8
Amandinea punctata	lichen	7
Porina leptalea	lichen	7
Peniophora lycii	fungus	6
Acrocordia gemmata	lichen	6
Arthonia didyma	lichen	6
Bacidia sabuletorum	lichen	6
Ramalina canariensis	lichen	6
Caloplaca cerinelloides	lichen	5
Enterographa crassa	lichen	5
Gyalideopsis anastomosans	lichen	5
Hypogymnia tubulosa	lichen	5
Micarea	lichen	5

Micarea cinerea	lichen	5
Micarea lignaria	lichen	5
Rinodina	lichen	5
Hypnum cupressiforme resupinatum	moss	5
Kindbergia praelonga	moss	5
Ascodichaena rugosa	fungus	4
Nectria	fungus	4
Nectria galligena	fungus	4
Pteridospora scoriodea	fungus	4
Colura calyptrifolia	hepatic	4
Arthopyrenia analepta	lichen	4
Arthopyrenia salicis	lichen	4
Bacidia	lichen	4
Bacidia laurocerasi	lichen	4
Lecania cyrtellina	lichen	4
Hypoxylon fuscum	fungus	3
Nectria episphaeria	fungus	3
Arthonia spadicea	lichen	3
Arthopyrenia fraxini	lichen	3
Buellia disciformis	lichen	3
Evernia prunastri	lichen	3
Lecanora pulicaris	lichen	3
Mycoblastus sterilis	lichen	3
Punctelia subrudecta	lichen	3
Ramalina fastigiata	lichen	3
Athelia arachnoidea	fungus	2
Stigmidium microspilum	fungus	2
Syzygospora physciacearum	fungus	2
Anisomeridium	lichen	2
Bacidia naegelii	lichen	2
Cyphelium inquinans	lichen	2
Lecanora conizaeoides	lichen	2
Lecanora jamesii	lichen	2
Loxospora elatinum	lichen	2
Melanelia exasperata	lichen	2
Melanelia glabratula	lichen	2

Mycomicrothelia confusa	lichen	2
Mycoporum antecellans	lichen	2
Normandina acroglypta	lichen	2
Pertusaria amara	lichen	2
Phaeophyscia orbicularis	lichen	2
Tephromela atra	lichen	2
Isothecium myosuroides	moss	2
Ulota phyllantha	moss	2
Arthopyrenia platypyrenia	fungus	1
Hysterium pulicare	fungus	1
Hysterographium fraxini	fungus	1
Peniophora incarnata	fungus	1
Phyllactinia fraxini	fungus	1
Phyllactinia guttata	fungus	1
Pleospora herbarum	fungus	1
Psammina stipitata	fungus	1
Cololejeunea minutissima	hepatic	1
Odontoschisma sphagni	hepatic	1
Arthonia	lichen	1
Arthopyrenia antecellans	lichen	1
Arthopyrenia cerasi	lichen	1
Bacidia adastra	lichen	1
Bacidia delicata	lichen	1
Buellia griseovirens	lichen	1
Caloplaca cerina	lichen	1
Candelaria concolor	lichen	1
Cladonia chlorophaea	lichen	1
Cliostomum grifithii	lichen	1
Diploicia canescens	lichen	1
Fellhanera vezdae	lichen	1
Graphis brittanica	lichen	1
Hypotrachyna sinuosa	lichen	1
Lecania erysibe	lichen	1
Lecanora confusa	lichen	1
Lecanora farinaria	lichen	1
Lecanora laevis	lichen	1
Lecanora piniperda	lichen	1

Melanelia exasperatula	lichen	1
Micarea adnata	lichen	1
Micarea alabastrites	lichen	1
Micarea denigrata	lichen	1
Micarea peliocarpa	lichen	1
Mycoglaena myricae	lichen	1
Opegrapha brevis	lichen	1
Opegrapha ochrocheila	lichen	1
Pannaria rubiginosa	lichen	1
Pertusaria coccodes	lichen	1
Pertusaria hymenea	lichen	1
Pertusaria pertusa	lichen	1
Phlyctis argena	lichen	1
Physcia semipinnata	lichen	1
Physconia distorta	lichen	1
Psoroglaena stigonemoides	lichen	1
Ramalina calicaris	lichen	1
Ramalina fraxinea	lichen	1
Thelotrema lepadinum	lichen	1
Trapeliopsis granulosa	lichen	1
Trapeliopsis pseudogranulosa	lichen	1
Xanthoria polycarpa	lichen	1
Hypnum jutlandicum	moss	1
Zygodon viridissimus	moss	1

REPRODUCTIVE PROPAGULES OF EPIPHYTES

The ecological traits of 180 epiphyte species detected in the EpiAir study were tabulated. It is considered that twig epiphytes are generally composed of pioneer species. The reproductive propagules of epiphytes are genets which land on twig bark, and from which new epiphyte thalli grow. Air Pollution has the likely effect to inhibit recruitment of these dispersal propagules.

The age of the bark surface on a twig identified to host was considered to be a potentially important environmental parameter with regard to epiphyte presence or absence. Epiphyte presence absence frequency counts on aged twigs should give a preliminary statistically informative overview of the success or failure of this natural ecological process.

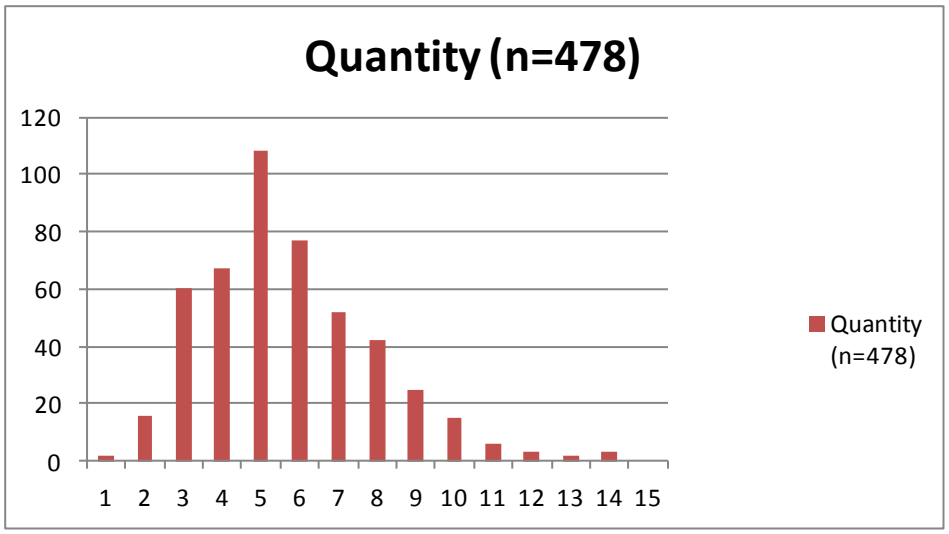
Epiphyte colonies might be recruited from ascospores, soredia etc. and other propagules on twig surfaces that are a few years old. There may be an initial stage on a young twig where the bark surface is hostile to epiphyte colonisation. This might become better for propagule growth, once some algal colonies are on the bark to resynthesize the lichen symbiosis in nature, under optimal microclimatic conditions.

Tree ring counts are a proxy for twig age. The ring count is detectable in cross sections of ring porous woods and is the maximum age of the bark based on the phenomenon of tree ring accrual in live twigs.

When twigs die, no tree ring accrual occurs, so this underestimates the age of dead attached twigs. It was not foreseen to control for this in our observations. We estimate that less than 20% of twigs were attached dead twigs when collected.

Age	Quantity (n=478)
1	2
2	16
3	60
4	67
5	108
6	77
7	52
8	42
9	25
10	15
11	6
12	3
13	2
14	3
15	0

This data is plotted below. More twigs than any were 5 years and majority of samples were in the range 3 years to 8 years.



The lichen diversity of plots was extracted and graphed by number of species. For each lichen diversity count total, the maximum age proxy for the twig was averaged over the number of replicates at that lichen diversity available in the twig sample set (n=478).

Plot of frequency of epiphyte diversity counts (0 to 21 species) against the average maximum ring count (4 to 8 years) of all aged twigs sampled. The peak at 19 is a statistical artefact of no significance caused a lack of replicates.

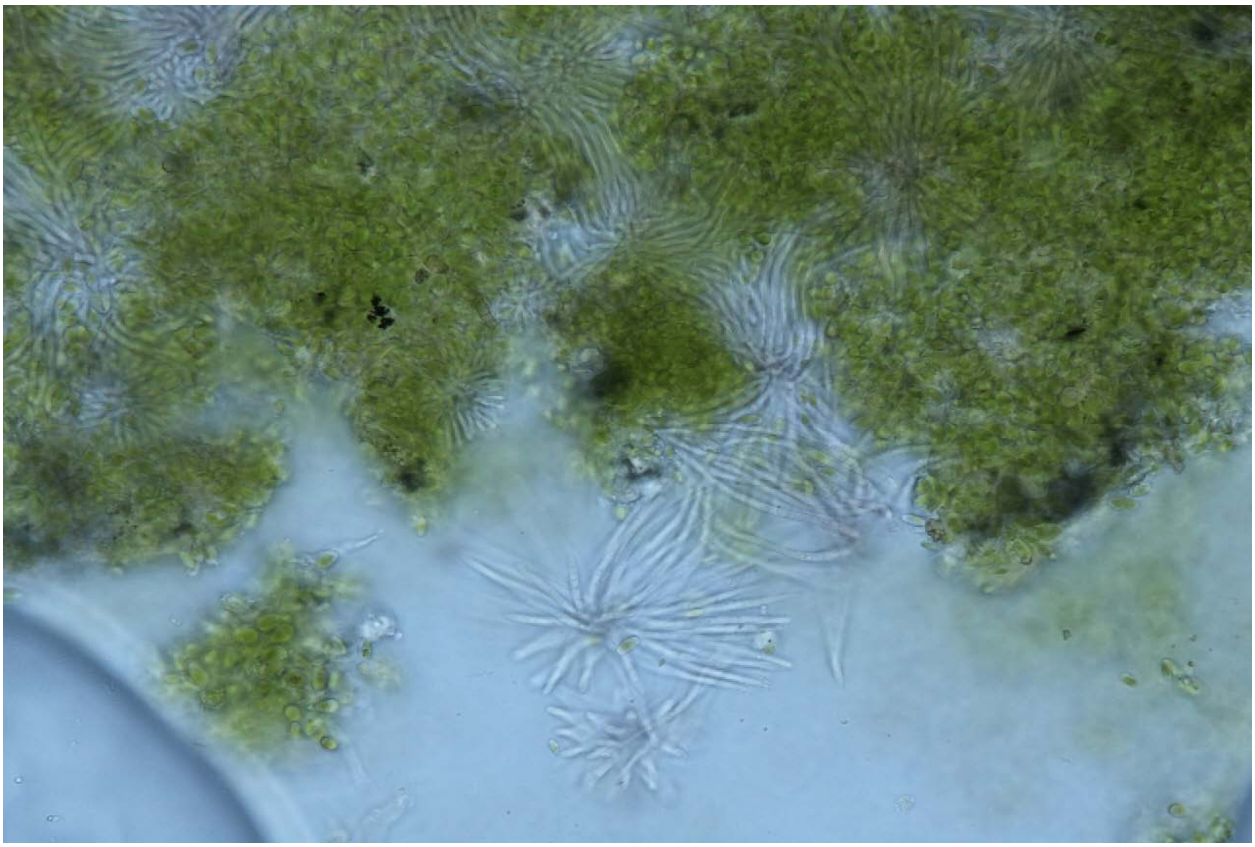
This trend of gradient demonstrates that lichen diversity increases with twig age in this pioneer epiphyte cohort.

The lichen diversity statistic behaves quite differently when trunk communities of young, mature and old trees are sampled, and lichen diversity declines with

community closure (which is competition effect) and diversity recruitment rates decline.

New to Ireland and notable records

New to Ireland - *Psammina stipitata* Lichenicolous fungus parasitising algal cells.



Twig key

The basis for the identification of twigs from experience from our handling large number of twigs (12.385 kg and 4209 counted from 480 samples) is difficult to communicate. We have probably made some errors as the morphological properties of some twigs are very similar indeed, but we have tried to keep these to a minimum.

The correct botanical identification of the woody plants in the field in the winter months is very challenging and difficult. Fieldwork began in September 2011 and continued to November 2011. It resumed in March 2012 until early June 2012 and was concluded in September 2012. Tree leaves are absent for 7 months in the year, and essential botanical properties needed for species identification of the woody plant are best displayed in a balanced consideration of the whole plant from multiple views of the shoots, thorns, leaves, flowers, fruits, seeds. Tree leaves were available to assist identification for the small portion of sampling undertaken between mid May and early June.

The OCAE consultants field teams and the EpiAir screening team, the agreement at taxonomic species level is 33%. At the taxonomic level of botanical family, the agreement increases significantly to 50%.

As only 25 grams (4 gr ams min. to 51 gramsmax.) of woody material was presented, the morphological expression in the hand sample was restricted. The EpiAir team identified the woody twig hosts on the basis of criteria of the surface of the bark, lenticels, epidermal hairs, lenticels, thorns and bud characters in the sample presented. There is a pressing scientific need to develop a quick, convenient and more reliable laboratory testing method for twig identification.

Biometric data was generated from a subset of 480 samples with the following woody plant attribution.

Acer campestre	2
Acer pseudoplatanus	24
Alnus glutinosa	54
Betula pubescens	17
Calluna vulgaris	1
Corylus avellana	46

Crataegus monogyna	49
Fagus sylvatica	9
Fraxinus excelsior	44
Ilex aquifolium	2
not determined	18
Picea abies	1
Picea sitchensis	27
Pinus contorta	3
Prunus avium	1
Prunus domestica	1
Prunus spinosa	20
Quercus petraea	4
Salix	133
Salix atrocinerea	2
Sambucus nigra	3
Sorbus aucuparia	15
Ulex europaeus	3
Viburnum opulus	1
	480

Genetics Results

The ITS (Inter Transcribed Spacer) region is a "barcode" segment of rRNA used in fungal genetics.

In order to access the genetic code of a fungal sample the following steps must be carried out successfully:

The sample must be isolated and digested

The sample must undergo a PCR (Polymerase Chain Reaction) process

The PCR product is checked using a Nanodrop® 2000 for DNA

The PCR product is visualised by Gel electrophoresis and using this method bands of DNA are elucidated

The PCR product is then purified and sent for sequencing of the ITS region

Genetic methods are described in detail in Chapter 2.

The results are listed below with the nearest species listed on GenBank using BLAST (Basic Local Alignment Sequencing Tool) analysis.

Areas of the sequence in Red signify the end of the 18S region and beginning of the 28S regions, white the ITS1 and ITS2 sequences and green the 5.8S region.

6009.1SP With Opegrapha vulgata (partial sequence)

CCTTCTTCGGTAGGCG..CGGGAGGAAGGATCATTA.CGAG.GTTGTACCTCT...AGGTTCCCACTTCCA.G.GTAA
GT.G.CTTTAA.CGTAGTTTAGG.GTACCTC.GACTTTAATCTTCC.ACCAGTATTTGGCCAAGGGTGTCACCTTCG
GGACGGCCGATCCTCACACTGGGTACCATTTTTGAATTATTATAACCGTAACA.AACTTTCAACAA.GGATCTGA
TTGGTCTCGCTTAGAAGAAGATCGCAGGGTAATGGGATAAGTAAAGTGAAAAGCAGAAT.CAGTAGA.TCCGA
ATCTCTG.ACGCACCCCGCGCCTC.AATTATTCAGAGAGGCACGCCCCC.GAGTGTCATAAAAAATCAAAATCAC.
GACTTTCATGACCTGGGGTGGACCCGGATTGGGGTGTTTCCCGTCCCCGGCTCCCCTCCAAAACCTTAGTAGG
GACCAG.CCGCCTT.CAGCGAGGTAAAATTATCG.TTACGTAGG..ACTGGGCCTCTCTCAGAAAA..CCAC....A...
GACCTCTAAT

BLAST sequence search gives the following as the first species name listed but the similarity level between these species is very low at 77% of only 197 base pairs – really covering the central and conserved 5.8S region separating ITS1 from ITS2:

Fellomyces polyborus 18S rRNA gene (partial), 5.8S rRNA gene, 26S rRNA gene (partial), ITS1 and ITS2, strain CBS 6643 = HB97

Sequence ID: emb|AJ608672.1|

Alignment statistics for match #1

Score	Expect	Identities	Gaps
129 bits(142)	3e-26	151/197(77%)	8/197(4%)

```
Query    189    TAACAAACTTTCAACAA-GGATCTGATTGGTCTCGCTTAGAAGAAGATCGCAGGGTAATG
247
            ||| |||||||||||||| ||||||  | | |||||| | || ||||| ||||| | ||||
Sbjct    143    TAAAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATG
202

Query    248    GGATAAGTAAAGTGAAAAGCAGAAT-CAGTAGATC--CGAATCTCTGA-CGCACCCCGCG
303
             ||||||||| |||||  ||||||| ||||  |||  ||||||| ||| ||||||  |||
Sbjct    203    CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCG
262

Query    304    CCTCAAT-TATTCAGAGAGGCACGCCCCC--GAGTGTCATAAAAAATCAAAATCACGACT
360
            ||||    ||||| |||||||| |||     ||||||||| |||||||||   | ||  |
Sbjct    263    CCTCTTGGTATTCCGAGAGGCATGCCTGTTTGAGTGTCATGAAAAATCAACCCCTCGGGT
322

Query  361  TTCATGACCTGGGGTGG  377
            ||   ||||||||||||
Sbjct  323  TTTCCGACCTGGGGTGG  339
```

6013.1SP With Lecanora chlarotera

CTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTTTACTGAGCTGT...T.CCCTCTCGGGGGATGTGCAC
GTCTTTCACTCATCTATACACCTGTGAACCTATGCGAGTTTAATGGGGGATTAGCGAAAGGGC.GAAA.CTTCC
GTAGGTGAACCTGCGGAAGGATCATTAC.TAGAGTTTGTAGA.TT.GGT.TGATACCTCTTACCCATGTCTTTTGA
GTACCTTCGTTTCCTCGGCGGGTCCGCCCGCCGATTGGACAACATTCAAACCCTTTGCAGTTGCAATCAGCGTA
TGAAAAAAACATAATAGTTTCAACTTTCAACAACGGATCTCTTGGTTCTGGCATTGATGAAGAACGCAGCGAA
ATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTA
TTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCT
CTGCGTGTAGACTCGCCTTAAATCAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTT
GCACTCATAA.GTCGACGTCCAAAAG..CAT...ACACTCTGACCTCTAATC

A MegaBLAST analysis gave the most common species in GenBank as:

Epicoccum nigrum strain Fk164 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
Sequence ID: gb|KC464342.1|

Score	Expect	Identities	Gaps
804 bits(435)	0.0	477/495(96%)	12/495(2%)

```
Query  140  TTCCGTAGGTGAACCTGCGGAAGGATCATTA-CTAGAGTTTGTAGA-TT-GGT-TGATAC  195
            |||||||||||||||||||||||||||||| ||||||||||||| || ||| || |||
Sbjct  23   TTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTTGTAGACTTCGGTCTGCTAC  82

Query  196  CTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCCCGCCGATTGGAC  255
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  83   CTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCCCGCCGATTGGAC  142

Query  256  AACATTCAAACCCTTTGCAGTTGCAATCAGCGTATGaaaaaaaCATAATAGTTTCAACTT  315
            |||||||||||||||||||||||||||||| || ||||||||||||||| ||||||
Sbjct  143  AACATTCAAACCCTTTGCAGTTGCAATCAGCGTCTG-AAAAAACATAATAGTTACAACTT  201

Query  316  TCAACAACGGATCTCTTGGTTCTGGCATTGATGAAGAACGCAGCGAAATGCGATAAGTAG  375
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  202  TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAG  261

Query  376  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTA  435
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  262  TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTA  321

Query  436  TTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGG  495
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322  TTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGG  381

Query  496  TGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTTAAATCAATTGGCAGCCGGCGTATTGAT  555
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  382  TGTTTGTCTCGCCTCTGCGTGTAGACTCGCCTTAAAACAATTGGCAGCCGGCGTATTGAT  441

Query  556  TTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAA-GTCGACGTCCAAAAG--CA---  609
            |||||||||||||||||||||||||||||||||||| | ||||||||||||  ||
Sbjct  442  TTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTACATTT  501

Query  610  -TACACTCTGACCTC  623
             |||||||||||||
```

```
Sbjct  502  TTACACTCTGACCTC  516
```

Epicoccum nigrum is a saprophytic mould that has world-wide distribution. Outdoors, it can be found in the soil, on canvas and cotton, and on any decaying vegetation. Indoors, Epicoccum has been isolated from carpets, wood flooring, mattress dust and exposed acrylic paint.

Epicoccum is frequently reported in air samplings. Studies have found 20 – 30% of atopic (having a predisposition to have allergies) patients had a positive test result to *Epicoccum*. Numerous allergenic proteins have been identified from *Epicoccum* and it has been linked to allergic fungal sinusitis (AFS) (Noble *et al.* 1997).

6031.13S With Lecidella elaeochroma

CCTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGATAGGGTTCCCTGAGCCCGACCTCCAACCCTTTGA
TTAAACCACTCTGTTGCTTCGGCCGGCCCGTCTTCTTCACGGAAGACCACCGGAGGTGTTCAGTCACCTCTGGT
CCGTGCTGGCCGGTAGCCCATTTTAATTCTTTTTTAAACGATGTTTGTCTGATGATTGAAAATCAATAATTAAAA
CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCATATTGCGCCCAAAGGTATTCCGAAGGGCATGCCTGTTCGA
GCGTCATAATCAACCAGTCAAGCCTGGCTTGTCATTGGGTCTCCATCGATCACACGATGGATCCGAAAGATAA
TGGCGGCGTGAAGATCGACCCCAGGTGCAGCGAGCTTTCCAAGCATACACTGAGGCGGTCGTCTTCACCCGG
TCGCACGATTTTCAGTA.GGTGACCT

A MegaBLAST analysis gave the most common species in GenBank as:
Phaeomoniella capensis strain CBS 123535 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gb|FJ372391.1|

Score	Expect	Identities	Gaps
516 bits(279)	1e-142	441/517(85%)	19/517(3%)

```
Query   3      TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGATAGGGTTCCCT-GAGCCCGACCT  61
              ||||||||||||||||||||||||||||||| ||| ||||||||  | |||||||| ||
Sbjct   111    TTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAGTTAGGGTTCTTTCGAGCCCGATCT  170

Query   62     CCAACCCTTTGATTAAACCA-CTCTGTTGCTTCGGCCGGCCCGTCTTCTTCACGGAAGAC  120
              |||||||||||  || ||| | ||  ||||||| ||| |||||||  |||| ||||  |||
```

```
Sbjct    171    CCAACCCTTTG-TTCAACTACCTTCGTTGCTTTGGCAGGCCCGT--TCTTTACGG--GAC
225


Query    121    CACCGGAGGTGTTCAGTCACCTCTGGTCCGTGCTGGCCGGTAGCCCATTTTAATTCTTTT
180

            | ||||||||||  |||||||||| ||||||  ||| ||||||||    |||||||
Sbjct    226    CGCCGGAGGTGTTAGGTCACCTCTGGCCCGTGCCTGCCAGTAGCCCACACAAATTCTT-C
284


Query    181    TTAAACGATGTTTGTCTGATGATTGAAAATCAA--TAATTAAAACTTTCAACAACGGATC
238

            |  ||  || ||||||| |  ||| || || ||  ||||||||||||||||||||||||
Sbjct    285    TACAA--AT-TTTGTCTAAGCATTAAATATAAATTTAATTAAAACTTTCAACAACGGATC
341


Query    239    TCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
298

            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    342    TCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
401


Query    299    ATTCAGTGAATCATCGAATCTTTGAACGCATATTGCGCCCAAAGGTATTCCGAAGGGCAT
358

            |||||||||||||||||||||||||||||| |||||||||   ||||||||||||||||
Sbjct    402    ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGAAGGGCAT
461


Query    359    GCCTGTTCGAGCGTCATAATCAACCAGTCAAGCCTGGCTTGTCATTGGGTCTCCA-TCGA
417

            |||| |||||||||||| ||| |||| ||||||| ||||||| ||||||||| |  |||
Sbjct    462    GCCTATTCGAGCGTCATTATC-ACCACTCAAGCCCGGCTTGTTATTGGGTCTTCTGTCGT
520


Query    418    TCACACGATGGATCCGAAAGATAATGGCGGCGTGAAGATCGACCCCAGGTGCAGCGAGCT
477

            | | | ||  || |||||||||||||||||| ||||  ||||||||||||||||||||
Sbjct    521    T-A-AGGACAGACCCGAAAGATAATGGCGGCGTCAAGAATGACCCCAGGTGCAGCGAGCT
578


Query  478  T-TCCAAGCATACACTGAGGCGGTCGTCTTCACCCGG  513
               | | |||||||||||||| ||| |||||  |||||
Sbjct  579  CATAC-AGCATACACTGAGGTGGTTGTCTTGTCCCGG  614
```

Phaeomoniella capensis because of its etymology is unlikely to be present in this sample. *Its* Name refers to the Cape Province of South Africa, where this fungus was first described. *Specimen examined*. South Africa, Western Cape Province, Kirstenbosch Botanical Garden, on living leaves of *Encephalartos altensteinii*, 22 May 2008, *A.R. Wood*, CBS H-20159, culture ex-type CPC 15416 = CBS 123535, CPC 15417–15418.

Eurotiomycetes sp. genotype 400 isolate FL0854 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gb|JQ760492.1|

Score	Expect	Identities	Gaps
542 bits(600)	1e-150	429/503(85%)	15/503(2%)

```
Query  3    TTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGATAGGGTTCCCTGAGCCCGACCTC  62
            |||||||||||||||||||||||||||||||| |||||||||| | |   ||||||||||
Sbjct  213  TTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAGATAGGGTCCTCGTGGCCCGACCTC  272

Query  63   CAACCCTTTGATTAAACCACTCT-GTTGCTTCGGCCGGCCCGTCTTCTTCACGGAAGACC  121
            |||||||||| ||||   || || ||||||| ||| ||||||||||     |||  ||||
Sbjct  273  CAACCCTTTGTTTAATTTACCCTTGTTGCTTTGGCAGGCCCGTCTTT----CGG--GACC  326

Query  122  ACCGGAGGTGTTCAGTCACCTCTGGTCCGTGCTGGCCGGTAGCCCATTTTAATTCTTTTT  181
             ||||||||| |   |||| ||||||| ||||| |||  ||||| |||  |||||| |||
Sbjct  327  GCCGGAGGTGATTCTTCACTTCTGGTCAGTGCTTGCCATTAGCCAATTGAAATTCTCTTT  386

Query  182  TAAACGATGTTTGTCTGATGATTGAAAATCAAT-AATTAAAACTTTCAACAACGGATCTC  240
             |||  |||   ||||||   || ||  | ||| ||||||||||||||||||||||||||
Sbjct  387  -AAATAATGCA-GTCTGAATCTTTAAT-TAAATCAATTAAAACTTTCAACAACGGATCTC  443

Query  241  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  444  TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAT  503

Query  301  TCAGTGAATCATCGAATCTTTGAACGCATATTGCGCCCAAAGGTATTCCGAAGGGCATGC  360
            |||||||||||||||||||||||||| |||||||||   ||||||||||||||||||||
Sbjct  504  TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGAAGGGCATGC  563

Query  361  CTGTTCGAGCGTCATAATCAACCAGTCAAGCCTGGCTTGTCATTGGGTCTCCATCGATCA  420
            ||||||||||||||| ||| |||  ||||||||||||||| | |||||||  |||| |||
Sbjct  564  CTGTTCGAGCGTCATTATCCACC-CTCAAGCCTGGCTTGTTAATGGGTCT-TATCGGTCA  621
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Query  421  CACGATGGATCCGAAAGATAATGGCGGCGTGAAGATCGACCCCAGGTGCAGCGAGCTTTC  480
             | ||| || |  |||||||||||||||| | ||| ||||||||||||||||||||||
Sbjct  622  AATGATAGACCTCAAAGATAATGGCGGCGTCATGATAGACCCCAGGTGCAGCGAGCTTT-  680

Query  481  CAAGCATACACTGAGGCGGTCGT  503
             |||||||||||||| ||||||
Sbjct  681  -AAGCATACACTGAGGTGGTCGT  702
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6031.14 With *Scoliciosporum umbrinum*

CTTCCGTTGGTGAAC.TG.GGAAGGATCATTA.TGAGTTAGGGTCTTATAGGCCCG...TCCAACC.TT.GAGCCCGT
TCCTTGAAATATAGGGACCGCCGGGGGTTTAATACCCTTGGTCCGTGCTCGTCGTATAGCCATTATTAAANTCT
TGATTAAATGTGTCGTGTAAGTAAACAAGCAAATAAAATAAAACTTTCAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGCGAATTGCAGAATCCGTGAGTCATCGAATCTTTGAACG
CATATTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTCGAGCGTCATTATCACCTCTCAAGCCTGGCTGGCT
GTTGGACTTTAGGTCTTGCTTAGGCATGCCCGGTCCCAAAGATAAGGAGGGCGTCTGTGAGGACCCCAGTAC
ATTGAGCTTTTATTAGCACGTACTAGGTGGGAGTCCAGGCCCGGTCTT.A.TATAACTT.T.AAGGTGACCT..AAT

A MegaBLAST analysis gave the most common species in GenBank as:
Capronia sp. 97002a 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gb|EU139150.1|

Score	Expect	Identities	Gaps
595 bits(322)	1e-166	407/448(91%)	8/448(1%)

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Query  63   GAGCCCGTTCCTTGAAATATAGGGACCGCCGGGGGTTTAATACCCTTGGTCCGTGCTCGT  122
             ||||||||  |||||||||||| | |||||||||||| |||||| | ||||||||||||
Sbjct  96   GAGCCCGTCTCTTGAAATATAGAGGCCGCCGGGGGTTTTATACCCCTAGTCCGTGCTCGT  155

Query  123  CGTATAGCCATTATTAAANTCTTGATTAAATGTGTCGTGTAAGT--AAACAAGCAAATAA  180
             || |||||| |||||||  ||||| |||| |||||||| |||||  |||||   || |||
Sbjct  156  CG-ATAGCCTTTATTAAGCTCTTGTTTAACTGTGTCGTCTAAGTAAAAACACTTAATTAA  214

Query  181  AATAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG  240
             |  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  215  ACCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG  274

Query  241  CGATAAGTAATGCGAATTGCAGAATCCGTGAGTCATCGAATCTTTGAACGCATATTGCGC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  275  CGATAAGTAATGCGAATTGCAGAATCCGTGAGTCATCGAATCTTTGAACGCATATTGCGC  334

Query  301  CCTTTGGTATTCCGAAGGGCATGCCTGTTCGAGCGTCATTATCACCTCTCAAGCCTGGCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  335  CCTTTGGTATTCCGAAGGGCATGCCTGTTCGAGCGTCATTATCACCTCTCAAGCCTGGCT  394

Query  361  GGCTGTTGGACTTTAGGTCTTGCTTAGGC-ATGCCCGGTCCCAAAGATAAGGAGGGCGTC  419
             |||||||||||| ||||| |||||  || | | ||||||||||||||| || ||||||
Sbjct  395  TGCTGTTGGACTTCAGGTCATGCTTTAGCTAAGACCGGTCCCAAAGATAATGACGGCGTC  454

Query  420  TGTGAGGACCCCAGTACATTGAGCTTTTATTAGCACGTACTAGGTGGGAGTCCAGGCCCG  479
            |||||||||||||||| |||||||||| |||||||||||||| ||||||||||||||||
Sbjct  455  TGTGAGGACCCCAGTACAATGAGCTTTTA-TAGCACGTACTAGGCGGGAGTCCAGGCCCG  513

Query  480  GTCTTATATAACTT-T-AAGGT-GACCT  504
            ||||||| ||  || | ||||| |||||
Sbjct  514  GTCTTATTTATTTTATCAAGGTTGACCT  541
```

Cladophialophora scillae strain CBS 116461 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gb|EU137329.1|

Score	Expect	Identities	Gaps
521 bits(282)	2e-144	395/449(88%)	12/449(2%)

```
Query  63   GAGCCCGTTCCTTGAAATATAGGGACCGCCGGGGGT-TTAATACCCTTGGTCCGTGCTCG  121
            ||||||||| |||||||||||| |||||||||||||| ||| ||||  ||||||||||||
Sbjct  73   GAGCCCGTTTCTTGAAATATAGAGACCGCCGGGGGTGTTATTACCTCTGGTCCGTGCTCG  132

Query  122  TCGTATAGCCATTATTAAANTCTTGATTAAATGTGTCGTGTAAGTAAACAAGCAAATAAA  181
            ||| ||||||| | ||||| ||||| |||| | |||||| ||| ||||  |   ||| ||
Sbjct  133  TCG-ATAGCCAAT-TTAAACTCTTGTTTAACTATGTCGTCTAAATAAAGCACTTAAT-AA  189

Query  182  ATAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC  241
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  ACAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC  249

Query  242  GATAAGTAATGCGAATTGCAGAATCCGTGAGTCATCGAATCTTTGAACGCATATTGCGCC  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  250  GATAAGTAATGCGAATTGCAGAATCCGTGAGTCATCGAATCTTTGAACGCATATTGCGCC  309
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Query  302  CTTTGGTATTCCGAAGGGCATGCCTGTTCGAGCGTCATTATCACCTCTCAAGCCTGGCTG  361
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  310  CTTTGGTATTCCGAAGGGCATGCCTGTTCGAGCGTCATTATCACCTCTCAAGCTTGGCTT  369

Query  362  GCTGTTGGACTTTAGGTCTT-GCTTAGGCATGCCCGGTCCCAAAGATAAGGAGGGCGTCT  420
            ||||||||| | |||||||  | |||    || ||||||| |||||||| || || ||||
Sbjct  370  GCTGTTGGA-TCTAGGTCTGAGTTTATCTCTGACCGGTCCTAAAGATAATGACGGTGTCT  428

Query  421  GTGAGGACCCCAGTACATTGAGCTTTTATTAGCACGTACTAGGTGGGAGTCCAGGCCCGG  480
             ||||||| |||||||||||||||||| | ||||||||| |||| | | || ||||||||
Sbjct  429  ATGAGGACTCCAGTACATTGAGCTTTT-TAAGCACGTACCAGGTAGAACTCTAGGCCCGG  487

Query  481  TCTTA-T-ATAACTT-T-AAGGT-GACCT  504
            ||||| | |||  || | ||||| |||||
Sbjct  488  TCTTAATCATATTTTATCAAGGTTGACCT  516
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In summary, lichen samples examined in this genetic study were very small due to the need for specimen purity. A reduced amount of digest chemicals was used in acknowledgement of small specimen volume. Unfortunately, the supernatant liquid retained the vast majority of DNA after the digest preparation for PCR. So in three out of four cases the supernatant sample from the digest was used for analysis, with two of these three samples being purified as explained to examine any difference in quality that might be attained.

Results did not feature lichen sequences but interesting surface or endophytic species of fungi.

Epicoccum nigrum shows a relatively high (96%) agreement across 495 base pairs of the ITS sequence from sample 6013.1SP. This is a small, yet harmful fungus that has been implicated in pulmonory problems in humans.

Sample 6031.14 shows a 91% correlation across 448 base pairs of ITS sequence of *Capronia* sp. Certain species of the *Capronia* genus have been identified as endophytes of lichens. While many species are represented in GenBank, the ones that we study here are among the least represented and so it is not unusual for microscopic fungal species to be absent. However, with some further work, there may be a number of exciting outcomes from this study.

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