

Scotland's Rural College

## Development and use of microsatellite markers to study diversity, reproduction and population genetic structure of the cereal pathogen *Ramularia collo-cygni*

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1 **Development and use of microsatellite markers to study diversity, reproduction**  
2 **and population genetic structure of the cereal pathogen *Ramularia collo-cygni*.**

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15 *Ramularia collo-cygni* (*Rcc*), Ramularia Leaf Spot (RLS), microsatellite (SSR), growth stage (GS), Quinone outside Inhibitors  
16 (Qols)

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25

26 **Abstract**

27 *Ramularia collo-cygni* (*Rcc*) is a major pathogen of barley that causes  
28 economically serious yield losses. Disease epidemics during the growing season are  
29 mainly propagated by asexual air-borne spores of *Rcc*, but it is thought that *Rcc*  
30 undergoes sexual reproduction during its life cycle and may also disperse by means  
31 of sexual ascospores. To obtain population genetic information from which to infer  
32 the extent of sexual reproduction and local genotype dispersal in *Rcc*, and by  
33 implication the pathogen's ability to adapt to fungicides and resistant cultivars, we  
34 developed ten polymorphic microsatellite markers, for which primers are presented.  
35 We used these markers to analyse the population genetic structure of this cereal  
36 pathogen in two geographically distant populations from the Czech Republic (n =30)  
37 and the United Kingdom (n =60) that had been sampled in a spatially explicit manner.  
38 Genetic diversity at the microsatellite loci was substantial,  $H_t = 0.392$  and  $H_t = 0.411$  in  
39 the Czech and UK populations respectively, and the populations were moderately  
40 differentiated at these loci ( $\Theta = 0.111$ ,  $P < 0.01$ ). In both populations the multilocus  
41 genotypic diversity was very high (one clonal pair per population, resulting in >96%  
42 unique genotypes in each of the populations) and there was a lack of linkage  
43 disequilibrium among loci, strongly suggesting that sexual reproduction is an  
44 important component of the life cycle of *Rcc*. In an analysis of spatial genetic  
45 structure, kinship coefficients in all distance classes were very low (-0.0533 to 0.0142  
46 in the Czech and -0.0268 to 0.0042 in the Scottish population) and non-significant ( $P$   
47 >0.05) indicating lack of subpopulation structuring at the field scale and implying  
48 extensive dissemination of spores. These results suggest that *Rcc* possesses a high  
49 evolutionary potential for developing resistance to fungicides and overcoming host

50 resistance genes, and argue for the development of an integrated disease  
51 management system that does not rely solely on fungicide applications.

52 **Keywords:** *Ramularia collo-cygni*, microsatellites, population structure, genetic  
53 diversity, evolutionary potential

54

## 55 **1. Introduction**

56 The last four decades have brought rapid intensification of agriculture, which  
57 has led to a twofold increase in food production. This has been made possible  
58 through a combination of several factors, including cultivation of highly yielding  
59 varieties, soil fertilization and irrigation and effective control of crop pathogens,  
60 insects and weeds with chemical products (Oerke & Dehne, 2004; Tilman, 1999).  
61 Effective chemical control of biotic stresses has significantly reduced the yield losses  
62 in crops (Oerke & Dehne, 2004) whereas the narrow genetic variability of crop  
63 species has created a suitable environment for the adaptation and emergence of new  
64 crop diseases (Stukenbrock & McDonald, 2008). Currently plant protection is  
65 challenged both by the evolution of pathogens that overcome host resistance genes  
66 and by the evolution of losses of sensitivity mutants to commonly used chemical  
67 control products (Hollomon & Brent, 2009).

68 To predict the capacity of pathogen populations to adapt to changing  
69 environments, including such control measures as fungicide application and resistant  
70 cultivars, it is essential to understand their evolutionary potential (McDonald & Linde,  
71 2002). A population genetic approach has been successfully used to assess the  
72 evolutionary potential of pathogens such as *Zymoseptoria tritici* (Banke & McDonald,  
73 2005; Linde *et al.*, 2002; Medini & Hatnza, 2008), *Melampsora larici-populina* (Barres  
74 *et al.*, 2012; Xhaard *et al.*, 2011) and *Rhynchosporium commune* (McDonald *et al.*,

75 1999; Zaffarano *et al.*, 2009). The important parameters from which evolutionary  
76 potential can be inferred are the genetic diversity of populations, the reproduction  
77 system controlling the ability to recombine genetic variants, and the dispersal ability  
78 of the pathogens. In the context of the evolution of fungicide resistance, the greater  
79 the genetic diversity of populations, the greater the standing genetic variation from  
80 which resistant variants may be selected. In terms of the mating system, populations  
81 undergoing sexual or mixed reproduction are more likely to develop resistance than  
82 population reproducing only clonally because sex facilitates recombination and  
83 allows new resistant mutations to become associated with genotypes at other loci  
84 that confer greatest fitness. Furthermore in sexually reproducing populations such  
85 resistant genotypes of high fitness can then be dispersed effectively by sexual as well  
86 as asexual spores, and the resistance phenotype can be rapidly established in the  
87 population. In asexually reproducing populations parasexualism can generate some  
88 recombination, but the process is much less effective than that involving sexual  
89 reproduction and does not generate sexual spores for dispersal (Barrett *et al.*, 2008;  
90 McDonald & Linde, 2002).

91 A very efficient way of establishing the genetic diversity of pathogen  
92 populations, and inferring their mating system and dispersal capacity is to analyse  
93 variation at selectively neutral molecular marker loci in spatially defined samples from  
94 natural populations. In this study we develop and used microsatellite (SSR) genetic  
95 markers to explore the genetic diversity, reproductive system and genetic structure of  
96 the emergent fungal pathogen of barley *Ramularia collo-cygni* (*Rcc*). We use these  
97 data to infer the potential of populations of this pathogen to evolve in response to  
98 management changes such as fungicide application and growth of resistant cultivars.

99           *Ramularia collo-cygni* is an ascomycete fungus causing Ramularia Leaf Spot  
100 (RLS) disease in both spring and winter barley (*Hordeum vulgare*), (Huss, 2004;  
101 Oxley *et al.*, 2002). It was reported for the first time in 1893 in Northern Italy (Cavara,  
102 1893). However it is only in the last 20 years that the disease has been associated  
103 with serious economic losses in countries across Europe, as well as other continents  
104 worldwide (Sachs *et al.*, 1998; Sachs, 2006). The significant inoculum sources of the  
105 disease are winter barley (Frei *et al.*, 2007) and infected barley seed (Havis *et al.*,  
106 2006; Havis *et al.*, 2014; Matusinsky *et al.*, 2011). Additionally some volunteers, crop  
107 debris and other grasses are considered to act as possible inoculum sources (Frei *et*  
108 *al.*, 2007; Huss, 2004; Salamati & Reitan, 2007; Walters *et al.*, 2008). Spores of *Rcc*  
109 are air-borne and sporulation events have been shown to occur after a certain  
110 amount of leaf wetness over the season (Frei *et al.*, 2007; Huss, 2004; Oxley &  
111 Havis, 2010; Salamati & Reitan, 2007). To the present moment the teleomorph stage  
112 of the pathogen has not been observed. As breeding for resistance in barley has not  
113 so far been successful (Havis *et al.*, 2012; Matusinsky *et al.*, 2013; Oxley & Havis,  
114 2010), RLS in barley is currently controlled by fungicide applications (HGCA, 2013).  
115 Therefore it is essential to establish the evolutionary potential of the pathogen to  
116 better assess the risk of fungicide resistance development and the potential success  
117 of future resistance breeding programmes.

118           Previous studies of population genetic structure in *Rcc* have used dominant,  
119 biallelic, AFLP markers to investigate its genetic and clonal diversity and the  
120 distribution of this diversity among countries, regions and individual fields (Hjortshoj  
121 *et al.*, 2013; Leisova-Svobodova *et al.*, 2012). While the AFLP technique used in  
122 these studies generates many markers in a cost effective manner, it suffers from the  
123 limitations that individual loci are anonymous, relies on the assumption that

124 fragments of identical size represent homologous regions of DNA, and generates  
125 results that are difficult to reproduce between laboratories. These limitations can be  
126 overcome by developing microsatellite markers that target particular regions of DNA,  
127 generate loci with many alleles possessing high information content, and produce  
128 results that can readily be compared between research groups (Jarne & Lagoda,  
129 1996; Oliveira *et al.*, 2006; Schlotterer, 2000; Selkoe & Toonen, 2006; Sunnucks,  
130 2000).

131 In this study we develop a set of ten primer pairs for SSR loci that can be  
132 applied reproducibly to investigate the population genetics of *Rcc*. We use these  
133 markers to measure detailed genetic diversity and genetic structuring of *Rcc* within  
134 two fields that have been sampled in a spatially explicit manner. Our aim is not only  
135 to make inferences about the role of sexual reproduction in *Rcc*, but also to explore  
136 the effectiveness of spore dispersal within populations by determining the extent of  
137 spatial clustering of genotypes within each field.

138

## 139 **2. Materials and methods**

### 140 **2.1. Microsatellite primers development**

141 Microsatellite primers were derived using data from a Scottish Government  
142 funded *Rcc* whole genome sequencing project at SRUC (McGrann *et al.*,  
143 unpublished data; genome browser: <http://ramularia.org/jbrowse>). Microsatellites  
144 were predicted using MISA (Thiel *et al.*, 2003) using the standard misa.ini file (i.e.  
145 definition: unit\_size, min\_repeats: 1-10 2-6 3-5 4-5 5-5 6-5; interruptions:  
146 max\_difference\_between\_2\_SSRs: 100). Both the genomic and transcriptomic  
147 sequences were used to predict microsatellites loci of different repeat motifs (di-, tri-  
148 tetra-, penta- and combined motifs). Perl scripts linked with the Primer3 program

149 (Untergasser *et al.*, 2012) were subsequently used to design primers from the  
150 flanking regions of the microsatellites. For the purpose of this study we chose ten  
151 sets of primers (Eurogentec) amplifying pentanucleotide microsatellite loci in the  
152 genomic sequence of *Rcc* (Table 1) and we tested them across ten *Rcc* strains  
153 originating from a SRUC worldwide collection. Loci were chosen from different  
154 contigs and with non-overlapping allele lengths to facilitate PCR multiplexing of loci  
155 and increase the chance of their location on different chromosomes. Each of the F  
156 primers was tailed at the 5' end with M13 universal primer and the M13 primer was  
157 labelled with 6-FAM dye at the 5' end as previously described by Schuelke (2000).

## 158 **2.2. Study populations and sampling strategies**

159 We analysed 90 isolates of *Rcc* collected from spring and winter barley  
160 cultivars in two European countries, the Czech Republic and the United Kingdom.  
161 Isolates from the Czech Republic (n =30) were supplied by Pavel Matusinsky from  
162 Agrotest Fito, Ltd, Kromeriz. Leaf samples (F-1 or flag leaves) were collected at  
163 Krenovice (49°19'30.986"N, 17°15'44.491"E) in June 2012 from a highly infected  
164 winter barley cultivar Traminer at growth stage (GS) 85 (Zadoks *et al.*, 1974). In total  
165 100 isolates from two transect lines separated by 16 meters, were obtained. Along  
166 each of two transect lines separated by 16 meters, a total of 50 locations were  
167 sampled at intervals of a meter. We used thirty randomly chosen isolates from this  
168 collection in this study (Figure A. 1).

169 In the UK we sampled a commercial crop of the spring barley cultivar Waggon  
170 at the end of the growing season (GS 85) at Bush Estate (55°52'15.449"N,  
171 3°12'9.787"W) in Boghall near Edinburgh in August 2012 following slightly modified  
172 procedure of McDonald *et al.* (1999). Sixty isolates derived from two transect lines,  
173 20 meters apart, were amassed (n =60). On each line three circles one meter in



174 diameter separated by 20 meters were sampled. At each location ten F-1 leaves from  
175 different plants around the circle were removed (Figure A. 2). This collection  
176 procedure enabled us to obtain hierarchically sampled isolates at the subpopulation  
177 level, defining each circle as one subpopulation.

### 178 **2.3. DNA extraction**

179 Single spore isolates of *Rcc* were obtained from sampled leaves and  
180 cultivated on PDA media amended with streptomycin 5 µg/ml and/or kanamycin 50  
181 µg/ml in a phytotron in the dark, at 15°C. After three to four weeks fungal material  
182 was collected in Eppendorf tubes, freeze dried overnight and ground to a fine powder  
183 in a tissue lyser (Tissue Lyser LT, Qiagen). DNA of *Rcc* isolates was extracted using  
184 Illustra Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare Life  
185 Sciences), according to the manufacturer's instructions. If high protein content was  
186 observed, a second step of DNA purification using a mixture of chloroform-phenol-  
187 isoamyl alcohol (Sigma-Aldrich) was performed.

### 188 **2.4. Multiplex PCR**

189 SSR primers were grouped into two mixes for multiplex PCR: MixI: SSR2,  
190 SSR4, SSR7, SSR11 and MixII: SSR1, SSR3, SSR5, SSR6, SSR8, SSR12.  
191 Amplification was carried out using the Multiplex PCR Kit (Qiagen). Each of the  
192 amplification reactions was composed of 1x Master Mix, 0.2 µM of a final  
193 concentration of each R primer and M13 primer and 0.5 µM of each F primer, 12.5 ng  
194 of DNA template and RNase free water to a total volume of 25 µl (Qiagen). The  
195 thermocycler conditions were as follows: initial denaturation at 95°C for 15 minutes,  
196 followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for  
197 1.5 minutes, extension at 72°C for one minute and final extension at 60°C for 30  
198 minutes.

## 199 **2.5. Microsatellite analysis**

200 PCR products were run on an ABI 3730 automated sequencer at DBS  
201 Genomics (Durham, UK). The sizes of DNA fragments were scored and alleles  
202 differing in size by a single base (and in very few cases by two) were manually  
203 binned for genetic analysis. Genotype input files were formed with CREATE v1.37  
204 (Coombs *et al.*, 2008). To identify clonal genotypes and calculate the probability of  
205 genotype identity (Woods *et al.*, 1999) GIMLET software v1.3.3 was used (Valiere,  
206 2002). After identification and removal of clonal replicates, mean gene diversities for  
207 each locus ( $H_{sk}$ ), overall gene diversity ( $H_t$ ), allelic richness ( $A$ ), linkage disequilibrium  
208 and genetic differentiation were calculated with FSTAT v2.9.3.2 (Goudet, 1995;  
209 Goudet, 2002). Linkage disequilibrium was estimated as the correlation between the  
210 pairs of loci (Weir, 1996).  $H_{sk}$  and  $H_t$  were estimated using Nei's (1987) unbiased and  
211 unweighted estimators respectively and Weir and Cockerham's (1984) estimator of  
212 the  $F_{st}$  ( $\Theta$ ) was used to measure genetic differentiation among populations.  
213 Significance of  $\Theta$  was based on 15000 bootstraps over loci while significance level  
214 for genotypic disequilibrium was based on 900 permutations with  $P$  values adjusted  
215 for 5% nominal level to  $P = 0.001$ . The spatial structure of *Rcc* populations was  
216 analysed using SPAGeDi v1.4 software (Hardy & Vekemans, 2002) based on  
217 pairwise kinship coefficients for co-dominant markers according to Loiselle *et al.*  
218 (1995). The data in the Czech population was divided into five pairwise distance  
219 classes, and in the Scottish population into four. Significance of the substructuring  
220 was based on 1000 random permutations.

221

222

223

## 224 **3. Results**

### 225 **3.1. Genetic diversity**

226 All ten pentanucleotide SSR loci chosen showed polymorphism in the total  
227 *Rcc* sample. Within individual populations 90% of loci were polymorphic with  
228 monomorphism at SSR5 and SSR11 in the Czech and Scottish populations  
229 respectively. The two populations showed similar level of genetic diversity across ten  
230 SSR loci (Table 2). The mean numbers of alleles per locus were  $A = 3.2$  and  $A = 3.7$   
231 and mean calculated gene diversities over all loci were  $H_t = 0.392$  and  $H_t = 0.411$  in  
232 the Czech and Scottish populations respectively.

### 233 **3.2. Genotypic diversity and reproduction system**

234 The genotypic diversity in the tested populations was very high and the  
235 majority of the isolates within and between tested populations had unique genotypes.  
236 Only one clonal pair was identified in each population; isolates CZ12Rcc031 and  
237 CZ12Rcc039 in the Czech population (Figure A. 1) and isolates GBS12Rcc090 (b1)  
238 and GBS12Rcc121 (e2) in the Scottish population (Figure A. 2). In the Czech  
239 population these clonal isolates were separated by 25.6 meters and in the Scottish  
240 population one clonal pair was identified among strains sampled approximately 21  
241 meters apart. The probability that these two pairs of identical genotypes have been  
242 formed independently via sexual reproduction was very low in both the Czech ( $P$   
243  $= 1.70 \times 10^{-7}$ ) and the Scottish ( $P = 5.06 \times 10^{-5}$ ) populations. The two populations  
244 shared only one multilocus genotype in common, the remainder of the genotypes  
245 being unique. The analysis of linkage disequilibrium demonstrated that there was no  
246 reason to reject the null hypothesis of random association between alleles at different  
247 loci ( $P > 0.001$ ) in either populations.

248

### 249 **3.3. Population differentiation and isolation by distance**

250 Significant moderate genetic differentiation ( $\Theta = 0.111$ ,  $P < 0.01$ ) was found  
251 between the two populations. To determine whether there was any genetic  
252 structuring within the field populations, spatial genetic analysis based on pairwise  
253 kinship coefficients (Loiselle *et al.*, 1995) was performed on both the Czech and the  
254 Scottish populations (Figure 1, Figure 2). The kinship coefficients in each distance  
255 class were quite low and not significantly different from zero ( $P > 0.05$ ), ranging from -  
256 0.0533 to 0.0142 in the Czech population and from -0.0268 to 0.0042 in the Scottish  
257 population of *Rcc*, indicating no spatial genetic structure within either population.  
258 Additionally within the Scottish population there was no detectable differentiation  
259 among the six subpopulations that had been sampled ( $\Theta = 0.013$ ,  $P > 0.05$ ).

260

### 261 **4. Discussion**

262 The microsatellite primer pairs designed in this study from *Rcc* genome  
263 sequence information (McGrann *et al.*, unpublished data) successfully amplified ten  
264 polymorphic microsatellite loci. Loci were sufficiently variable to allow detailed studies  
265 of clonal structure, genotypic diversity, linkage disequilibrium and spatial genetic  
266 structure. These markers are likely to be a valuable resource for further population  
267 genetic studies of *Rcc*, yielding data that can be readily shared and directly  
268 compared among different research groups. The benefits of using highly variable and  
269 reproducible microsatellite markers have already been well illustrated in comparative  
270 studies of a range of other pathogenic fungi (Barnes *et al.*, 2008; Barres *et al.*, 2012;  
271 Dilmaghani *et al.*, 2012; Gurung *et al.*, 2011; Gurung *et al.*, 2013; Linde *et al.*, 2005;  
272 Rieux *et al.*, 2013; Stefansson *et al.*, 2012; Tomsovsky *et al.*, 2013).

273

#### 274 **4.1. The role of sexual reproduction in *Rcc* populations**

275 The most striking result to emerge from the microsatellite analysis of genotypic  
276 diversity is that in both populations all except one of the multilocus genotypes found  
277 was sampled only once. Populations comprise a very diverse collection of genotypes,  
278 and no single genotype or small collection of genotypes dominates the population.  
279 This occurs despite the fact that in both populations sampling took place at the end of  
280 the season after a period of extensive asexual reproduction and spread. The result  
281 implies that differences in fitness among genotypes are not large, for if this were the  
282 case the population would quickly be dominated by the fitter genotypes. Previous  
283 studies using AFLP markers have also found very high genotypic diversity within a  
284 range of *Rcc* populations across Europe (Hjortshoj *et al.*, 2013; Leisova-Svobodova  
285 *et al.*, 2012). The situation contrasts markedly with that found in other pathogens  
286 such as *Phytophthora infestans* where single genotypes may increase rapidly in  
287 frequency and come to dominate large geographic areas (Cooke *et al.*, 2012).  
288 Although our findings of high genotypic diversity agree with the results of previous  
289 studies on *Rcc*, we failed to detect any linkage disequilibrium among the  
290 microsatellite loci scored, a result that is at variance with previous reports of linkage  
291 disequilibrium among AFLP loci in *Rcc* populations (Hjortshoj *et al.*, 2013; Leisova-  
292 Svobodova *et al.*, 2012). Linkage disequilibrium is likely to be generated in *Rcc*  
293 populations by differential asexual reproduction of multilocus genotypes through the  
294 growing season (Frei *et al.*, 2007; Huss, 2004; Salamati & Reitan, 2007). If there is  
295 sexual reproduction, this linkage disequilibrium will be broken down at a rate that is  
296 proportional to the recombination fraction between the loci.

297 A number of reasons could account for discrepancies in the level of linkage  
298 disequilibrium found here and in the previous studies. The first relates to the nature of

299 the markers studied. In our study markers were chosen from different linkage groups,  
300 thus maximising the rate of decay of linkage disequilibrium following sexual  
301 reproduction. In contrast the genomic location of AFLP markers is unknown, and if  
302 pairs of markers were tightly linked this would reduce the effect of sexual  
303 reproduction in reducing linkage disequilibrium.

304 The second reason for a difference in the result is that patterns of selection  
305 could be different between the studies, with stronger differential selection among  
306 genotypes in populations previously studied. The third possibility is that sexual  
307 reproduction may have been more frequent in the populations studied here than in  
308 previous studies, leading to a reduction in the level of linkage disequilibrium. Finally it  
309 is also possible that we failed to detect linkage disequilibrium due to relatively small  
310 sample sizes used in this study ( $n = 30$  in Czech Republic and  $n = 60$  in Scotland). It  
311 was shown previously that in most of the cases quite large sample sizes were  
312 required to detect linkage disequilibrium in the populations (Brown, 1975).

313 Whatever the reason for the observed discrepancies, it should be noted that  
314 even when linkage disequilibrium is detected its level is low, and we can conclude  
315 that sexual reproduction has or is occurring in *Rcc*, at least to the extent required to  
316 prevent the build-up of extensive linkage disequilibrium. We cannot however exclude  
317 the possibility that currently the role of sex in *Rcc* is minor but large population size  
318 and possibly more frequent sexual reproduction in the past has generated the  
319 observed results. A follow on project is underway to directly investigate the  
320 reproductive biology and crossing potential of *Rcc*. Previous data has suggested that  
321 a cryptic sexual stage does exist (Kaczmarek, personal communication). Similar  
322 population genetics studies to ours have provided evidence for sexual reproduction in

323 the causal agent of barley scald, *Rhynchosporium commune*, which was originally  
324 thought to reproduce only asexually (McDonald *et al.*, 1999).

#### 325 **4.2. Effectiveness of spore dispersal within populations**

326 We found no isolation by distance and significant amounts of gene flow within  
327 both field populations, indicating extensive spore dispersal of *Rcc* across the field  
328 (Barrett *et al.*, 2008; Biek & Real, 2010). Individuals showed a low genetic  
329 relatedness even in the shortest distance class (6.6 meters for the Czech population  
330 and 8.7 meters for Scottish population). In the Scottish population of *Rcc*, clonal  
331 genotypes were separated by around 21 meters and in the Czech population by  
332 around 25.6 meters. Thus the distance that the asexual spores of *Rcc* can be  
333 disseminated is at least this distance. However given the limited number of samples  
334 analysed in this study as well as the relatively small spatial scale of this experiment,  
335 potential dissemination distances for spores could easily be longer.

336 A similar lack of spatial structure was previously described for *Mycosphaerella*  
337 *fijiensis*, a causal agent of banana and plantain (Rieux *et al.*, 2013), whose  
338 ascospores were shown to have the capability for long-distance dispersal (Burt *et al.*,  
339 1998). In contrast in *Dothistroma septosporum* (an ascomycete pathogen closely  
340 related to *Rcc*) genetic structuring at the local scale, up to a distance of 8-12 meters  
341 was found (Kraj & Kowalski, 2013). The asexual conidia of the pathogen, that are  
342 rain-splashed over short distances, were shown to be the most important source of  
343 infection over the season and the sexual stage of the fungus is thought to be less  
344 frequent and also to occur for a shorter period of time (Gibson, 1972; Karadzic, 1989;  
345 Peterson, 1973). Similar results have also been found in the ascomycete canker  
346 pathogen of pine, *Gremeniella abietina* (Wang *et al.*, 1997). Thus is it very likely that  
347 the extensive gene flow and the lack of isolation by distance within field populations

348 of *Rcc* result from the extensive dissemination of air-borne asexual spores over the  
349 epidemic season coupled with possible long distance dispersal of ascospores  
350 following sexual reproduction.

#### 351 **4.3. Genetic differentiation and the mode of possible dispersal between *Rcc*** 352 **populations**

353 We found moderate genetic differentiation ( $\Theta = 0.111$ ,  $P < 0.01$ ) between the  
354 two sampled locations in the Czech Republic and Scotland. A previous study using  
355 AFLP markers described genetic differentiation of  $F_{st} = 0.123$  ( $P < 0.001$ ) between 19  
356 locality population in Central Europe (Leisova-Svobodova *et al.*, 2012). In Northern  
357 Europe the fixation index between two distinct populations from Denmark and  
358 Scotland based on AFLP markers was  $G_{st} = 0.031$  ( $P = 0.01$ ) suggesting that the  
359 populations were genetically fairly similar. Higher differences in allele frequencies  
360 were found among two Danish subpopulations ( $G_{st} = 0.135$ ,  $P = 0.001$ ), however no  
361 significant differentiation was indicated among Scottish subpopulations ( $G_{st} = 0.042$ ,  $P$   
362  $= 0.096$ ), (Hjortshoj *et al.*, 2013). Furthermore both of the studies indicated that most  
363 of the genetic variation was distributed on a small scale (within field, plots, and  
364 localities) rather than over larger geographical areas (Hjortshoj *et al.*, 2013; Leisova-  
365 Svobodova *et al.*, 2012), which confirms the results presented here using SSR  
366 markers.

367 The observed genetic differentiation between the two studied populations of  
368 *Rcc* from the Czech Republic and Scotland suggests that these populations either  
369 had the same origin but due to the occurrence of genetic drift in each of the  
370 populations, genetic discontinuity was observed, or that gene flow occurs through  
371 either long-distance dispersal of pathogen spores for several hundred kilometres or  
372 through the transport of infected barley seeds. *Ramularia collo-cygni* is a seed-borne



373 pathogen and could be introduced to new territories through the transport of infected  
374 seed (Havis *et al.*, 2006; Havis *et al.*, 2014; Matusinsky *et al.*, 2011), a likely scenario  
375 which has been previously described by Hjortshoj *et al.* (2013) and Leisova-  
376 Svobodova *et al.* (2012) and which was shown to be responsible for migration events  
377 in another barley pathogen *Rhynchosporium commune* (Linde *et al.*, 2009).

378 On the other hand the asexual spores of *Rcc* are thought to be air-borne and  
379 already Huss (2004) proposed that they could be disseminated to new locations with  
380 wind currents which could contribute to recent disease establishment events. Thus it  
381 is possible that gene flow among *Rcc* populations occurs through a combination of  
382 long-distance spore dispersal and the transport of infected seeds, which brings a  
383 need for an effective seed control method especially in territories where the pathogen  
384 is currently absent. Furthermore, spores of the pathogen are likely to be dispersed  
385 over a long distance which indicates the threat of a rapid spread of both fungicide  
386 resistance alleles and potential resistance genes bred into cultivars within field  
387 populations, as well as between more distant localities. However it is also possible  
388 that there may have been little time for differentiation to have built up among  
389 populations following the recent dispersal of the species to diverse areas through  
390 seed movement or spore dispersal and because of that moderate or no differentiation  
391 between population is observed in this study as well as in the previous ones  
392 (Hjortshoj *et al.*, 2013; Leisova-Svobodova *et al.*, 2012).

#### 393 **4.4. Conclusions**

394 From an evolutionary perspective pathogen populations that are characterised  
395 by a large population size, a high level of gene flow, a high mutation rate and that  
396 undergo both sexual and asexual reproduction cycles over the season are  
397 considered to be the most risky in terms of their adaptive potential for overcoming

398 resistance to such control measures as fungicide applications and cultivars resistant  
399 to the pathogen (McDonald & Linde, 2002). The genetic parameters that we  
400 estimated in this study suggest that populations of *Rcc* possess a great adaptive  
401 potential and pathogen associated risk from the perspective of biological  
402 characteristics should be considered as high. To date, *Rcc* has only developed wide  
403 spread resistance to the Quinone outside Inhibitors (QoIs) group of fungicides  
404 (Fountaine & Fraaije, 2009; Matusinsky *et al.*, 2010). This study suggests that,  
405 especially with a lack of resistant cultivars, an integrated management system which  
406 reduces reliance on fungicides and hence prolongs their effective life time is required.  
407 Further genetic studies using microsatellite markers used at different time points  
408 during the growing season and including more populations are needed to give a  
409 better insight into the evolutionary forces shaping the genetic structure of this  
410 damaging pathogen of barley. Additionally this study indicated the lack of genetic  
411 structuring at the field scale and for future experiments a simple sampling design, not  
412 taking into account hierarchical sampling, should be sufficient to obtain the necessary  
413 information.

414         Although there has been an increase in studies of *Rcc* epidemiology in recent  
415 years, we still lack the critical knowledge of the population biology of the species in  
416 many areas. Our indirect population genetic study provides important insight into the  
417 biology of the fungus and the evolutionary forces that shape its population structure.  
418 However it is important to remember the limitations of this approach, and further  
419 direct studies of *Rcc* population biology are required and expected in the coming  
420 years.

421

422

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427 in this study. We would also like to thank Edinburgh Genomics, and in particular Urmi  
428 Trivedi, for bioinformatic assistance with microsatellite development.

429

430 **Table 1 Final list of ten sets of primers used to amplify SSR loci in populations of *Rcc*.**

SSR number	Primers name	Sequence 5'-3'	SSR type	Allele size range in studied populations <sup>a</sup>
1	PentaSSR_1_223nt_F1	GCGTCAAACCTGACGAATGAG	(GGCAT) <sub>5</sub>	228-253
	PentaSSR_1_223nt_R1	ACATCCTTCCAAACACCAGC		
2	PentaSSR_2_233nt_F1	CTCATGTTGCAGAGAGCGAG	(CTTCA) <sub>5</sub>	245-260
	PentaSSR_2_233nt_R1	AATTTCCGACGTGGATTGAG		
3	PentaSSR_3_253nt_F1	TAGGACAGGAAGACCCGAGA	(GACAG) <sub>6</sub>	260-275
	PentaSSR_3_253nt_R1	ACCTCGACACCTGAACCTTG		
4	PentaSSR_4_266nt_F1	AGGAGATTAGGGATGCGGTT	(GTCCT) <sub>5</sub>	285-305
	PentaSSR_4_266nt_R1	TAAATCCATCGAGCCCGTAG		
5	PentaSSR_5_243nt_F1	ATCAACACATCGCGATCAAA	(CAGCA) <sub>5</sub>	255-275
	PentaSSR_5_243nt_R1	GACAATTGCGGAGTTCCATT		
6	PentaSSR_6_202nt_F1	CTAGGAGAAGAGTGCCGTGG	(CACAG) <sub>5</sub>	207-217
	PentaSSR_6_202nt_R1	AACACGACGACGATTGTGAA		
7	PentaSSR_7_171nt_F1	GTCCACACATTGACCGAGTG	(GGTGT) <sub>5</sub>	174-209
	PentaSSR_7_171nt_R1	GGGTTCGCCTGATCTACTGA		
8	PentaSSR_8_131nt_F1	AGATCGCAAAGGATGGATTG	(GGAAG) <sub>5</sub>	143-148
	PentaSSR_8_131nt_R1	ATTCATTGCACGTTGGTTGA		
11	PentaSSR_11_213nt_F1	TATTCCACACAACGCTCCAA	(CCTTA) <sub>5</sub>	230-235
	PentaSSR_11_213nt_R1	ATCTCACGCCTCAACGATTC		
12	PentaSSR_12_292nt_F1	ATCAGATCAGCTCCATTGCC	(ATCCA) <sub>8</sub>	292-347
	PentaSSR_12_292nt_R1	GGTCGGCGATGACATTACTT		
-	M13_For_(-20)_FAM	ACTGTAAAACGACGGCCAGT	-	-

431 <sup>a</sup> All sizes comprise M13 primer length

432 **Table 2 Allelic richness (*A*) and gene diversity (*H*) per locus and population at ten SSR loci in**  
 433 **the Czech Republic and the Scottish populations of *Rcc*.**

Loci	Czech population (n <sup>c</sup> =28)		Scottish population (n =58)	
	<i>A</i>	<i>H<sub>sk</sub></i> <sup>a</sup>	<i>A</i>	<i>H<sub>sk</sub></i>
SSR1	3.000	0.474	4.998	0.693
SSR2	3.000	0.442	3.998	0.334
SSR3	3.000	0.659	3.998	0.641
SSR4	4.000	0.585	3.998	0.504
SSR5	1.000	0.000	3.996	0.134
SSR6	2.000	0.138	3.000	0.473
SSR7	4.000	0.373	4.998	0.256
SSR8	2.000	0.071	2.000	0.479
SSR11	2.000	0.349	1.000	0.000
SSR12	8.000	0.844	5.000	0.596
All over the loci ( <i>H<sub>t</sub></i> <sup>b</sup> )	3.200	0.392	3.699	0.411

434 <sup>a</sup> *H<sub>sk</sub>* =unbiased Nei's (1987) estimator of mean gene diversities for each locus

435 <sup>b</sup> *H<sub>t</sub>* =unweighted Nei's (1987) estimator of overall gene diversity

436 <sup>c</sup> n =clone corrected sample size

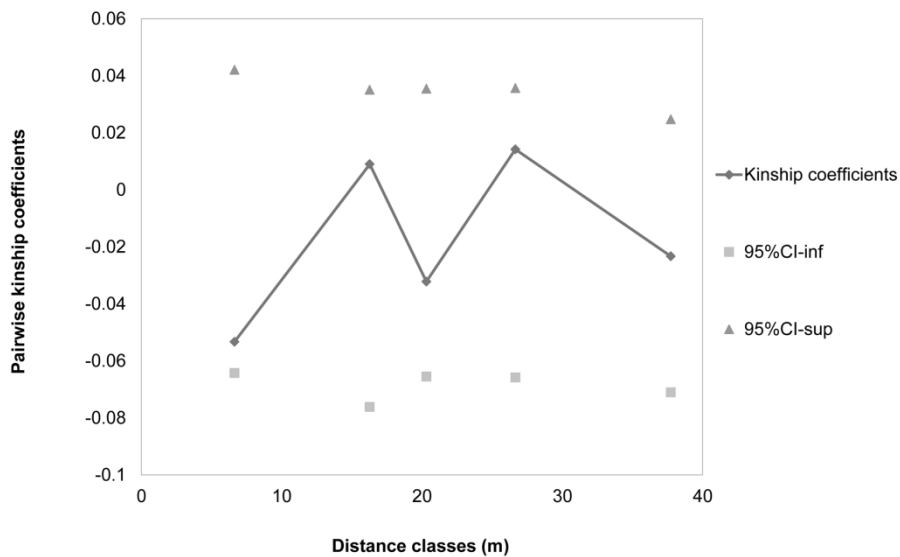
437

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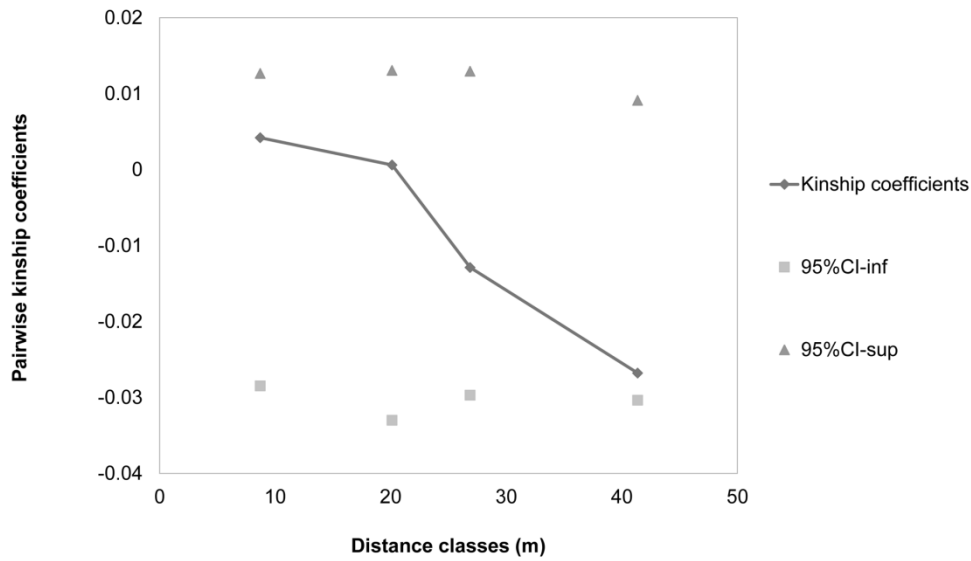
440

441



442

443 **Figure 1 Spatial structure of Czech population divided into five distance classes and plotted**  
 444 **against pairwise kinship coefficients according to Loiselle *et al.* (1995), (CI- confidence**  
 445 **intervals, inf- inferior, sup- superior).**



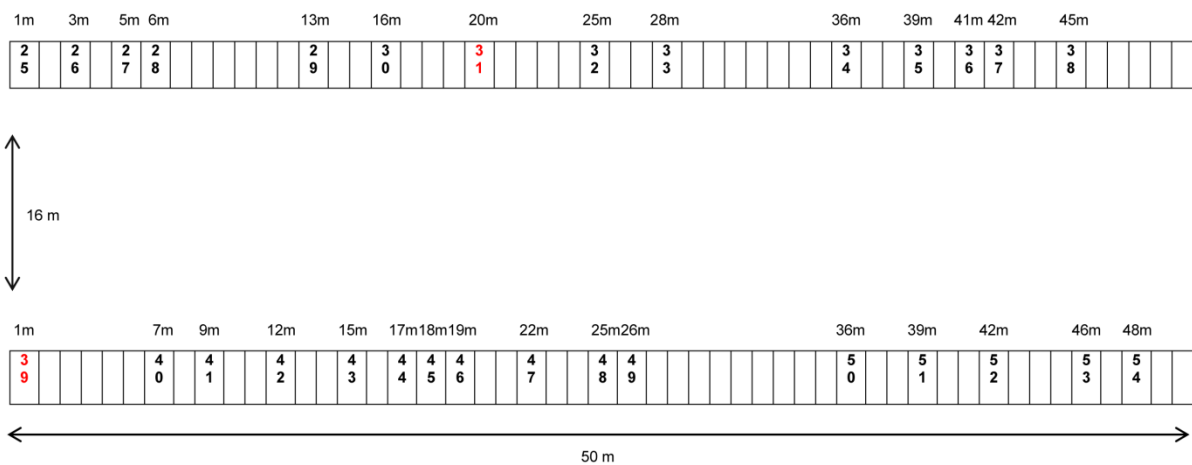
446

447 **Figure 2 Spatial structure of Scottish population divided into four distance classes and plotted**  
 448 **against pairwise kinship coefficients according to Loiselle *et al.* (1995), (CI- confidence**  
 449 **intervals, inf- inferior, sup- superior).**

450

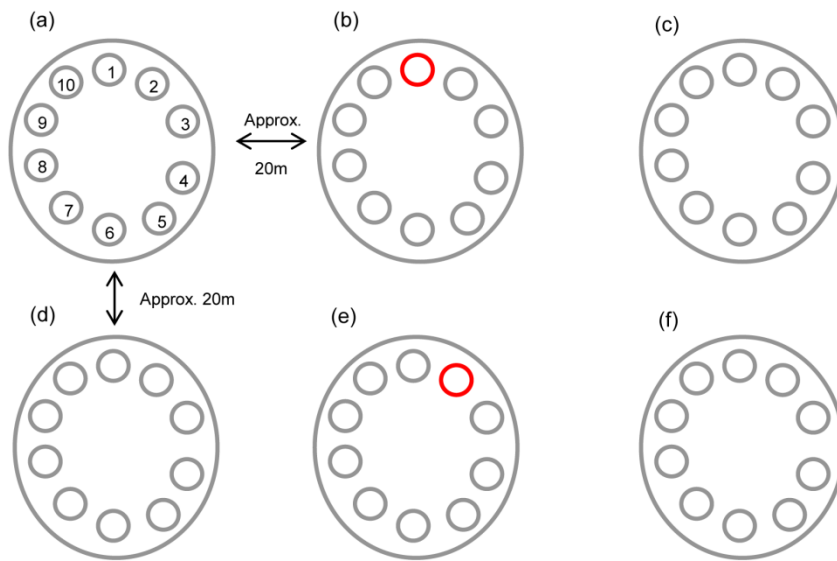
451

452 **Supplementary figures**



453

454 **Figure A. 1 Sampling strategy in the Czech Republic. Samples were collected in two transect**  
 455 **lines. In each line 50 isolates, each one meter apart, were sampled. Fourteen leaves from the**  
 456 **first line and 16 from the second line were used in the study. The numbers in red indicate**  
 457 **clonal genotypes.**



458

459 **Figure A. 2 Sampling strategy in Scotland. Sixty isolates in two transect lines were amassed. In**  
 460 **each line three circles and then ten leaf samples around the circle, as indicated by numbers 1-**  
 461 **10, were collected. Red circles indicate clonal genotypes.**

462

463

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