Eaton, S. , Zuniga, C., Czyzewski, J., Ellis, C., Genney, D. R., Haydon, D., Mirzai, N. and Yahr, R. (2018) A method for the direct detection of airborne dispersal in lichens. *Molecular Ecology Resources*, 18(2), pp. 240-250. There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

Eaton, S. , Zuniga, C., Czyzewski, J., Ellis, C., Genney, D. R., Haydon, D., Mirzai, N. and Yahr, R. (2018) A method for the direct detection of airborne dispersal in lichens. *Molecular Ecology Resources*, 18(2), pp. 240-250. (doi:10.1111/1755-0998.12731) This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

http://eprints.gla.ac.uk/151181/

Deposited on: 12 November 2017

MS. SALLY EATON (Orcid ID : 0000-0001-8680-4592)

Title page

Title: A method for the direct detection of airborne dispersal in lichens

Authors: Eaton, S.[1], Zuniga, C.[1], Czyzewski, J.[2], Ellis, C.J.[1], Genney, D.[3], Haydon, D.[2], Mirzai, N.[2], and Yahr, R[1].

Postal addresses: [1] Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh, EH3 5LR; [2] Graham Kerr building, College of Medical, Veterinary & Life Sciences (MVLS), University of Glasgow, University Avenue, Glasgow G12 8QQ; [3] Great Glen House, Leachkin Road, INVERNESS, IV3 8NW.

Corresponding author: Eaton, S.; address - Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh, EH3 5LR; email - seaton@rbge.ac.uk.

Running title: Direct detection of dispersal in lichens

Abstract:

This paper sets out a novel method to determine dispersal distances in lichens. Direct measurement of dispersal often remains difficult for lichens and other small inconspicuous species because of the need to track microscopic reproductive propagules, which even if they can be captured, cannot be identified using traditional morphological approaches.

A low-cost device (<£200) was developed in order to trap the reproductive propagules of lichens, capable of sampling around $0.1m^3$ of air per minute. In parallel, molecular techniques were developed to enable species specific detection of propagules caught by the devices, with identification using novel species-specific primers and optimization of a standard DNA extraction and nested PCR protocol. The methods were tested for both their sensitivity and specificity against a suite of lichen epiphytes, differing in their reproductive mechanisms, dispersal structures, and rarity.

Sensitivity tests showed that the molecular techniques could detect a single asexual propagule (soredium or isidium), or as few as 10 sexual spores. As proof of concept, propagule traps were deployed into a wooded landscape where the target epiphytes were present. Extractions from deployed propagule traps were sequenced, showing that the method was able to detect the presence of the target species in the atmosphere.

As far as we are aware, this is the first attempt to use mechanized propagule traps in combination with DNA diagnostics to detect dispersal of lichens. The tests carried out here point the way for future dispersal studies of lichen epiphytes and other passively-dispersed microscopic organisms including fungi or bryophytes.

Key-words: air-borne propagules, nested PCR, rotating arm propagule trap, species specific primers

1. Introduction

Dispersal has become a major point needing clarification in the ecology of many small inconspicuous organisms such as spore-forming fungi, lichens, bryophytes, protists and bacteria (e.g. Bell *et al* 2005; Whitfield 2005; Foissner 2006; van der Gast 2015). Dispersal studies are problematic for these species because of their microscopic life history stages; these frequently lack definitive morphological features, thus limiting the usefulness of conventional tracking and identification methods. As a result, there is a paucity of dispersal data for these groups, despite the promise of recent technological advances such as genetic identification. The impact of these data gaps is exacerbated for rare and endangered species. For example, while connectivity is an important parameter within conservation projects (Öster *et al* 2009), certain species such as lichen epiphytes operate as patch-tracking meta-populations (Snäll *et al* 2005), such that knowledge of dispersal distance and frequency become particularly important to inform management plans. However, a lack of accurate information on dispersal means that these species are often neglected in forest ecology and conservation planning (Scheidegger & Stofer 2015).

Most estimates of dispersal distance for case-study species such as lichen epiphytes have been made indirectly. Data have been collected through investigations of the spatial arrangement of colonized trees (e.g. Dettki *et al* 2000; Belinchón *et al* 2011; Gjerde *et al* 2015), through population genetic studies (e.g., Walser 2004; Lättman 2009; Jüriado *et al* 2011; Hilmo *et al* 2012; Scheidegger *et al* 2012), through modelling approaches (e.g. Wagner *et al* 2006; Johansson *et al* 2012), or time series observations of colonization events (e.g. Öckinger *et al* 2005; Fedrowitz *et al* 2012). As these studies rely on data from established lichen thalli, they are unable to apportion the effect of dispersal from that of establishment, and therefore make estimates of effective dispersal only (Scheidegger & Werth 2009). In addition, varying methods produce conflicting results that depend in part on their assumptions. For example, the dispersal distance of the epiphytic *Lobaria pulmonaria* has been shown to be efficient at a regional scale in some contexts (Wagner *et al* 2006; Werth *et*

al 2006a; Hilmo *et al* 2012; Otálora *et al* 2015), or limited to local distances of less than 100m in others (Öckinger *et al* 2005; Jüriado *et al* 2011).

Direct measures of dispersal distance in species such as lichen epiphytes, though more likely to draw robust conclusions, are less frequent because of difficulties in tracking and identifying reproductive propagules (note, the term propagules is applied here when referring to sexual spores or asexual soredia or isidia), coupled with the low concentration of such propagules in the environment. Such studies must comprise two parts, first any airborne propagules present must be extracted from the air/ground, and second, techniques must be employed to identify whether the target species is present in the sample. Early studies were based on passive sampling methods often under experimental conditions, combined with morphological identification (e.g. Bailey 1966; Armstrong 1994). More recently highly efficient propagule traps, capable of capturing minute concentrations of biological particles in the atmosphere have been used, which greatly increase the chances of capturing the target species should it be present. To date, such technology has been combined with morphological identification techniques in lichen studies (Marshall 1996; Favero-Longo *et al* 2014), thereby restricting investigations to experimental conditions or to species that produce distinctive propagules.

Genetic techniques (DNA extractions combined with PCR techniques) enable identification of a greater range of species propagules (subject to the relevant primers being available), as well as being highly sensitive e.g. being able to detect the presence of a single fungal spore in a complex sample (Schwarzott & Schüßler 2001; McCartney *et al* 2003). However, direct estimates of lichen epiphyte dispersal utilizing DNA-based diagnostics have yet to be combined with propagule trap technology. Other organism groups of commercial importance have been subjected to these combined technologies however, particularly in studies of plant fungal pathogens (Williams *et al* 2001; Calderon *et al* 2002; McCartney *et al* 2003; Schweigkofler *et al* 2004; Peccia & Hernandez 2006; West *et al* 2008; Brittain *et al* 2013), which have led to evidenced-based predictions of disease risk, rate of spread and early

warning systems in crop and forestry systems (West *et al* 2008).

The technology to measure the dispersal distance of lichen epiphytes exists in theory, but in practice the cost of propagule traps is high, leading to a tendency towards passive sampling (e.g. Walser *et al* 2001). This issue of cost is exacerbated by the large numbers of traps required by dispersal studies which are often inherently large in scale and require replication for statistical testing. In addition, the resources to develop species specific primers are often not available, leading to a focus on well-studied species (e.g. *Lobaria pulmonaria* (Hilmo *et al* 2012; Walser *et al* 2001; Werth *et al* 2006a)).

To address these issues on behalf of lichen epiphytes and other small inconspicuous organisms (see above), here we develop a low-cost propagule trap and optimize existing DNA-based diagnostics to enable sensitive detection of dispersed propagules. As very little is known about dispersal in lichen epiphytes, the method was developed to explore dispersal in species exhibiting a range of ecological strategies (including both common and rare species, as well as those reproducing sexually and asexually, or both); the species selected for study are *Lobaria pulmonaria, Nephroma laevigatum, Nephroma parile, Pannaria conoplea, Pannaria rubiginosa, Parmelia saxatilis sensu lato* and *Pectenia* spp. The inclusion of rare species means that detection methods must concentrate efforts on target species, to prevent swamping of the desired signal. Propagule sizes (see Supplementary Material 1) range from 5-11µm x 15-30 µm in the case of the studied sexual spores (comprising the fungal partner only), and 40-240µm x 40-480µm in the case of asexual soredia and isidia (which include both symbiotic partners). Specificity and sensitivity tests are presented, along with the results of a field trial as proof of concept. The trapping method could easily be combined with other molecular diagnostics including, for example, metabarcoding or high-throughput sequencing in order to various expand or limit the taxonomic range of organisms under study.

2. Materials and Methods

2.1 Propagule trap design

A propagule trap was designed to be capable of trapping reproductive propagules of epiphytic lichens, ranging from c.500µm³ to over 2,000,000µm³, or 10.6µm to 800µm in their aerodynamic diameter (AED (Di-Giovanni 1998), using the settling velocity calculation of McCubbin 1944. The propagules are expected to be emitted throughout the year at a rate of between zero and many hundred per day (Bailey 1966; Armstrong 1992). The trap was designed to meet the following specifications. It must be:

- Capable of trapping air borne particles on impact,

- Capable of trapping hydrophobic fungal propagules,

- Able to process a constant and high throughput of air,

- Capable of trapping continuously over short periods (up to 24hrs) or intermittently for longer periods (up to 72 hrs),

- Portable and able to withstand the outdoor field environment,

- Relatively cheap to construct (material costs <£200 per unit), allowing many units to be deployed at once, and

- Compatible with DNA-based diagnostics with minimal transfer loss.

The final design was based on a rotating arm prototype, run from an electronic microcontroller enabling continuous or timed intermittent sampling. Power was provided by sealed lead acid batteries. Capable of sampling 0.1m³ (100L) of air per minute over a total surface area of 2 x 1.5cm², the rotating motion pulls air in from both above and below the device (Di-Giovanni 1998). Airborne particles were then trapped into a thin layer of petroleum jelly that coats the front-most face of the sampling arms. Impactor type traps such as this are suited to sampling airborne particles with an AED of over 10 µm (West and Kimber 2015), with smaller particles being deflected around the rotating arms (Di-Giovanni 1998). Post deployment, the layer of petroleum jelly can be transferred into a DNA

extraction tube using a spatula; alternatively, the sampling arms may be removed and transferred directly into the tubes. Full details of the designed trap can be found in Supplementary Material 2 (note fabrication of the device is likely to require a skilled technician).

2.2 Primers

Pre-existing specific primers were available for just one of the seven species under study; *Lobaria pulmonaria* (Walser *et al* 2001). For the remaining six species, *Nephroma laevigatum, Nephroma parile, Pannaria conoplea, Pannaria rubiginosa, Parmelia saxatilis* sensu lato. and *Pectinia atlantica*, specific primers were designed for the ITS region (ITS1-5.8S-ITS2).

For each species, an ITS sequence set was generated from sequence data deposited in GenBank (see Supplementary Material 2) and, in the case of those species that were poorly represented, new accessions were generated from at least 2 individuals per lichen species from different populations across Scotland. For each specimen, DNA was isolated from 20 mg of dried thallus material using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions modified by increasing initial lysis incubation to 1h at 65°C to ensure maximum yield. From the extracted DNA, the ITS region was amplified and sequenced from both directions using consensus primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990).

Each sequence set included those of the target species aligned against those of closely related non-target species. Primers were manually selected over regions of specificity and their melting temperatures, hairpin stability and self and hetero-dimer formation were later checked in Primer3 (Untergrasser *et al* 2012) and Oligo Analyzer 3.1 (available at: www.idtdna.com/calc/analyzer). In cases where there was a high chance of hairpin and/or dimer formation, a single mutation was introduced in the primer sequence, with the aim of disrupting the stability of the secondary structure. Specificity of the primers was checked manually by submitting them to a Basic Local Alignment Search Tool analysis (BLAST)

adjusted for short input sequences to determine interactions with non-target sequences. Initially, more than 15 primers were designed and tested *in silico*; only those that presented no relevant matches with the GenBank database were selected as having potentially good specificity and chosen for further laboratory analyses (Table 1). Universal primers ITS4 (White *et al* 1990) and ITS1F (Gardes & Bruns 1993) were used in combination with the forward and reverse specific primers, respectively.

To determine the optimal annealing temperature of each primer pair that guaranteed a specific reaction, PCR reactions were performed using the HotStarTaq™ Master Mix Kit (QIAGEN, Hilden, Germany) at different annealing temperatures ranging from 55°C to 68°C on a selection of DNA products from closely related lichen species; Pcn1F and Prb1F were tested on *P. conoplea* and *P. rubiginosa*, Nla1F and Npa2F were tested on *N. parile, N. laevigatum* and *N. tangeriense*, Dg1R was tested on *P. cyanoloma, P. atlantica* and *P. plumbea*, and Psax3F was tested on *P. saxatilis, P. sulcata, P. discordans, P. omphalodes, P. serrana* and *P. ernstiae*. PCR reactions (21µl) contained 3.5µl of template DNA, 10µl HotStarTaq ™ Master Mix (QIAGEN, Hilden, Germany), 4µl 5x Combinatory Enhancer Solution (CES; 2.7M betaine, 6.7% DMSO and 55µg/ml BSA), 2µl CoralLoad Concentrate (QIAGEN, Hilden, Germany), 0.35µM ITS1F (Gardes & Bruns 1993) and 0.35µM ITS4 (White *et al* 1990). Cycling conditions were as follows: initial 5 min denaturation step at 95 °C, 30 amplification cycles of 94 °C for 1 minute, species specific annealing temperature for 1 minute, 72 °C for 90 s, and a final extension step of 72 °C for 5 minutes. Samples were held at 8°C on completion of the PCR. In all cases, the PCR products were analysed by running 5µl on a 1% agarose gel containing SYBR safe in 1X TBE buffer, and were visualized and photographed on a UV transilluminator. Optimum annealing temperatures (see Table 1) were identified when they produced a band under the UV transilluminator for the specific species in question only.

2.3 DNA extraction protocol

The method used for extraction and purification of propagule DNA from the collection medium was adapted from that described by Calderon *et al* (2002). Possible contamination was recognized through the addition of controls (samples containing petroleum jelly which had not been exposed to the species under study). Samples were transferred into 2 ml FastPrep™ Lysing Matrix C tubes (MP Biomedicals United Kingdom) along with 220µl IGEPAL CA630 (Sigma, USA) and 0.2g 0.1mm zirconia beads. The tubes were heated to 65ºC in a water bath until the petroleum jelly liquefied, then immediately transferred to a FastPrep-24™ 5G Instrument (MP Biomedicals United Kingdom) where they were shaken to release cell contents. The minimum disruption period capable of releasing cell contents (found through microscopic examination of experimental samples) was found to be 3 periods of 40s at 6ms^{-1}. A five-minute interval between runs in the FastPrep machine was implemented to prevent overheating of the machine, during this time the samples were transferred to the water-bath to re-liquefy the petroleum jelly.

DNA was purified from the resulting suspensions using the method of Doyle & Doyle (1987), modified by the addition of 20ng glycogen (Roche Diagnostics Ltd., Lewes, UK) at the isopropanol precipitation step, as per Williams *et al* (2001). Further modifications were made to the protocol as follows: due to the volume of petroleum jelly added to the tubes only 750ml of CTAB was used, the sample was incubated for 1 hour at 65ºC on an Eppendorf™ ThermoMixer C Temperature Control Device (Fisher Scientific, United Kingdom) during the cell lysis and chloroform extraction stages, and the samples were left in the freezer for 3 days during the isopropanol precipitation step. The final DNA pellet was re-suspended in 50µl of TE after centrifugation.

2.4 Nested PCR protocol

A nested PCR approach was implemented as this has been shown to be more sensitive than single-step PCR in the detection of airborne fungal spores (Williams *et al* 2001). Fungal

DNA was amplified using the highly conserved fungal rDNA gene primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al* 1990) using the HotStarTaq™ Master Mix Kit (QIAGEN, Hilden, Germany). PCR reactions were the same as those set out in the PCR reactions described in section 2.2 above. The PCR thermal protocol consisted of an initial 5 min denaturation step at 95 °C, 30 amplification cycles of 94 °C for 1 minute, 57 °C for 1 minute, 72 °C for 90 s, and a final extension step of 72 °C for 5 minutes. Samples were held at 8°C on completion of the PCR. In all cases, the PCR products were analysed by running 5μl on a 1% agarose gel containing SYBR safe in 1X TBE buffer, and were visualized and photographed on a UV transilluminator.

The PCR product was then taken to a new PCR chamber (i.e. laminar flow hood) to decrease contamination risk. Here it was diluted 1:100 in distilled water to be used in the second round of PCR, this time using species-specific primers. PCR reactions were as above, though amended by using 2μl of diluted PCR product and 4.5μl CES, giving an overall reaction volume of 20μl. The PCR thermal protocol was dependent on the optimum annealing temperature (as described in section 2.2, see Table 1 for final annealing temperatures used). Samples were held at 8°C on completion of the PCR and visualized as above.

2.5 Sensitivity tests

Sensitivity testing begins with a known number of propagules placed into extraction tubes. In the case of asexual species (*Lobaria pulmonaria, Nephroma parile, Pannaria conoplea, Parmelia saxatilis, Pectenia atlantica*), isidia/soredia were first dislodged from their respective thalli (using a brush or a spatula) under a dissecting microscope and transferred to a sheet of paper for counting. In this way, three replicates of; 1, 2, 3, 5, 10 and 20 soredia/isidia, were transferred to individual labelled tubes for extraction.

For sexually reproducing/dispersing species (*Lobaria pulmonaria, Nephroma laevigatum, Pectenia cyanoloma, Pectenia rubiginosa*) spores were extracted from their apothecia using a method adapted from Yoshimura *et al* (2002). Several apothecia were carefully removed

from cleaned thalli, placed into a muslin bag, and left in a 250ml beaker of water under a running tap for around 4hrs. Several soaked apothecia were then immediately transferred to a single Petri dish, where they were attached to the inside of the upper lid using petroleum jelly. Fifteen millilitres of distilled water were added to the bottom of the Petri dish (water was used a collecting medium rather than a microscope slide as initial tests showed it was difficult to dislodge emitted spores once they had adhered to the glass) and the dishes then sealed with Parafilm to be left on a window ledge for 72 hrs. After each 24hr period, a water drop was pipetted onto individual apothecia and the dishes re-sealed. The water from the agar plate was then spun down in a centrifuge and the supernatant removed, leaving a small amount (approx. 1ml) of liquid remaining. This suspension was agitated then transferred into a 1.5ml Eppendorf with 10µl of Tween 20 (Sigma, USA). The tubes were then agitated to ensure that any spores in the solution were well dispersed and an Improved Neubauer Bright Line haemocytometer (Blau Brand) was used to determine the spore concentration under a compound microscope. From this initial solution, dilutions were made to gain approximate known concentrations of spores of 1000/ml (where spore abundance allowed), 100/ml, 10/ml and 1/ml. The presence of packets of spores (anywhere between 2 to 8 spores, viewed in the haemocytometer) rendered spore concentrations approximate.

Three samples of each solution were made up per species. In addition to the pure propagule samples, equivalent sensitivity tests were performed with the addition of 0.1g of petroleum jelly, giving a total of 6 samples per species/concentration. DNA extractions and PCR reactions were then performed using the methods detailed above, with the PCR product again analysed on an agarose gel.

To assess the effect of non-target species on the assay, thallus fragments from a mixture of 4 lichen epiphytes (*Lobaria virens, Parmeliella triptophylla, Peltigera collina* and *Sticta fuliginosa*) were combined with propagule suspensions of the target species (approx. 10 spores or 1 soredium/isidium of each species) into a single extraction tube. This was then subject to DNA extraction detailed above, and the DNA product tested for the presence of

each of the target species through nested PCR (as detailed above). The PCR products were then cleaned using 2 µl ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific) for 5µl of PCR product, and each sent to the University of Edinburgh to be sequenced in two reactions (using the two specific PCR primers) following Big Dye Terminator v. 3.1 chemistry (Applied Biosystems, Warrington, UK). Sequences were then assembled and aligned using Sequencher v. 3.7 (GeneCodes Corp., Ann Arbor, Michigan, USA) and queried against nucleotide sequence databases using BLAST-N.

2.6 Proof of concept

Three propagule traps were deployed in Glen Creran, Argyll on the west coast of Scotland (56.565489˚N, -5.2338723˚W). Each trap was set up at least 100m from a woodland edge (see Figure 1), where large populations of the target species are known to be found (Eaton *et al* 2017a). Trap A was set up at the mouth of Loch Creran, just above the high tide line, Trap B was set up in the bottom of the glen in an area of grazing marsh, and Trap C was set up at the head of the glen in an area of recently cleared non-native plantation woodland. This distance was selected to balance capturing both estimated long-distance dispersal events and a background rain of abundant species.

At each trapping location, a single propagule trap was erected on a pole approximately 1m above ground level. The front-most facing sides of the 2 x 1.5cm² sampling arms were covered with a thin layer of petroleum jelly (approx. 0.1g). Prior to their use the sampling arms had been autoclaved, and were sealed and not exposed to outdoor conditions until arrival at the trapping locations. The traps were then operated on an intermittent setting (3 mins on/off) for a period of 5 days from the 10th October 2015. The traps were re-visited every 24hrs to ensure that they were still functioning and after a period of 72hrs the batteries were replaced.

At the end of the trapping period, the petroleum jelly was transferred from the pair of collecting arms directly into the extraction tubes in-situ at the trap site, using a sterile scalpel blade. Sampling was then repeated (again for 5 days) using a new set of sterilized sampling

arms.

The environmental samples were then subject to DNA extraction and nested PCR as described above. An additional control was added to the sample line up comprising petroleum jelly which had been applied to a pair of sterilized collecting arms and then transferred to an extraction tube in the laboratory.

As a final check of specificity, all positive samples from the rarest species in the landscape (those thought to be present in the lowest concentrations in the atmospheric samples) were sent to the University of Edinburgh for sequencing. Sequencing of both strands of the final PCR product was performed using specific primers as described above.

The number of positive trappings per species was then compared to the percentage of colonized trees in the landscape (as estimated in Eaton *et al* 2017a).

3. Results

3.1 Primer specificity

Designed primers were tested against a wide range of non-target lichen species that were either closely related to the study species, or that also occurred within the woodland study site. In the case of *L. pulmonaria, N. laevigatum, N. parile, P. conoplea* and *P. rubiginosa* the primers were found to be species specific (see Table 2). In the case of the primers designed for *P. atlantica* and *P. cyanoloma*, it was not possible to identify a primer pair capable of separating the two species. In the case of *P. saxatilis* sensu lato, the exact species of *Parmelia* present at the test site was unknown (though likely including *P. saxatilis* s. str., *P. ernstiae*, and probably *P. serrana*), therefore the primer pair was designed to identify a range of *Parmelia* species making up the *P. saxatilis* sensu lato complex.

3.2 Sensitivity of DNA diagnostics

Sensitivity testing found that the DNA-diagnostic system could identify a single propagule in the case of asexual species*, P. atlantica, L. pulmonaria N. parile, P. conoplea* and *P. saxatilis* (see Figure 2).

In the case of sexually reproducing species, the method was reliably sensitive to 10 spores and occasionally sensitive to a single spore (see Figure 2 for frequency of positive result and see Figure 3 for gel of the example species *L. pulmonaria*).

It was not possible to determine whether the occasional sensitivity to a single spore was due to a poorly dispersed suspension (i.e. the presence of more than a single spore in some samples, and none at all in others), or a lower sensitivity in the method itself. The presence of collecting medium in the samples was not found to affect the sensitivity of the method for asexual species even when only a single propagule was present in the samples. The collecting medium had no impact on sensitivity of 10 spore suspensions in sexual species. At more dilute concentrations, there was an apparent decrease in sensitivity when collecting medium is added to the samples, with 9 of 12 positive samples, decreasing to 7 of 12 positive samples.

The presence of non-target species in a 10-spore sample was not found to affect the sensitivity or specificity of the method, with amplification of target DNA successful, even when extracted with excess non-target DNA. All resultant nucleotide sequences had a high (≥98%) identity across full-length ITS and high BLAST match scores with Scottish specimens of the target species.

3.3 Field tests

The field tests showed that the propagule traps were capable of collecting sexual and asexual lichen propagules under field conditions (see Figures 4 and 5). Propagules of *P. saxatilis* sensu lato were found in 4 of the 6 samples, *L. pulmonaria* and *N. laevigatum* in 3 of the 6 samples, *P. conoplea* in 2 of the 6 samples, and *P. rubiginosa* in 1 of the 6 samples.

Nephroma parile and *P. atlantica/P. cyanoloma* were not detected in the traps over the 10-day period. The controls were all negative.

The frequency of positive tests was found to be positively correlated with the species abundance (percentage of trees in landscape colonized by the species) in the case of *P. saxatilis, N. parile, P. conoplea,* and *P. rubiginosa* (see Figure 6, marked as o). *Lobaria pulmonaria* and *N. laevigatum* (marked as x in Figure 6) were detected more frequently by the traps than would be expected according to this relationship. As the *Pectenia* spp result does not represent a single species, it was not included in this analysis.

The eight samples sent for sequencing (positive samples of *L. pulmonaria, P. conoplea* and *N. laevigatum*) confirmed that the PCR products giving positive bands under the UV transilluminator matched nucleotide sequences for the target species with high BLAST match scores with Scottish specimens as expected.

4. Discussion

Direct measures of dispersal distance in lichens are rare, and to date have been confined to a small number of species (Heineken 1999; Walser *et al* 2001; Hilmo *et al* 2012; Favero-Longo *et al* 2014) and/or experimental conditions (Bailey 1966; Armstrong 1994). We present a highly sensitive technique that is capable of being used in field conditions, for a variety of lichen species. The results of the field trial clearly demonstrate that lichen reproductive propagules can be sampled by rotating arm propagule traps, and that DNA diagnostics can be used to detect their presence. As far as we are aware, this is the first attempt to use propagule traps in combination with specific primers to detect the presence of airborne reproductive propagules in lichens. Against a background of propagules of other fungal guilds like saprotrophs or pathogens or even common species in the same guild, propagules of rare species of lichen fungi may be swamped at any of several experimental stages by more abundant templates without a targeted approach. The sensitivity of the technique combined

with efficient trapping however, suggest that high-throughput sequencing techniques could easily be applied, using either meta-barcoding or shotgun sequencing should the facilities and expertise be available.

On the other hand, it is important to balance cost with investment, and for ecological or landscape-scale studies, the large number of samples required (in the hundreds) may mean that relatively cheap traps and detection methods (e.g. PCR only) may be advantageous (e.g. Smart *et al.* 2016).

4.1 Propagule trap design

Rotating arm samples were first developed ca. 60 years ago, (Perkins 1957), and were originally combined with microscopy for the detection and quantification of airborne spores and pollen (Magill *et al* 1968). Such traps offered an improved alternative to passive sampling for two reasons. First, they allow highly dilute particulate matter in the atmosphere to be captured and concentrated, thus enabling detection of even very rare and intermittent particles, and second, they enable even very small particles (such as fungal spores) to be captured, as the speed at which the collecting arms rotate increases particle momentum, thus leading to impaction rather than deflection. The trap designed here allows over $0.1m^3$ of air to be processed per minute. At a speed of $1800m/s^{-1}$ the collecting arms ensure that lichen propagules can be captured (Di-Giovanni 1998). Though a variety of other trap types are available for atmospheric sampling (e.g. Hirst-type traps, Cascade impactors), rotating arm traps are relatively simple to construct and are robust to the outdoor environment. However, in common with commercially available traps the device designed here does require a skilled technical person to fabricate, which may be seen as a limitation of the design. The low cost of the trap, being approximately one tenth of the price of many commercially available traps however, enables multiple traps to be deployed simultaneously, thus increasing their usefulness in future dispersal experiments.

4.2 DNA diagnostics and sensitivity of method

Nested PCR is commonly used when concentrations of the target organism (or its DNA) are low and the objective is to increase sensitivity of detection (Wilson *et al* 1991); this method has been shown to detect minute quantities of target DNA, e.g. from a single spore (Schwarzott & Schüßler 2001; Williams *et al* 2001). The methodology employed here allowed reliable detection of a single asexual propagule (soredia/isidia) and occasional detection of a single sexual spore; though detection was only consistent when 10 spores were present. The sensitivity reported here is very similar to that reported by other (non-lichen) fungal dispersal studies that use nested PCR methods (e.g. Williams *et al* 2001; Calderon *et al* 2002), and improves on those for epiphytic lichen dispersal studies which detect single asexual propagules of *L. pulmonaria* in 50% (Hilmo *et al* 2012) and 33% (Walser *et al* 2001) of samples tested (sensitivity tests using standard PCR).

Though the sensitivity of spore detection reported here was variable (between 1 and 10 spores), it is not clear whether this was due to the sensitivity of the method itself or due to poorly dispersed suspensions of spores. Poorly dispersed spores in suspension occur as a result of groups or packets of spores being released simultaneously from an ascus (Yoshimura *et al* 2002). Such grouping was observed in the spores of all the sexual species used in this study during an earlier experiment (see Eaton *et al* 2017b). Therefore, it is possible that the variability in detection is due to variability in the concentration of spores in the suspensions; this suggests that the positive results from single spore dilutions actually result from higher spore concentrations.

Although technology would enable quantification of propagules through qPCR, the usefulness of such data in the case of wind dispersed propagules remains equivocal. First, sampling efficiency is known to be affected by variable environmental factors such as wind speed and humidity, which can affect the release of propagules from apothecia (spores) or thallus surfaces (isidia/soredia) (Scott 1959; Ostrofsky & Denison 1980). Second, sampling efficiency is known to be affected by species/propagule specific factors, such as impaction

efficiency, retention and bounce-off (Di-Giovanni 1998; Peel *et al* 2014) e.g. rotating arm samplers have been found be 85% efficient when trapping 50µm particles (May *et al* 1976), and just 50% efficient when trapping 7µm particles (May & Clifford 1967). In addition to variables affecting the number of propagules a trap may catch under different conditions, there is also variation to be found at a molecular level, for example the unknown variability in template DNA copy number within and among species or the potential for bias in recovery from PCR and nested PCR or, all of which would require careful standardisation experiments to be carried out. Therefore, rather than quantifying the number of propagules caught in a trap (which could be subject to species specific factors listed above), it was felt that frequency of positive trappings would provide a more comparable metric of dispersal across species.

Last, although high-throughput sequencing not requiring specific primers may appear more straightforward, such an approach carries the risk of missing rare species (as detailed above). In addition, the apparent economy of high-throughput sequencing can quickly be lost when balancing the need for screening many separate samples to understand landscape-scale processes. In that case, one needs not specific primers, but rather indexed primers which can be separated bioinformatically after the sequencing run, to account for multiple samples. Labs which already have access to a panel of indexed primers however can take advantage of the other aspects of the approach here to maximize DNA yield from spores and simultaneously achieve robust landscape-scale sampling. Here, the caveat about rare species still applies; these may represent small enough fractions of total sequence outputs that they may be lost in cleaning and quality checking steps without very careful controls to test for sensitivity of recovery by spiking experiments.

4.3 Field trial

The field trial showed that after 5 days there was a variable detection rate for propagules of the contrasting epiphyte species. Here, the frequency of positive trappings was broadly explained by species abundance (Eaton *et al* 2017a), apart from in the case of *Lobaria pulmonaria* and *Nephroma laevigatum*. These species were caught more frequently by the

traps than would be expected by their abundance in the landscape. Both these species have been found to produce very high numbers of spores per apothecia during emission experiments (Eaton *et al* 2017a), which could explain this discrepancy. In addition, these species produce the smallest propagules of all the species in this study (see Supplementary material 1). Small reproductive propagules are thought to travel further than larger propagules due to their lower settling velocity (Jongejans & Telenius 2001) and therefore higher time airborne (though this relationship is less clear for very small particles such as lichen spores). The combination of longer time airborne and high overall numbers could explain the frequency of positive catches despite their low abundance in the landscape.

Inter- and intra-species variability in propagule capture/detection have been associated with numerous factors however, such as weather (e.g. rainfall (Scott 1959; Kofler & Bouzon 1960), temperature (Bailey & Garrett 1968; Pyatt 1969; Ostrofsky & Denison 1980) and/or humidity (Garrett 1971; Ostrofsky & Denison 1980; Armstrong 1991), and wind speed and direction (Armstrong 1987; 1991)), the concentration of propagules in the air (and the amount of time traps are deployed), trap configuration and distance to source (it is possible that the propagules of some species do not travel very far from their parent thallus (Armstrong 1994; Jüriado *et al* 2011)), and/or seasonality of propagule production (Des Abbayes 1951; Verseghy 1955; Pyatt 1969). Through the methods described here, there is much scope to further explore these factors.

Though the field trial here was limited, the results demonstrate the potential for using rotating arm propagule traps in combination with DNA diagnostics, to investigate dispersal dynamics in lichen epiphytes. It is hoped that these methods will enable further exploration of direct dispersal in lichen epiphytes and other inconspicuous organisms in the future.

Acknowledgements

grant from Scottish Natural Heritage (no. 14503).

Data Accessibility

Trap design – see Supplementary material 2

DNA sequences – see Supplementary material 3 for Genbank accessions

Author Contributions

SE, CE, RY, DH and DG conceived the initial ideas for this project. NM, JC and SE designed the propagule traps, JC built the propagule traps, CZ designed the species-specific primers with support and guidance from RY, SE optimized the DNA extraction and nested PCR protocol with support and guidance from RY, SE conducted sensitivity, specificity and proof of concept tests with support and guidance from RY. SE led the writing of the manuscript with support and guidance from RY and CE.

References

Armstrong, R. A. (1987). Dispersal in a population of the lichen *Hypogymnia physodes*. Environmental and Experimental Botany, 27, 357-363. doi: 10.1016/0098-8472(87)90046-3

Armstrong, R. A. (1991). The influence of climate on the dispersal of lichen soredia. Environmental and Experimental Botany, 31, 239-245. doi: 10.1016/0098-8472(91)90076-Z

Armstrong, R. A. (1992). Soredial dispersal from individual soralia in the lichen *Hypogymnia physodes* (L.) Nyl. Environmental and Experimental Botany, 32, 55-63. doi: 10.1016/0098-8472(92)90030-6

Armstrong, R. A. (1994). Dispersal of soredia from individual soralia of the lichen *Hypogymnia physodes* (L.) Nyl. in a simple wind tunnel. Environmental and Experimental Botany, 34, 39-45. doi: 10.1016/0098-8472(94)90007-8

Bailey, R. H., 1966. Studies on the dispersal of lichen soredia. Botanical Journal of the Linnean Society, 59, 479-490. doi: 10.1111/j.1095-8339.1966.tb00074.x

Bailey, R. H. & Garrett R. M. (1968). Studies on the discharge of ascospores from lichen apothecia. Lichenologist, 4, 57-65. doi: 10.1017/S0024282968000083

Belinchón, R., Martínez, I., Aragón, G., Escudero, A. & De la Cruz, M., 2011. Fine spatial pattern of an epiphytic lichen species is affected by habitat conditions in two forest types in the Iberian Mediterranean region. Fungal Biology, 115, 1270-1278. doi: 10.1016/j.funbio.2011.09.003

Bell, T., Newman, J. A., Thompson, I. P., Lilley, A. K., & van der Gast, C. J. (2005) Bacteria and island biogeography. Science 309, 1998–1999. doi: 10.1126/science.309.5743.1997

Brittain, I., Selby, K., Taylor, M. & Mumford, R. (2013). Detection of plant pathogen spores of economic significance on pollen trap slides. Journal of Phytopathology, 161, 855-858. doi: 10.1111/jph.12129

Calderon, C., Ward, E., Freeman, J. & McCartney, A. (2002). Detection of airborne fungal spores sampled by rotating-arm and Hirst-type propagule traps using polymerase chain reaction assays. Journal of Aerosol Science, 33, 283-296. doi: 10.1016/S0021-8502(01)00179-3

Des Abbayes, H. (1951). Traite de Lichenologie. Encyclopedia of Biology, 41, 1-2.

Dettki, H., Klintberg, P. & Esseen, P.A. (2000). Are epiphytic lichens in young forests limited by local dispersal? Ecoscience, 7, 317-325. doi: 10.1080/11956860.2000.11682601

Di- Giovanni, F. (1998). A review of the sampling efficiency of rotating- arm impactors used in aerobiological studies. Grana, 37, 164-171. doi: 10.1080/00173139809362661

Doyle J. J. & Doyle J. L. (1987) CTAB DNA extraction in plants. Phytochemical Bulletin 19:11–15

Eaton S, Ellis C. J., Genney D., Thompson R., Yahr R., & Haydon D. (2017a). Adding small species to the big picture: species distribution modelling in an age of landscape scale conservation. Biological Conservation. Accepted for publication.

Eaton S., Ellis C. J. & Yahr R. (2017b). How many? Spore emission experiments in epiphytic lichens. The Lichenologist. Accepted for publication.

Favero-Longo, S. E., Sandrone, S., Matteucci, E., Appolonia, L. & Piervittori, R. (2014). Spores of lichen-forming fungi in the mycoaerosol and their relationships with climate factors. Science of the Total Environment, 466, 26-33. doi:10.1016/j.scitotenv.2013.06.057

Fedrowitz, K., Kuusinen, M. & Snäll, T. (2012). Metapopulation dynamics and future persistence of epiphytic cyanolichens in a European boreal forest ecosystem. Journal of Applied Ecology, 49, 493-502. doi: 10.1111/j.1365-2664.2012.02113.x

Foissner, W. (2006) Biogeography and dispersal of microorganisms: a review emphasizing protists. Acta Protozoologica 45, 111–136.

Gardes, M. & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes- application to the identification of mycorrhizae and rusts. Molecular Ecology, 2, 113-118. doi: 10.1111/j.1365-294X.1993.tb00005.x

Garrett, R. M. (1971). Studies on some aspects of ascospore liberation and dispersal in lichens. The Lichenologist, 5, 33-4. doi: 10.1017/S0024282971000070

Gjerde, I., Blom, H. H., Heegaard, E. & Sætersdal, M. (2015). Lichen colonization patterns show minor effects of dispersal distance at landscape scale. Ecography, 38, 939-948. doi: 10.1111/ecog.01047

Heineken, T. (1999). Dispersal patterns of terricolous lichens by thallus fragments. Lichenologist, 31, 603-612. doi: 10.1017/S0024282999000791

Hilmo, O., Lundemo, S., Holein, H., Stengrundet, K. & Stenoien, H. K. (2012). Genetic structure in a fragmented Northern Hemisphere rainforest: large effective sizes and high connectivity among populations of the epiphytic lichen *Lobaria pulmonaria*. Molecular Ecology, 21, 3250-3265. doi: 10.1111/j.1365-294X.2012.05605.x

Johansson, V., Ranius, T. & Snäll, T. (2012). Epiphyte metapopulation dynamics are explained by species traits, connectivity, and patch dynamics. Ecology, 93, 235-241. doi: 10.1890/11-0760.1

Jongejans, E., & Telenius A. (2001). Field experiments on seed dispersal by wind in ten umbelliferous species (Apiaceae). Plant Ecology, 152, 67–78. doi: 10.1023/A:1011467604469

Jüriado, I., Liira, J., Csencsics, D., Widmer, I., Adolf, C., Kohv, K. & Scheidegger, C. (2011). Dispersal ecology of the endangered woodland lichen *Lobaria pulmonaria* in managed hemiboreal forest landscape. Biodiversity and Conservation, 20, 1803-1819. doi: 10.1007/s10531-011-0062-8

Kofler, L. & Bouzon F. (1960). Emission et germination des spores chez quelques champignons des lichens. In 85eme Congres des Societes Savantes (pp. 389-399)

Lättman, H., Lindblom, L., Mattsson, J. E., Milberg, P., Skage, M. & Ekman, S. (2009). Estimating the dispersal capacity of the rare lichen *Cliostomum corrugatum*. Biological Conservation, 142, 1870-1878. doi: 10.1016/j.biocon.2009.03.026

Magill, P. L., Lumpkins, E. D. & Arveson, J. S. (1968). A system for appraising airborne populations of pollens and spores. American Industrial Hygiene Association Journal, 29, 293-298.

Marshall, W. A. (1996). Aerial dispersal of lichen soredia in the maritime Antarctic. New Phytologist, 134, 523-530. doi: 10.1111/j.1469-8137.1996.tb04370.x

May K. R., Pomeroy N. P. & Hibbs S. (1976). Sampling techniques for large windborne particles. Journal of Aerosol Science 7, 53-62. doi: 10.1016/0021-8502(76)90009-4

May, K. R. & Clifford, R., (1967). The impaction of aerosol particles on cylinders, spheres, ribbons and discs. Annals of Occupational Hygiene, 10, 83-95. doi: 10.1093/annhyg/10.2.83

McCartney, H. A., FÖster, S. J., Fraaije, B. A. & Ward, E. (2003). Molecular diagnostics for fungal plant pathogens. Pest Management Science, 59, 129-142. doi: 10.1002/ps.575

McCubbin, W. A., 1944. Relation of spore dimensions to their rate of fall. Phytopathology, 34, 230-234.

Öckinger, E., Niklasson, M. & Nilsson, S.G. (2005). Is local distribution of the epiphytic lichen *Lobaria pulmonaria* limited by dispersal capacity or habitat quality? Biodiversity and Conservation, 14, 759-773. doi: 10.1007/s10531-004-4535-x

Öster, M., Ask, K., Cousins, S. A. & Eriksson, O. (2009). Dispersal and establishment limitation reduces the potential for successful restoration of semi- natural grassland communities on former arable fields. Journal of Applied Ecology, 46, 1266-1274. doi: 10.1111/j.1365-2664.2009.01721.x

Ostrofsky, A. & Denison, W. C. (1980). Ascospore discharge and germination in *Xanthoria polycarpa*. Mycologia, 1171-1179.

Otálora, M. A., Belinchón, R., Prieto, M., Aragón, G., Izquierdo, P. & Martínez, I. (2015). The threatened epiphytic lichen *Lobaria pulmonaria* in the Iberian Peninsula: genetic diversity and structure across a latitudinal gradient. Fungal Biology, 119, 802-811. doi: 10.1016/j.funbio.2015.05.004

Peccia, J. & Hernandez, M. (2006). Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: a review. Atmospheric Environment, 40, 3941-3961. doi: 10.1016/j.atmosenv.2006.02.029

Peel, R. G., Kennedy, R., Smith, M. & Hertel, O. (2014). Relative efficiencies of the Burkard 7-day, Rotorod and Burkard personal samplers for *Poaceae* and *Urticaceae* pollen under field conditions. Annals of Agricultural and Environmental Medicine, 21. doi: 10.5604/12321966.1129927

Perkins, W. A. (1957). The rotorod sampler, Second semi-annual report, Aerosol Laboratory, Department of Chemistry and Chemical Engineering, Stanford University, Palo Alto, CA, pp. 66.

Pyatt, F. B. (1969). Studies of periodicity of spore discharge and germination in lichens. Bryologist, 72, 48-53. doi: 10.1639/0007-2745(1969)72[48:SOTPOS]2.0.CO;2

Scheidegger, C. & Werth, S. (2009). Conservation strategies for lichens: insights from population biology. Fungal Biology Reviews, 23, 55-66. doi: 10.1016/j.fbr.2009.10.003

Scheidegger, C., Bilovitz, P. O., Werth, S., Widmer, I. & Mayrhofer, H. (2012). Hitchhiking

with forests: population genetics of the epiphytic lichen *Lobaria pulmonaria* in primeval and managed forests in south-eastern Europe. Ecology and Evolution, 2, 2223-2240. doi: 10.1002/ece3.341

Scheidegger, C. & Stofer, S. (2015). Bedeutung alter Wälder für Flechten: schlüsselstrukturen, vernetzung, ökologische Kontinuität. Swiss Journal of Forestry, 166, 75-82. doi: 10.3188/szf.2015.0075

Schwarzott, D. & Schüßler, A. (2001). A simple and reliable method for SSU rRNA gene DNA extraction, amplification, and cloning from single AM fungal spores. Mycorrhiza, 10, 203-207. doi: 10.1007/PL00009996

Schweigkofler, W., O'Donnell, K. & Garbelotto, M. (2004). Detection and quantification of airborne conidia of *Fusarium circinatum*, the causal agent of pine pitch canker, from two California sites by using a real-time PCR approach combined with a simple propagule trapping method. Applied and Environmental Microbiology, 70, 3512-3520. doi: 10.1128/AEM.70.6.3512-3520.2004

Scott, G. D. (1959). Observations on spore discharge and germination in *Peltigera praetextata* (Flk.) Vain. The Lichenologist, 1, 109-111. doi: 10.1017/S0024282959000263

Smart, A. S., Weeks, A. R., van Rooyen, A. R., Moore, A., McCarthy, M. A. and Tingley, R. (2016). Assessing the cost-efficiency of environmental DNA sampling. Methods in Ecology and Evolution, 7, 1291–1298. doi:10.1111/2041-210X.12598

Snäll, T., Pennanen, J., Kivistö, L. & Hanski, I. (2005). Modelling epiphyte metapopulation dynamics in a dynamic forest landscape. Oikos, 109, 209-222. doi: 10.1111/j.0030-1299.2005.13616.x

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3—new capabilities and interfaces. Nucleic Acids Research, 40, 115. doi: 10.1093/nar/gks596

van der Gast, C. J. (2015). Microbial biogeography: the end of the ubiquitous dispersal hypothesis. Environmental Microbiology, 17, 544-546. doi: 10.1111/1462-2920.12635

Verseghy, K. (1965). Effect of dry periods on spore production of lichens. Acta Biol. Acad. Sci. Hung. 16, 85-104.

Wagner, H. H., Werth, S., Kalwij, J. M., Bolli, J. C. & Scheidegger, C. (2006). Modelling forest recolonization by an epiphytic lichen using a landscape genetic approach. Landscape Ecology, 21, 849-865. doi: 10.1007/s10980-005-5567-7

Walser, J. C., (2004). Molecular evidence for limited dispersal of vegetative propagules in the epiphytic lichen *Lobaria pulmonaria*. American Journal of Botany, 91, 1273-1276. doi: 10.3732/ajb.91.8.1273

Walser, J. C., Zoller, S., Büchler, U. & Scheidegger, C. (2001). Species-specific detection of *Lobaria pulmon*aria (lichenized ascomycete) diaspores in litter samples trapped in snow cover. Molecular Ecology, 10, 2129-2138. doi: 10.1046/j.1365-294X.2001.01353.x

Werth, S., Wagner, H. H., Gugerli, F., Holderegger, R., Csencsics, D., Kalwij, J. M. & Scheidegger, C. (2006) Quantifying dispersal and establishment limitation in a population of an epiphytic lichen. Ecology, 87, 2037–2046. doi: 10.1890/0012-9658(2006)87[2037:QDAELI]2.0.CO;2

West, J. S., Atkins, S. D., Emberlin, J. & Fitt, B. D. (2008). PCR to predict risk of airborne disease. Trends in microbiology, 16, 380-387. doi: 10.1016/j.tim.2008.05.004

West, J. S. & Kimber, R. B. E. (2015). Innovations in air sampling to detect plant

pathogens. Annals of Applied Biology, 166, 4-17. doi: 10.1111/aab.12191

White, T. J., Bruns, T., Lee, S. J. W. T. & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18, 315-322.

Whitfield, J. (2005) Biogeography: is everything everywhere? Science 310, 960–961. doi: 10.1126/science.310.5750.960

Williams, R. H., Ward, E. & McCartney, H. A. (2001). Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. Applied and Environmental Microbiology, 67, 2453-2459. doi: 10.1128/AEM.67.6.2453-2459.2001

Wilson, I. G., Cooper, J. E. & Gilmour, A. (1991). Detection of enterotoxigenic Staphylococcus aureus in dried skimmed milk: use of the polymerase chain reaction for amplification and detection of staphylococcal enterotoxin genes entB and entC1 and the thermonuclease gene nuc. Applied and Environmental Microbiology, 57, 1793-1798.

Yoshimura, I., Yamamoto, Y., Nakano, T. & Finnie, J. (2002). Isolation and culture of lichen photobionts and mycobionts. In Protocols in Lichenology (pp. 3-33). Springer Berlin Heidelberg.

Tables and Figures

Table 1. PCR specific primers designed for the ITS region used in this study.

Primer[a]	Sequence (5'-3')[b]	Target species	Annealing temperature (°C)
Prb1F	CCGATGTGAAATTACCCGTA	*P. rubiginosa*	55
Pcn1F	CCACTGTcGAATTACCTTATG	*P. conoplea*	55
Nla1F	TCACTAGACATCCCCGGAGA	*N. laevigatum*	55
Npa2F	GCGTGGTGGTCAAGTATTCT	*N. parile*	61
Dg1R	GaAGCGACGTGGTCTACTACTA	*P. atlantica, P. cyanoloma*	55
Psax3F	ACCCCTCTCGTCGATCTACCG	*P. saxatilis* sensu lato	57

[a] *F* and *R* indicate forward and reverse primers, respectively [b] lower case letters correspond to single mutations intentionally introduced in the primer sequence to disrupt secondary structures.

Table 2. Specificity of designed primers.

Primer pair[a]	Specificity
ITS1F, Dg1R	*Pectenia atlantica, P. cyanoloma*
ITS1F, nuITSII-137-3[b]	*Lobaria pulmonaria*
Nla1F, ITS4R	*Nephroma laevigatum*
Npar1F, ITS4R	*Nephroma parile*
Pcn1F,ITS4R	*Pannaria conoplea*
Prb1F,ITS4R	*Pannaria rubiginosa*
Psax3F, ITS4R	*Parmelia saxatilis, P. serrana, P. ernstiae, P. omphalodes*

[a] *F* and *R* indicate forward and reverse primers   [b] see Walser et al. (2001).

Figure 1. Map on left shows location of Glen Creran in relation to Scotland, map on right shows approximate locations of the three propagule trap sites © Crown Copyright and Database Right [2017]. Ordnance Survey (Digimap Licence).
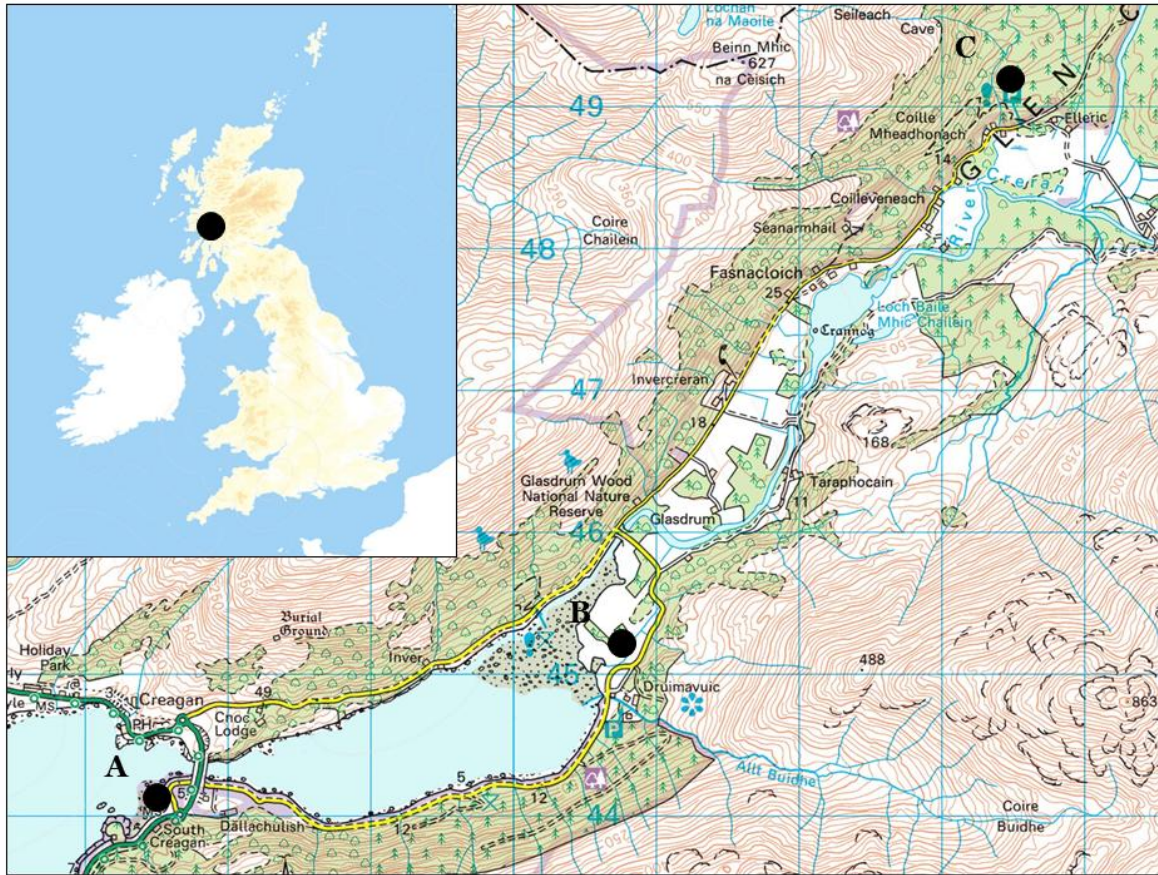

Figure 2. Sensitivity test results. The results of 1 and 10 propagule sensitivity tests both with (+m) and without (-m) collecting medium, showing the proportion of the 3 replicates which gave a positive result. S = Sexually reproducing individual/species, A = Asexual reproducing individual/species.
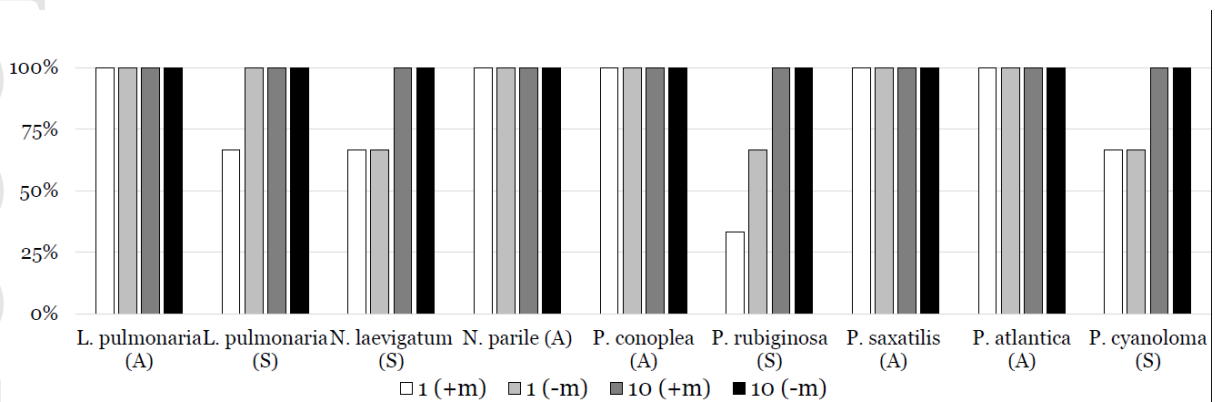
Figure 3. Sensitivity test gel. Example *L. pulmonaria* sensitivity test. Left to right: 1 spore, 10 spores, 100 spores, 1000 spores, negative control, positive control, 1 isidium/soreidum, 2 isidia/soreida, 3 isidia/soredia, 5 isidia/soredia.
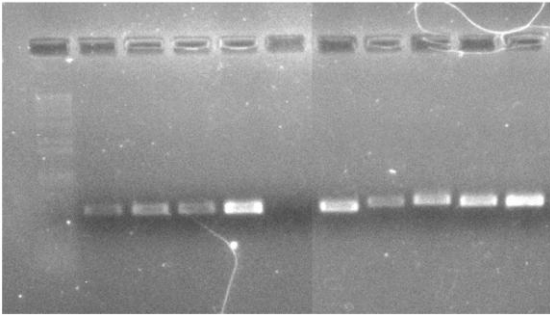

Figure 4. Field test results: Column 1 *P. saxatilis,* Column 2 *N. laevigatum,* Column 3 *L. pulmonaria,* Column 4 *P. conoplea,* Column 5 *P. rubiginosa,* Column 6 *Pectenia* spp., Column 7 *N. parile,* Column 8 Negative control (different target species per row). Row 1 Head of Glen 1, Row 2 Head of Glen 2, Row 3 Glen bottom 1, Row 4 Glen bottom 2, Row 5 Glen Mouth 1, Row 6 Glen Mouth 2.
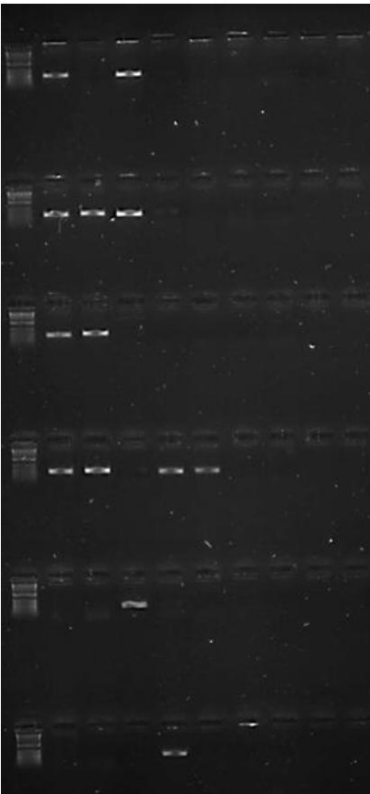
Figure 5. Field test results as proof of concept; showing the frequency, and location, of the positive results for each species.
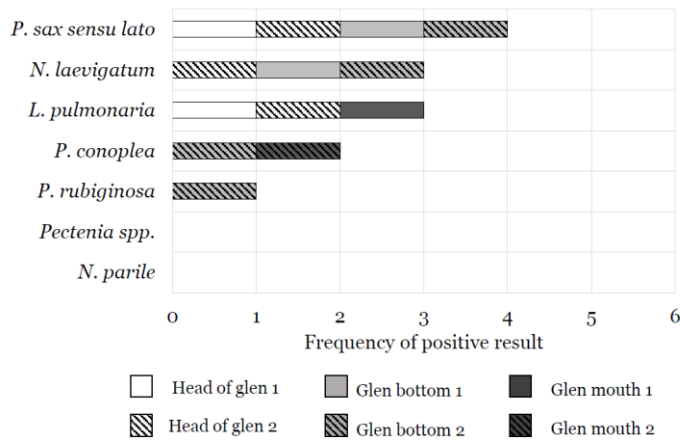

Figure 6. Species abundance and frequency of trapping. Exponential relationship ($y=1.19e^{0.80x}$ $r^2=0.98$) between the percentage of colonized trees in the landscape (as predicted in Eaton *et al* 2017a) and the frequency of positive trappings. Crosses represent outliers.