Analysis of the species diversity of leaf pathogens in Icelandic barley fields

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ABSTRACT

For the past 50 years the importance of barley in Icelandic agriculture has increased, not least as a result of environmental change and new cultivars. Following this the impact of pathogens is expected to increase as well as the importance of knowing which pathogens affect barley in Iceland. Presented here is the first analysis of the diversity of barley fungal pathogens in Iceland. Samples collected from around the country were analysed using molecular methods. At least twelve species of fungi were identified, including *R. secalis,* a known pathogen in Iceland, as well as fungi not previously recorded on barley in Iceland, including *Microdochium nivale*, *Fusarium avenaceum*, and *Epicoccum nigrum*. Three species, *Pyrenophora teres* f. *teres*, *Didymella exitialis*, and *Itersonilia perplexans*, are new to the Icelandic funga. The results show that diversity of barley fungal pathogens in Iceland is greater than previously thought and this will have implications for future breeding projects.

Keywords: Barley, fungal pathogens, species identification

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Greining á tegundafjölbreytni sveppa sem sýkja bygg í íslenskum ökrum

Síðastliðin 50 ár hefur mikilvægi byggræktar aukist í íslenskum landbúnaði, ekki síst vegna veðurfarsbreytinga og nýrra byggyrkja. Í kjölfar þessarar aukningar er viðbúið að skaðvaldar valdi meira tjóni í ræktun. Því er brýnt að afla þekkingar á hvaða skaðvaldar geta haft áhrif á bygg við íslenskar aðstæður. Rannsóknin sem kynnt er hér er sú fyrst þar sem tegundafjölbreytni sveppasýkla á byggi er greind á Íslandi. Sýnum var safnað umhverfis landið og þau greind með sameindaerfðafræðilegum aðferðum. Að minnsta kosti tólf tegundir sveppa voru greindar, þar með talið *Rhyncosporium secalis*, þekktur sjúkdómsvaldur í íslenskum byggökrum, en auk þess fundust sveppir sem ekki hafa fundist áður á byggi á Íslandi, svo sem *Microdochium nivale*, *Fusarium avenaceum* og *Epicoccum nigrum*. Þrjár tegundir, *Pyrenophora teres* f. *teres*, *Didymella exitialis* og *Itersonilia perplexans* eru nýjar í fungu Íslands. Niðurstöðurnar sýna að tegundafjölbreytni sveppa sem sýkja bygg við íslenskar aðstæður er meiri en áður var talið og eru því mikilvægt innlegg til framtíðar ræktunarverkefna.

INTRODUCTION

The early Icelandic settlers, believed to have arrived in Iceland around or somewhat before 874 AD, most likely brought with them Scandinavian barley varieties and started barley cultivation in Iceland. Barley is known to have been grown at several locations until the 14th century, when its cultivation ceased. Different hypotheses have been put forth to explain this, one suggesting that a drop in the price of imported barley from Scandinavia caused Icelandic farmers to shift from home grown to a cheaper imported barley (Hermannsson 1993). Another hypothesis suggests that the management of soils, rather than climatic or economic factors, caused the end of barley cultivation (Simpson et al. 2002). Whatever the reason for the end of barley cultivation, it was not until 1923 that barley was successfully grown in Iceland again for several years in a row. In 2010 barley was cultivated on approximately 4200 hectares of land with a total production of over sixteen thousand tons. Production has increased more than 30-fold since 1991 and if conditions in Iceland continue to favour barley cultivation it has been estimated that the annual production could double in the next ten years (Hermannsson & Björnsson 2008). As conditions for barley cultivation in Iceland continue to improve and the annual production increases, the infectious load can be expected to increase and additional pathogens can emerge (Hakala et al. 2011). Under such a scenario active monitoring of the diversity of fungal species in Iceland will be important in order to respond to changes in a timely manner and to strengthen breeding projects aimed at increasing pathogen resistance.

Categorization of plant diseases is usually either based on the cause of infection (bacteria, fungi, viruses or nematodes) or on which part of the plant is infected (root, stem, leaf, or spike). The study presented here focuses on fungal leaf diseases in Icelandic barley fields. So far, several pathogenic fungal species have been identified in Iceland including: *Claviceps purpurea* (causing ergot), *Pyrenophora graminea* (causing barley stripe), *Erysiphe graminis* (causing powdery mildew), *Pseudoseptoria stomaticola* (causing halo spot), *Rhynchosporium secalis* (causing barley scald), *Ustilago hordei* (causing covered smut), and *Ustilago tritici* (causing loose smut) (Hallgrimsson & Eyjolfsdottir 2004). In Iceland only *R. secalis* has been identified as a pathogen of serious economic importance, with reports of 10-20% average yield loss and a maximum yield loss of up to 36% (Hermannsson 2004). In Scandinavia, the source of seed imported into Iceland, many fungal pathogens cause serious barley diseases. Barley fungal pathogens in Scandinavia include species reported in Iceland, i.e. *E. graminis*, *R. secalis*, *U. hordei*, and *P. graminea*, as well as species that have not been reported in Iceland such as *Pyrenophora teres*, *Ustilago nuda*, *Puccinia hordei*, *Gaeumannomyces graminis*, and *Ramularia collo-cygni* (Emmerman et al. 1988, Hofsvang & Heggen 2005, Serenius et al. 2005, Jalli et al. 2011).

Several methods are used for the control of barley disease. Crop rotation is a natural approach to restrain disease proliferation, especially when pathogens overwinter on barley stubble and infect sprouting plants the next spring. Application of fungicides for disease control is a strategy widely used in many barley growing areas as well as the use of seed dressing. Despite field trials in Iceland showing yield increase of up to 24% when fungicides were used to control *R. secalis* this method has not been used to the same extent in Iceland as in neighbouring countries (Hermannsson & Sverrisson 2003). Although fungicides have been of great benefit in agriculture, there are disadvantages that need to be considered. The main concern in recent years has been an increased occurrence and spread of fungicide-resistant strains (Menzies 2008, Chen & Zhou 2009). Fungicide use increases the selective pressure on pathogens which can lead to increased frequency of resistant mutants, especially in pathogen populations with high evolutionary potential (McDonald & Linde 2002). This is supported by reports of increased frequency of highly resistant pathogen strains

under the application of a particular type of fungicide (Taggart et al. 1999).

The use of resistant cultivars is also an important method for controlling barley disease. There are two types of resistance, major gene or gene-for-gene resistance and minor gene or quantitative resistance. In major gene resistance pathogens produce elicitor molecules that are recognized by specific resistance-gene encoded receptors in the plant. The plant receptors trigger several defence reactions which can lead to the death of the infected plant cells and inhibition of the pathogen. In this case, where the resistance relies on a single gene, the appearance of a mutant virulent strain leads to a rapid breakdown in resistance. Quantitative resistance describes the effects of genes that are considered to have limited individual importance and rarely provide total resistance but lead to a quantitative resistance response that tends to be effective against all strains of a pathogen population (McDonald & Linde 2002). Population studies can reveal the evolutionary potential of pathogen populations which gives an important insight into which pathogens pose the greatest risk of breaking down host resistance and can help in choosing the right strategy for durable disease resistance breeding. The knowledge of pathogen species diversity and population genetic structure can be used to make predictions regarding the relative risk posed by different pathogens (McDonald & Linde 2002).

The objective of this study was to increase the knowledge of barley pathogens in Iceland in order to strengthen the basis of future breeding projects for durable disease resistance in barley cultivars adapted to local conditions. This was done by collecting field samples from 27 locations around the country and to subsequently identify barley fungal pathogens using molecular methods, such as species-specific PCR and ribosomal gene sequencing.

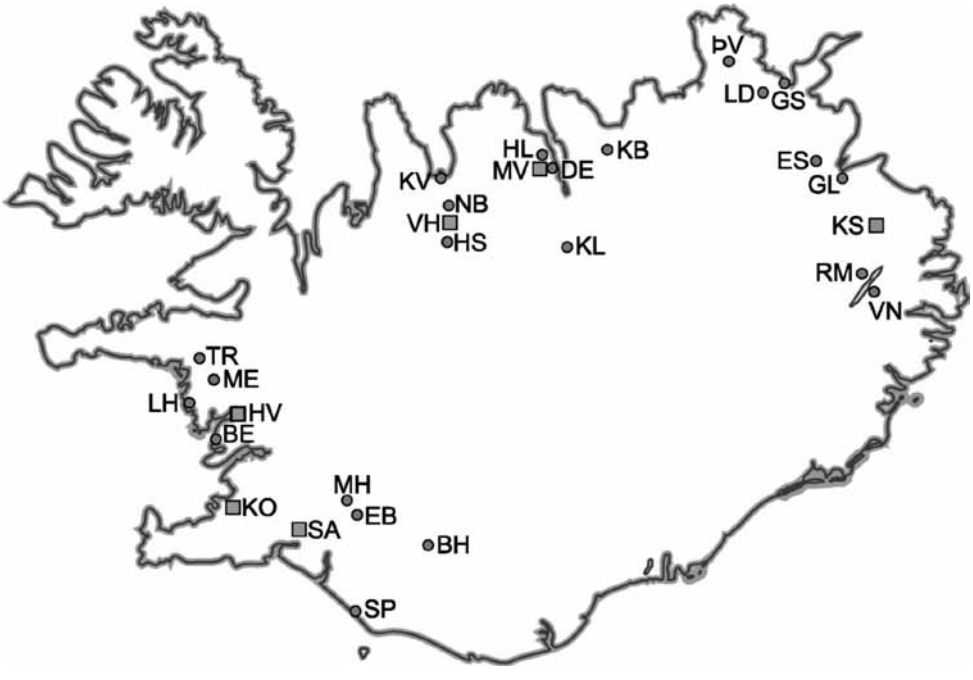

Figure 1. Sampling locations. Main sampling locations are shown as squares and minor sampling locations as circles. See Table 1 for full names of sampling locations.

MATERIALS AND METHODS

Sample collection

Leaves with signs of infection were collected in naturally infected barley fields at 27 locations in Iceland in August 2007. Sixty leaves were collected at the six main sampling sites (squares in Figure 1) using a hierarchical sampling strategy (McDonald et al. 1999). The remaining leaves were collected randomly from 21 different locations, one leaf at each sampling site (circles in Figure 1). Each plant was sampled only once. Each leaf from the sample collection was placed in an 8 × 16 cm paper bag, dried at room temperature for 2-3 days and kept at -23°C until fungal isolation.

Geographical locations and names of sampling sites as well as information on barley varieties are given in Table 1.

Fungal culture and DNA isolation

For fungal culture leaf segments of 5-10 mm were cut from the leaves and surfaced sterilized in 70% ethanol for 5-10 sec followed by 60-90 sec in a 0.5-2% NaOCl solution, then rinsed for 10 sec in sterile water, and finally dried between layers of autoclaved filter paper. After sterilization single leaf pieces were put on Wheat Germ Agar (WGA) plates (recipe after Xue (1990)) and incubated at 16°C in the dark for 10-14 days (Salamati & Tronsmo 1997). A total of 372 barley leaf segments were processed and incubated. Fungal myce-

Table 1. Names of sampling locations with abbreviations, geographic co-ordinates, and information on barley varieties where available.

Sampling location	Coordinates	Barley variety
Belgsholt (BE)	64°25'N, 21°59'W	unknown
Birtingaholt (BH)	64°04'N, 20°25'W	unknown
Dagverdareyri (DE)	65°45'N, 18°10'W	unknown
Efri-Brúnavellir (EB)	64°02'N, 20°31'W	unknown
Einarsstadir (ES)	65°37'N, 15°03'W	unknown
Graenilaekur (GL)	65°39'N, 14°56'W	unknown
Gunnarsstadir (GS)	66°09'N, 15°25'W	unknown
Hladir (HL)	65°46'N, 18°11'W	unknown
Hofsstadir (HS)	65°41'N, 19°22'W	unknown
Hvanneyri (HV)	64°33'N, 21°45'W	Olsok and Voitto
Keflavík (KV)	65°44'N, 19°29'W	unknown
Klauf (KL)	65°33'N, 18°04'W	unknown
Kleppjárnsstadir (KS)	65°28'N, 14°25'W	unknown
Korpa (KO)	64°09'N, 21°44'W	Kría and Olsok
Kvíaból (KB)	65°49'N, 17°33'W	unknown
Laxárdalur (LD)	66°09'N, 15°30'W	unknown
Laxárholt (LH)	64°35'N, 22°18'W	unknown
Melur (ME)	64°42'N, 22°04'W	unknown
Miklaholt (MH)	64°10'N, 20°32'W	unknown
Mödruvellir (MO)	65°46'N, 18°14'W	Lavrans, Skúmur, Teista, and Tiril
Nautabú (NB)	65°44'N, 19°12'W	unknown
Refsmýri (RM)	65°13'N, 14°33'W	unknown
Selpartur (SP)	63°48'N, 20°44'W	unknown
Stóra-Ármót (SA)	63°59'N, 20°56'W	Filipa
Thverá (ÞV)	66°08'N, 16°25'W	unknown
Tröd (TR)	64°49'N, 22°15'W	unknown
Vallanes (VN)	65°11'N, 14°32'W	unknown
Vindheimar (VH)	65°30'N, 19°21'W	Erkki, Kríló, Voitto, Olsok, Ven, Skúmur II, and Judit

Table 2. PCR primer pairs used for analysis of fungal species diversity.

Name	Primer sequence (5'-3')	Species	References
ITS1	TCCGTAGGTGAACCTGCGG	All species	White et al. 1990
ITS4	TCCTCCGCTTATTGATATGC	All species	White et al. 1990
RS8	TTGTTTTTAGTGATGTCTGAG	*R. secalis*	Lee et al. 2001
RS9	AGGCACCGCCACTGATTTTAGGG	*R. secalis*	Lee et al. 2001
PTTF	CTTGATGCGCTGGAGTGAGA	*P. teres* f. *teres*	Leisova et al. 2006
PTTR	TGCATTTCCACCTACTGGTATGTAC	*P. teres* f. *teres*	Leisova et al. 2006
PG2F	CTTCTTAGCTGGGGCTACCGTC	*P. graminea*	Bates et al. 2001
PG2R	ACCGACTCGGGAAAAGAGCA	*P. graminea*	Bates et al. 2001
CladF	CCKGGATGTTCATAACCCTTTG	*Cladosporium* spp.	Dean et al. 2005
CladR	CCCGAACACCCTTTAGCG	*Cladosporium* spp.	Dean et al. 2005

lium and spores were scraped off the plates and total DNA was extracted using a Microbial DNA Isolation Kit (MoBio, cat. no. 12224).

Primers and PCR conditions

Following extraction of total DNA, PCR amplification was used for identification of fungal species using species specific primers. Species-specific primers for *R. secalis*, *P. teres* f. *teres*, *P. gramineae*, and *Cladosporium* spp. (Table 2) were used to screen all samples and the presence of a specific PCR fragment was taken as a positive identification. PCR amplification and sequencing of rRNA ITS (Internal Transcribed Spacer) regions were used to assign a total of 78 samples to fungal species.

The PCR amplifications were performed in a 20 µL volume containing 2 µL of 10×PCR Buffer (New England Biolabs), 3 mM final concentration of $MgCl_2$, 0.8 mM of dNTP, 0.3 µM of each primer, 1 U of Taq polymerase (New England Biolabs), and 1 µL of undiluted DNA. The PCR amplifications were performed under different conditions (Table 3) for different primer pairs in a Thermo Px2 Thermal Cycler.

Species identification

A 10 µL sample from each PCR amplification was analysed by gel electrophoresis on 1.5% agarose gel run in 1×TAE buffer and stained with ethidium bromide. DNA fragments were visualized using GE ImageQuant 300 for the

Table 3. PCR amplification conditions used for analysis of fungal species diversity.

Species	Denaturation	Amplification cycle	Final extension	Cycles
ITS regions	6 min at 95°C	30 sec at 95°C 45 sec at 55°C 60 sec at 72°C	10 min at 72°C	33
R. secalis	6 min at 95°C	30 sec at 95°C 30 sec at 55°C 30 sec at 72°C	10 min at 72°C	30
P. teres f. *teres*	6 min at 96°C	30 sec at 96°C 45 sec at 58°C 60 sec at 72°C	10 min at 72°C	34
P. gramineae	6 min at 96°C	30 sec at 96°C 45 sec at 58°C 60 sec at 72°C	10 min at 72°C	34
Cladosporium spp.	6 min at 96°C	30 sec at 96°C 30 sec at 54°C 60 sec at 72°C	10 min at 72°C	34

presence of species-specific and ribosomal fragments. When needed, ITS fragments were excised from the agarose gel and DNA extracted using a Nucleospin Extract II kit (Macherey-Nagel). Sequencing was done by Eurofins MWG Operon and sequencing results compared to data from the NCBI database using BLAST (Basic Local Alignment Search Tool) (www.ncbi.nlm.nih.gov/blast).

RESULTS

Separate results for the six major sample locations and combined results for all locations are shown in Table 4. Out of the 372 leaf samples initially processed, fungal identification was possible on 277 leaves; 72 samples were a mix of two fungal species or a fungal species and bacteria, giving a total of 349 positive identifications. *Cladosporium* spp. were identified on 59% of the 277 leaves (164 positives). *R. secalis* and *P. teres* f. *teres* were found on 19% (53 positives) and 16% (46 positives) of the identified leaves, respectively. Other fungal species included *P. graminea* (18 positives), several individuals of *Stagonospora* species (12 positives not assigned to species), *Botryotinia fuckeliana* (3 identifications), *Epicoccum nigrum* (2 identifications), and single identifications of *Microdochium nivale*, *Fusarium avenaceum*, *Didymella exitialis*, and *Itersonilia perplexans*. The remainder, a total of 34 positives, were classified as unidentified endophytes based on ITS sequencing results. The frequency of different fungal species was different between different parts of the country.

DISCUSSION

Traditional identification methods of fungal species rely on years of training and experience, as well as the ability to identify disease symptoms and subtle morphological differences between closely related pathogens. Although these methods are fundamental in fungal diagnostics they can lead to problems in identification, resulting in inaccurate diagnosis, even when carried out by experienced personnel (Atkins & Clark 2004). Several factors can affect the correct identification of fungal pathogens of barley. For example, visual scoring of *R. secalis* symptoms does not detect early-season symptomless infections (Fountaine et al. 2007) and different barley cultivars react differently to infections depending on stages of growth (Oxley et al. 2003). Disease symptoms of several fungal leaf pathogens are similar and lesions caused by, for example, *P. teres* f. *teres* can be mistakenly identified as *P. teres* f. *maculata* and vice versa (Williams et al.

Table 4. Results of species identification. Separate results for the six main locations and combined results for all other locations (Other) are shown, as well as combined results for all locations. SA (Stóra-Ármót), KO (Korpa), HV (Hvanneyri), VH (Vindheimar), MO (Möðruvellir), and KS (Kleppjárnsstadir).

	SA	**KO**	**HV**	**VH**	**MO**	**KS**	**Other**	**TOTAL**
Cladosporium ssp.	23	4	27	30	54	24	2	**164**
Rhyncosporium secalis	12	20		10	6		5	**53**
Pyrenophora teres	3	1	4	11	4	19	4	**45**
Pyrenophora gramineae			4	4	9	1		**18**
Stagonospora ssp.	1	4	1			4	2	**12**
Botryotinia fuckeliana			2			1		**3**
Didymella exitialis	1							**1**
Epicoccum nigrum			1					**1**
Fusarium avenaceum	1							**1**
Itersonilia perplexans							1	**1**
Microdochium nivale		1						**1**
Unidentified endophyte	1					31	2	**34**
Bacteria	1	4			5	4	1	**15**
TOTAL	**42**	**34**	**44**	**55**	**73**	**84**	**17**	**349**

2001b), even though the species have different virulence patterns and trigger a different defence response from the host (Scott 1992, Ho et al. 1996). Because the resistance to the two forms of *P. teres* is inherited independently it is important that the pathogen is correctly identified, especially if the identification is to form the basis of a resistance breeding project (Williams et al. 2001a, Leisova et al. 2005). In agriculture, incorrect species identification will lead to an incorrect selection of disease control strategies. In recent years several new methods have emerged for diagnosing fungal pathogens, as well as other species, the most prolific field being PCR technology coupled with DNA sequencing giving rise to a promising new approach to species identification (McCartney et al. 2003).

At least twelve fungal species were identified including three major barley pathogens that were represented by more than ten samples: *R. secalis*, *P. teres* f. *teres*, and *P. graminea*. *R. secalis* and *P. graminea* have been reported on barley in Iceland (Hallgrimsson & Eyjolfsdottir 2004) although this study is the first to positively identify *P. graminea* as a barley pathogen. There are no previous reports of *P. teres* f. *teres* in Iceland, but *P. teres* infected leaves were found in five out of six major sampling locations, indicating that it has spread to most barley growing areas. Six of the fungal species identified in the study were only found in three or fewer samples. *M. nivale*, *F. avenaceum*, and *E. nigrum* are reported causal agents of barley diseases (Mathre 1997, Ioos et al. 2004) and have been found previously in Iceland on soil or plant residue but not on barley (Hallgrimsson & Eyjolfsdottir 2004). *D. exitialis* (*Ascochyta* spp.), the causal agent of Ascochyta leaf scorch on barley (Punithalingam 1979), is new to the Icelandic funga, as well as *I. perplexans*, which has not been previously described as a pathogen or endophyte of barley. *B. fuckeliana* has been reported on several plant species in Iceland (Hallgrimsson & Eyjolfsdottir 2004) and is a well known pathogen of several plant species and causes, for example, a serious disease in grapevine (Elad 1994). Unidentified fungi were found in 34 samples, including 31 from the same location, and ITS sequencing showed a close relation to *Aureobasidium pullulans*, a cosmopolitan saprophyte (Castoria et al. 2001). There are no previous reports of *A. pullulans* as a plant endophyte in Iceland.

The study also revealed species of two fungal genera, *Stagonospora* and *Cladosporium*. Several *Stagonospora* species are known to cause disease on barley and other cereals. These include *Stagonospora avenae*, the causal agent of speckled leaf blotch, and *Stagonospora nodorum*, the causal agent of leaf and glume blotch (Mathre 1997). *S. nodorum* has been found in Iceland (Hallgrimsson & Eyjolfsdottir 2004) but *S. avenae* has not been previously recorded. Sequencing results were insufficient for conclusive species identification within the *Stagonospora* genus. A surprisingly high number of *Cladosporium* samples were found here. *Cladosporium* species are found on a number of different plants all over the world but are not known to infect barley, suggesting other explanations for the leaf lesions. Lesions on barley leaves can be caused by means other than living pathogens. Leaf spots not caused by fungi, bacteria or viruses are sometimes referred to as physiological leaf spots (PLS) and include non-parasitic leaf spots, cultivar specific spots, and spots caused by nutrient deficiency or by the mlo resistance gene (Behn et al. 2004). It is possible that the high levels of *Cladosporium* species detected in this study could be explained by *Cladosporium* species outgrowing the lesion-causing fungal pathogens during the fungal isolation process.

Several factors must be taken into consideration when the species identification results are interpreted. The samples analysed here were collected in the third week of August. Up to that time the growing season had been quite unique because of relatively high temperatures and limited precipitation in all parts of the country (Jonsson 2008). Optimum conditions for both *R. secalis* and *P. teres* include high humidity levels and mild temperature (Steffen-

son & Webster 1992, Zhang et al. 1992). Conditions in Iceland during the 2007 growing season might therefore have favoured the proliferation of fungi other than R. secalis and *P. teres* f. *teres*. The homogenous growing conditions during the fungal isolations process, with respect to growth media, temperature, lighting, leaf sterilization, and inoculation period, also need to be considered. These conditions were adapted from an isolation protocol for *R. secalis* (Salamati & Tronsmo 1997) and do not necessarily represent optimum growing conditions for other fungi found on barley in Iceland, possibly affecting the frequency of the different fungi reported here. Also, the PCR method and the primers selected could affect the results by skewing the frequency of identification.

CONCLUSIONS

This is the first study on species diversity of fungal barley pathogens carried out in Iceland and the results show a considerably more complex situation than previously reported. Although the sample collection was limited to a single growing season the results offer an important insight into the funga of Icelandic barley fields. Future sampling at fixed locations over longer periods of time is required to estimate accurately the prevalence of pathogens and possible differences in geographical distribution of particular pathogens. The results revealed several fungal species, including a number of pathogenic fungi previously not recorded on barley in Iceland. At least twelve species of fungi were identified on 277 successfully analysed leaves. These included two important and widespread barley pathogens, *R. secalis* and *P. teres* f. *teres*. This study is the first report of *P. teres* in Iceland and out of the twelve species identified only *R. secalis* and *P. gramineae* have been reported on barley in Iceland before. The next rational step in the analysis of fungal pathogens in Iceland would be an analysis of the genetic diversity of the most frequent pathogens, as genetic diversity has been shown to affect the ability of pathogens to break genetic resistance of barley and would be, as such, a threat to barley in Iceland.

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