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ORIGINAL ARTICLE

Virulence of *Rhynchosporium commune* isolates collected in Iceland

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Abstract

Various fungal species continue to be one of the most difficult challenges faced by farmers, and hence societies in whole, when it comes to securing plentiful and wholesome food for a rapidly growing human population. Understanding the biology of pathogenic fungi in detail, both at the population and molecular levels, combined with continued emphasis on resistance breeding of important crops, offers the most obvious sustainable solution to this pressing problem. Here we present results of virulence testing and microsatellite analysis on a collection of Icelandic *Rynchosporium commune* isolates to test whether the previously demonstrated genetic diversity observed translated into functional diversity in the virulence of these isolates. Our results show considerable diversity in the virulence of the Icelandic *R. commune* samples with each isolate having a unique virulence spectrum on the 15 near-isogenic barley lines used for screening. Our findings have practical implications, showing that even with short continuous barley cultivation and isolation by geographical distance, breeding for Icelandic, and likely other remote or isolated locations, still needs to consider the importance of disease resistance in breeding decisions and variation in local pathotypes. Moreover, our analysis is the first step to focused breeding for disease resistance for Icelandic conditions, an important step in the ongoing Icelandic barley breeding project.

Keywords *Rhychosporium commune* · Scald of barley · Pathogen variation · Near-isogenic lines (NILs)

Introduction

Cultivated barley (*Hordeum vulgare* ssp. *vulgare*) is a diploid annual cereal grain of the family Poaceae and the domesticated form of the wild barley *H. vulgare* ssp. *spontaneum* (Stein and Muehlbauer 2018). Barley has been adapted to a great range of climates and daylengths and is cultivated from arid areas of North Africa (Bothmer et al. 1991) to subarctic areas near the arctic circle (Hilmarsson et al. 2017). Worldwide in 2017 it ranked fourth of cereal crops for area of cultivation and quantity produced, with 47 million hectares yielding 147 million metric tons, compared to 772 million metric tons of wheat (FAOSTAT 2020).

When Iceland was settled in the ninth century CE the settlers likely brought with them barley from Scandinavia and/or the British Isles (Karlsson 2009). Barley cultivation was then practiced in Iceland for several centuries, but eventually decreased drastically or ceased completely. This most likely happened around the turn of the fourteenth century, possibly due to environmental changes or changes in the price of imported barley (Karlsson 2009) although other sources suggest that changes in population density led to the cessation of barley cultivation in Iceland (Júlíusson 2018). It was not until 1923 that barley was successfully grown in Iceland again for several years in a row. Since 1991 barley production in Iceland has increased considerably, with around 5,000 hectares of land yielding up to 16,000 tons per year, an average yield of 3.2 t/ha (Hilmarsson et al. 2017).

Although several pathogenic fungi have been previously described on barley in Iceland (Hallgrímsson and Eyjolfsdottir 2004; Stefansson and Hallsson 2011), only *Rhynchosporium commune* (syn. *R. graminicola,* previously *R. secalis*), the

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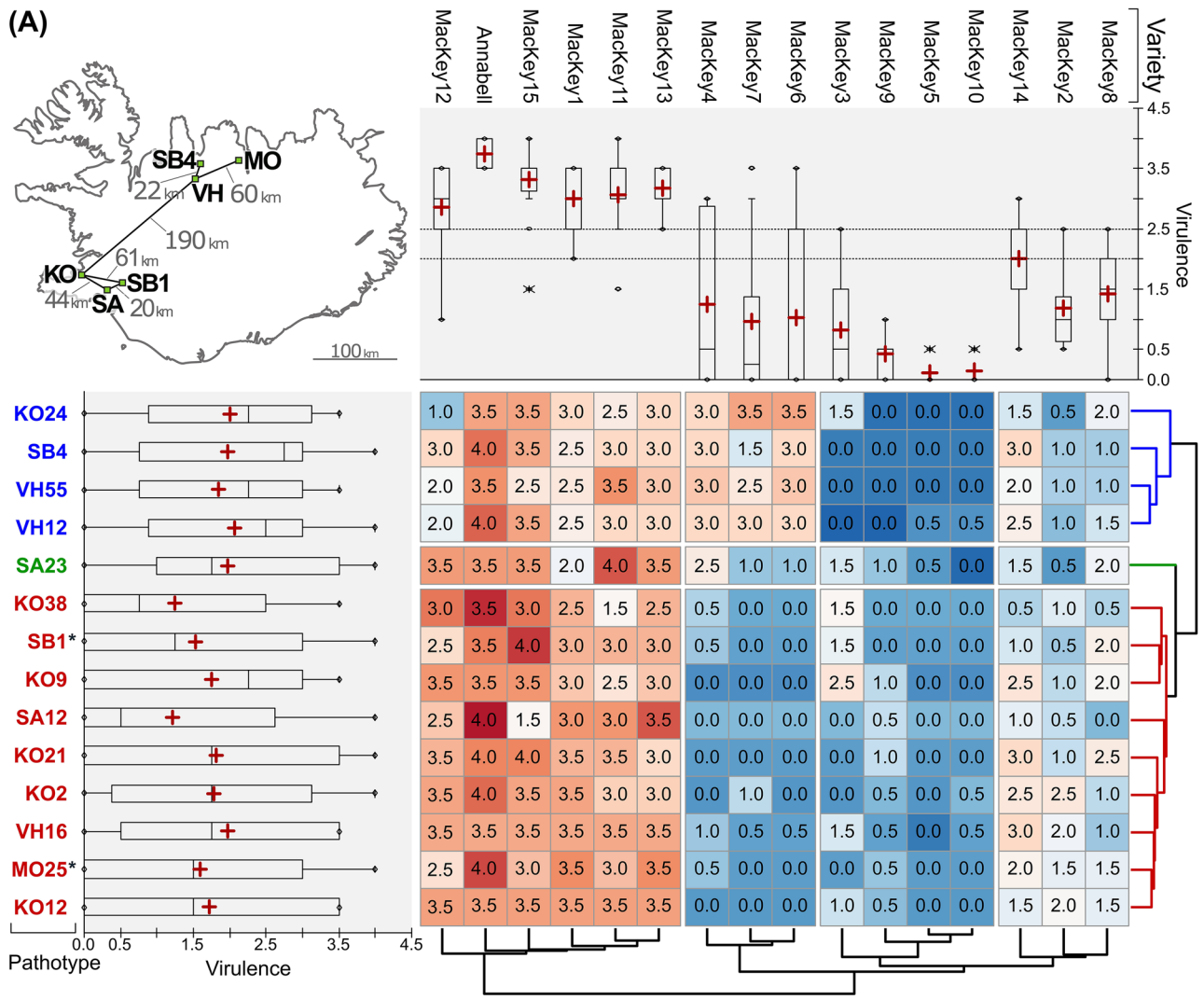
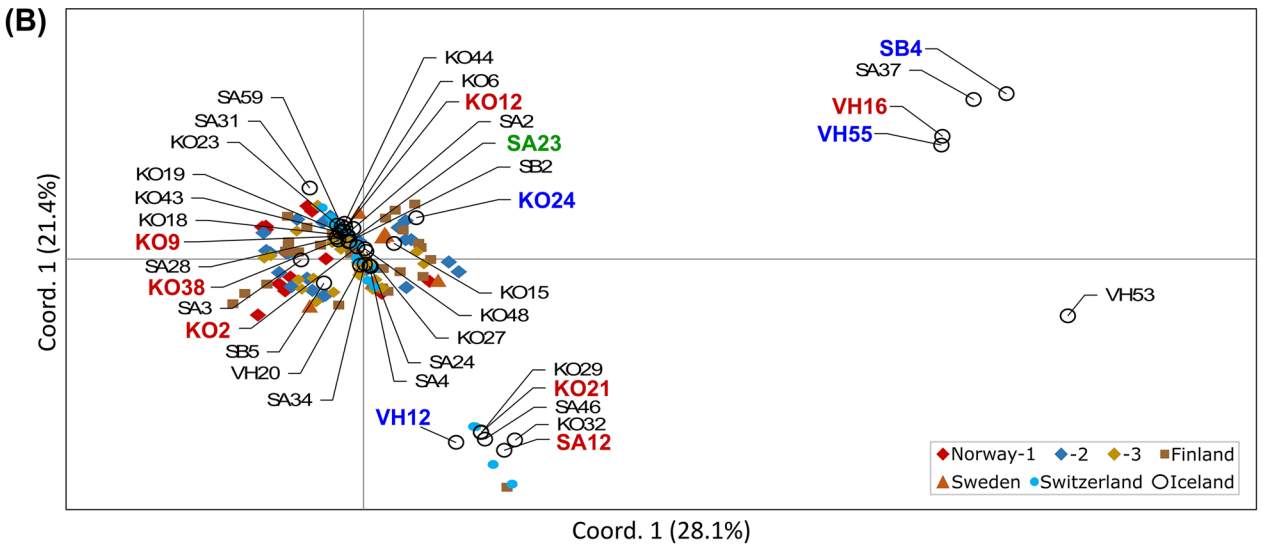

(A)
SB4
MO
22 km
VH 60 km
190 km
61 km
KO
SB1
44 km
SA 20 km
100 km
MacKey12
Annabell
MacKey15
MacKey1
MacKey11
MacKey13
MacKey4
MacKey7
MacKey6
MacKey3
MacKey9
MacKey5
MacKey10
MacKey14
MacKey2
MacKey8
Variety
Virulence
KO24
SB4
VH55
VH12
SA23
KO38
SB1*
KO9
SA12
KO21
KO2
VH16
MO25*
KO12
Pathotype
Virulence


(B)
KO44
KO6
KO12
SA2
SA23
SB2
KO24
KO15
KO48
KO27
SA24
SA4
KO29
KO21
SA46
KO32
SA12
VH12
SA34
VH20
SB5
KO2
SA3
KO38
SA28
KO9
KO18
KO43
KO19
KO23
SA31
SA59
SB4
SA37
VH16
VH55
VH53
Norway-1
-2
-3
Finland
Sweden
Switzerland
Iceland
Coord. 1 (21.4%)
Coord. 1 (28.1%)

◂**Fig. 1** Hierarchical clustering and genetic distance between Icelandic *R. commune isolates*. (**A**) A hierarchical cluster dendrogram for 14 Icelandic *R. commune* isolates based on phenotypic data of 15 barley differential lines after seedling inoculation, the map shows sampling locations for the *R. commune* isolates and the distance between locations, (**B**) PCoA of genetic distances between individual *R. commune* samples, with fungal isolates used for virulence testing color coded as in (**A**)

causal agent of barley scald (Zaffarano et al. 2011), has been suggested as a serious pathogen of economic importance, with reports of up to 36% yield loss and 10–20% average yield loss (Hermannsson 2004).

R. commune, a haploid fungus found in all major barley-growing regions of the world, is a serious disease in cool, semi-humid areas especially where leaves remain wet for long periods (Zhang et al. 1992). Scald symptoms appear as irregular shaped lesions, with the green color of the leave turning chlorotic with brown edges, as the infection develops the lesions can eventually merge. Yield losses are reported to range between 10 and 45% (Avrova and Knogge 2012). Studies on *R. commune* populations, from different countries, have revealed both genetic and pathogenic variation (Jorgensen and Smedegaard-Petersen 1995; McDermott et al. 1989; McDonald 2015; McDonald et al. 1999; Mohd-Assaad et al. 2018; Salamati et al. 2007; Stefansson et al. 2012, 2013, 2014; Tekauz 1991; Williams et al. 2003; Kequan Xi et al. 2002). Methods used have included virulence tests (Araz and Maden 2006; Kequan Xi et al. 2002), ribosomal DNA (Newton et al. 2001), isozymes (Goodwin et al. 1993), and DNA marker analyses (Bouajila et al. 2007; Kiros-Meles et al. 2005; McDonald et al. 1999). Isolates secured from different scald lesions taken from the same plant or even different spores isolated from the same lesion may vary significantly for virulence and molecular markers (Brown 1985; McDonald et al. 1999). This pathogenic diversity means that *R. commune* populations can potentially change rapidly in response to selection exerted by the introduction of new resistance genes or fungicides (Zhan et al. 2008) and rapidly render them ineffective (Newton et al. 2001; Oxley et al. 2003; Kegnan Xi et al. 2003).

The source of the genetic diversity in *R. commune* has been the subject of several studies leading to suggestions including asexual genetic exchange (Forgan et al. 2007; Newman and Owen 1985), frequency-dependent selection (Goodwin et al. 1993; McDermott et al. 1989), spontaneous mutations and migration (Goodwin et al. 1994; Williams et al. 2003), and sexual reproduction (Linde et al. 2003; McDonald et al. 1999; Salamati et al. 2007). Additional factors influencing genetic diversity in *R. commune* populations could be a large effective population size (McDermott et al. 1989), gene flow within regions by air-borne ascospores (Zhan et al. 2008), and gene flow between regions by seed trade (Zaffarano et al. 2007). Although a teleomorph has not yet been recognized research has shown gametic equilibrium of alleles within populations (Burdon et al. 1994; Salamati et al. 2007), but the degree of equilibrium can be used as an indirect measure of the significance of genetic exchange and recombination in presumably asexual populations (Chen and McDonald 1996; Linde et al. 2003). Linde et al. (2003) studied the frequency of mating type alleles in *R. commune* populations from six countries and found equal frequencies in most of the populations, suggesting frequency dependent selection consistent with sexual reproduction. Both mating types were frequently found occupying the same lesions or leaf, providing opportunity for isolates of opposite mating types to reproduce sexually (Linde et al. 2003).

Stefansson et al. (2012) have previously shown that Icelandic *R. commune* isolates are genetically polymorphic and distinct from populations in Scandinavia, but no virulence tests have been carried out with these Icelandic isolates. The aim of this study was to characterize virulence of Icelandic isolates of *R. commune*, and compare those results to population structure using microsatellite markers. The virulence tests were carried out on a set of near isogenic barley lines with resistance genes for *R. commune,* with the aim of informing further resistance breeding efforts in Iceland and beyond.

Materials and methods

Fungal isolates

Infected leaves with visual scald symptoms were collected from six locations in Iceland (Fig. 1 and Table 1) as previously described (Stefansson et al. 2012; Stefansson and Hallsson 2011). Each leaf was placed in a paper bag and dried at room temperature for 2–3 days. Leaves were then sterilized in 70% ethanol for 10 s and in 0.5% NaOCl for 90 s, rinsed twice with distilled water and dried on filter paper. Sterilized leaf segments were placed on wheat germ agar (WGA) plates covered with aluminum foil and kept at room temperature in the dark for seven days. Mycelium from the lesions was transferred onto new WGA plates and kept in the dark at room temperature for another 14 days. Pieces of WGA with *R. commune* culture were transferred to Eppendorf tubes containing 0.75 mL distilled water, the tubes shaken, and contents poured onto WGA plates. When mycelium growth became visible through a microscope, single spore cultures were picked using a wire inoculation loop, dissolved in water, and spread onto new WGA plates and stored for 14 days in the dark at 18 °C. This yielded 39 single spore *R. commune* isolates (Table 1) that were then used for both microsatellite analysis and greenhouse trials.

For inoculum preparation, conidia were scraped from the agar with a microscope glass and suspended in 10 mL of

distilled water. Conidia were counted using a hemocytometer and the concentration adjusted to 1×10^6 conidia per mL.

For fungal DNA extraction, single spore isolates were transferred on to WGA plates and incubated at 16 °C in the dark for 10 to 14 days. Fungal mycelium and spores were scraped off the plates and total DNA extracted using Microbial DNA isolation Kit (MoBio, cat. No. 12224). Fungal species were identified as described by Stefansson and Hallsson (2011).

Table 1 Icelandic *R. commune* samples used for microsatellite analysis and/or in virulence tests

Isolate ID		Sampling location	Analysis[a]	Cultivar
1	KO2	Korpa (64°09'N, 21°44'W)	M + V	Kría
2	KO6	Korpa (64°09'N, 21°44'W)	M	Kría
3	KO9	Korpa (64°09'N, 21°44'W)	M + V	Kría
4	KO12	Korpa (64°09'N, 21°44'W)	M + V	Kría
5	KO15	Korpa (64°09'N, 21°44'W)	M	Olsok
6	KO18	Korpa (64°09'N, 21°44'W)	M	Olsok
7	KO19	Korpa (64°09'N, 21°44'W)	M	Olsok
8	KO21	Korpa (64°09'N, 21°44'W)	M + V	Olsok
9	KO23	Korpa (64°09'N, 21°44'W)	M	Olsok
10	KO24	Korpa (64°09'N, 21°44'W)	M + V	Olsok
11	KO27	Korpa (64°09'N, 21°44'W)	M	Olsok
12	KO29	Korpa (64°09'N, 21°44'W)	M	Olsok
13	KO32	Korpa (64°09'N, 21°44'W)	M	Olsok
14	KO38	Korpa (64°09'N, 21°44'W)	M + V	Kría
15	KO43	Korpa (64°09'N, 21°44'W)	M	Kría
16	KO44	Korpa (64°09'N, 21°44'W)	M	Kría
17	KO48	Korpa (64°09'N, 21°44'W)	M	Kría
18	MO25	Möðruvellir (65°46'N, 18°14'W)	V	Swå02220
19	SA2	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
20	SA3	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
21	SA4	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
22	SA12	Stóra-Ármót (63°59'N, 20°56'W)	M + V	Filippa
23	SA23	Stóra-Ármót (63°59'N, 20°56'W)	M + V	Filippa
24	SA24	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
25	SA28	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
26	SA31	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
27	SA34	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
28	SA37	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
29	SA46	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
30	SA59	Stóra-Ármót (63°59'N, 20°56'W)	M	Filippa
31	VH12	Vindheimar (65°30'N, 19°21'W)	M + V	Swå01448
32	VH16	Vindheimar (65°30'N, 19°21'W)	M + V	Erkki
33	VH20	Vindheimar (65°30'N, 19°21'W)	M	Olsok
34	VH53	Vindheimar (65°30'N, 19°21'W)	M	Ven
35	VH55	Vindheimar (65°30'N, 19°21'W)	M + V	Kría
36	SB1	Efri-Brúnavellir (64°02'N, 20°31'W)	V	Kría
37	SB2	Einarsstaðir (65°37'N, 15°03'W)	M	Kría
38	SB4	Hofstaðir (65°41'N, 19°22'W)	M + V	Kría
39	SB5	Gunnarsstaðir (66°09'N, 15°25'W)	M	Kría

[a]*M* Microsatellite analysis (37 samples), *V* Virulence test (15 samples)

Plant material and greenhouse trials

Sixteen barley differential lines, consisting of 15 near-isogenic lines (NILs), each with a different resistance allele against *R. commune,* and the susceptible check 'Annabell', a German two rowed spring barley cultivar (Table 2), were used for virulence tests of 14 *R. commune* isolates (Goodwin et al. 1990; Patil et al. 2002). Two seeds per genotype were sown together with eight or nine genotypes per pot in two replications. Seeds were sown in nutrient supplemented peat and pots placed in the greenhouse at 18–20 °C during the day and 15 °C during the night with a 16 h/8 h photoperiod, with a relative humidity of 60–80%. Two weeks after sowing, at the 2–3 leaf stage, plants were spray inoculated with a homogenized conidial suspension using 0.4 mL per genotype and 3.2 mL per pot. After inoculation, the pots were kept in closed plastic bags for 48 h, with a relative humidity of 100%. Subsequently the bags were opened and water sprayed three times per week. Leaf symptoms were scored on the second leaf 21 days after inoculation using the 0–4 scale of Jackson and Webster (1976), with 0: no symptoms, 1–2: very small to small lesions within leaf margin, 3: large coalescing lesions, 4: collapse of leaf. Virulence patterns on a susceptible reaction (score ≥ 2.5) were used to group isolates as pathotypes with a method adapted from Tekauz (1991). Dendrograms showing the clustering of both barley varieties and fungal isolates were rendered in ClustVis (Metsalu and Vilo 2015) using Euclidean distance and average linkage.

Table 2 MacKey's near-isogenic lines (recurrent parent 'Ingrid') with resistance to *R. commune*. Lines provided by NordGen (www.nordgen.org)

NIL	Cultivar	Accession No.	Resistance type*
MacKey1	-	-	-
MacKey2	Hudson	CI 8067	*Rrs3* (*Rh1*)
MacKey3	Modoc	CI 7566	*Rh2, rh6*
MacKey4	-	CI 8162	*Rh3, rh6*
MacKey5	La Mesita	CI 7565	*Rh4, Rh10*
MacKey6	Turk	CI 14,400	*Rh5, rh6*
MacKey7	Brier	CI 7157	*Rh1, rh6*
MacKey8	Abyssinian	CI 668	*Rh9, Rhx*
MacKey9	Atlas 46	CI 7323	Rh2, Rh3
MacKey10	Hispont	CI 8828	*Rh* (unknown)
MacKey11	Steudelli	CI 2226	Rh6, Rh7
MacKey12	Quinn	CI 1024	*Rh* (unknown)
MacKey13	Abyssinian	CI 1233	*Rh* (unknown)
MacKey14	Nigrinudum	CI 2222	*Rh8*
MacKey15	-	-	-
Annabell	-	-	-

*Adapted from Goodwin et al. (1990) and Patil et al. (2002)

Rhynchosporium commune microsatellite analysis

Thirty-seven of the 39 *R. commune* isolates were analyzed with 14 polymorphic microsatellite markers (Linde et al. 2005) as previously described (Stefansson et al. 2012). Due to a low recovery rate for SSR marker *Rhyncho_14* in the Icelandic population it was omitted from further analysis. For comparison to the Icelandic isolates, isolates from Norway, Sweden, Finland, and Switzerland were used (for details see Stefansson et al. 2012 and references therein). Genetic distances between both individual isolates and groups were calculated using GenAlEx 6.5 (Peakall and Smouse 2005, 2012) using the 'Haploid-SSR' option under "Genetic Distance". A Principal Coordinates Analysis (PCoA) was then used to visualize the results using the 'Covariance-Standardized' option. Two samples used in the virulence testing (SB1 and MO25) failed in microsatellite typing and could therefore not be included in the genetic analysis.

Results

Virulence and population structure of Icelandic *R. commune* isolates

The susceptibility of the barley genotypes ranged from an average score of 0.1 for genotypes MacKey5 and MacKey10 to an average score of 3.7 for 'Annabell'. All genotypes were susceptible (max score ≥ 2.5; dotted line in Fig. 1A) to at least one isolate except genotypes 'MacKey5', 'MacKey9', and 'MacKey10'. All genotypes were resistant (min score ≤ 2) to at least one isolate except genotypes MacKey13 and 'Annabell' (Fig. 1A). Ten genotypes were considered resistant (average score ≤ 2) to the Icelandic *R. commune* isolates and six genotypes were considered susceptible.

The virulence spectrum of each of the *R. commune* isolates was unique, however, the average virulence only ranged from 1.2 for the isolate SA12 to 2.1 for the isolate VH12. Each isolate was both completely virulent and non-virulent on genotypes of the differential set. Different virulence patterns on a susceptible reaction (score ≥ 2.5) were used to group isolates as pathotypes resulting in eleven pathotypes, with isolates KO12, MO25 and SB1, and isolates KO24 and VH55 grouping together.

Hierarchical clustering separated *R. commune* isolates into three groups based on their virulence pattern (Fig. 1A). Group 1 (blue) comprised four isolates from three regions (*Hofstaðir*, *Vindheimar,* and *Korpa*). The second group (green) comprised only a single isolate from *Stóra-Ármót* (SA23). Group three (red) comprised the remaining nine isolates from three locations. Group three was the least virulent group of isolates with a mean virulence score of 1.6 across all NILs, although the other two groups had only slightly higher average virulence scores of 2.0 (Fig. 1A). Group one was more virulent on lines MacKey4, MacKey6 and MacKey7 (score 2.9) compared to groups two and three with mean scores of 1.5 and 0.2, respectively.

Analysis of genetic and geographical distances between the isolates tested did not reveal a separation between individuals in the three different virulence clusters (Fig. 1B).

Discussion

The fifteen NILs for differentiating *R. commune* isolates date back to 1977, when James MacKey started crossing 23 barley donor lines with partly known genes for scald resistance with the cultivar 'Ingrid' as a recurrent parent (Bjørnstad et al. 2002; Patil et al. 2002). Patil et al. (2002) screened nine of the 23 NILs along with their recurrent parent and donor lines with two fungal isolates ('4004' and '2'). Three NILs in this study where resistant (R) against both the isolates used by Patil et al. (2002) and all 14 Icelandic isolates: MacKey5, MacKey9 and MacKey10, which are NILs of the donor lines 'La Mesita', 'Atlas 46', and 'Hispont', respectively. It is interesting to note, that the resistance in these three lines, is based on different resistance genes, namely *Rh4* and *Rh10* in MacKey5, *Rh2* and *Rh3* in MacKey9 and an unknown resistance gene in MacKey10 (Table 2). MacKey2 was R against ten Icelandic isolates, moderately resistant (MR) against one, and moderately susceptible (MS) against three, and showed MR against '4004' and R against isolate '2'. NILs of 'Modoc' exhibited MR and R reactions against isolates '4004' and '2', respectively, with MacKey3, the corresponding line in the present study, R against eight isolates, MR against six isolates, and MS against one isolate. MacKey4 was R and MS to susceptible (S) against 9 and 5 Icelandic isolates, MR against '4004' and MR-MS against '2'. MacKey6 was R against isolate '4004' and ten Icelandic isolates and S towards isolate '2' and four Icelandic isolates. NILs of 'Brier' and corresponding line MacKey7 were resistant towards '4004' and ten Icelandic isolates, MR towards one Icelandic isolate and MS-S towards isolate '2' and three Icelandic isolates. NILs of 'Abyssinian' and respective line MacKey8 were R towards isolate '2' and six Icelandic isolates, MR towards '4004' and seven Icelandic isolates, and MS towards one Icelandic isolate.

Hierarchical clustering grouped the Icelandic *R. commune* isolates into three groups, with groups 2 and 3 closely connected. Isolates belonging to group 1 were collected at locations in the North (Hofstaðir and Vindheimar) and the West (Korpa). No clear connections between location and pathotype can be seen in such a small sample, with four isolates in group 3 being collected at locations in the Northern, Western, and Southern parts of Iceland. Among

the 14 *R. commune* isolates eleven pathotypes were identified. Isolates KO12 (group 3), MO25 (group 3) and SB1 (group 3) belonged to the same pathotype but were collected at different locations. Korpa (KO12) and Efri-Brúnavellir (SB1) in the western part of Iceland are about 80 km apart. Möðruvellir (MO25) is in the North and about 300 and 350 km from Efri-Brúnavellir and Korpa, respectively. Isolates KO24 and VH55 belonged to the same pathotypes and the same group (group 1). Korpa (KO24) and Vindheimar (VH55) are located about 200 km apart. Compared to the number of isolates, a very high number of pathotypes was detected. It shows that even when isolates were collected from the same regions or even within the same fields (see isolates from Korpa) they show a high variability in their pathogenicity. However, the same pathotypes can also occur at regions located far part from, concurrent with previous studies. For example, Tekauz (1991) tested 111 isolates from five locations in Canada on a set of 14 barley differentials and identified 45 different pathotypes. With 'CAN 1', the most common pathotype, found in all five regions. Xi et al. (2002) collected 256 isolates from nine locations in Alberta, Canada, and identified 52 pathotypes, many of which were comprised of only one isolate. RFLP analysis with 265 Australian isolates from five locations revealed 214 distinct genotypes (McDonald et al. 1999). The same study was able to show that *R. commune* populations are highly variable, and most of the genetic variation is found within fields, which supports the findings in the present study. Considering the low total number of isolates tested here this study should not be considered a final word in the analysis of pathogenicity and diversity of scald in Iceland, rather a first step to better understanding a complex population structure dictated by various genetic and environmental factors, such as sexual reproduction, climate change, fluctuating weather conditions, and flow of genetic variation from outside of Iceland. Even considering the short-comings of the study presented her the practical implications of our findings are that even with short continuous barley cultivation and isolation by geographical distance, breeding for remote locations, still needs to consider the importance of disease resistance and possible differences in pathotypes in localized breeding projects. Also, here we take the first steps to identify relevant resistance alleles that could be of use under Icelandic conditions, an important step in the ongoing Icelandic barley breeding project. Based on our analysis a good starting point would be to take a closer look at the resistance alleles found in the MacKey 3, MacKey 5, MacKey 9, and MacKey 10 NILs, as they show a broad resistance to all the Icelandic isolates tested here.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest The authors declare no conflict of interest.

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