

1 **Differences in *MAT* Gene Distribution and Expression between *Rhynchosporium* Species**  
2 **on Grasses**

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13 Running title: *MAT* genes in *Rhynchosporium* species

14

15 **Abstract**

16

17 Leaf blotch is a globally important disease of barley crops and other grasses that is caused by  
18 at least five host-specialised species in the fungal genus *Rhynchosporium*. The pathogen *R.*  
19 *commune* (specialised to barley, brome-grass and Italian ryegrass) has long been considered  
20 to reproduce only by asexual means, but there has been accumulating evidence for  
21 recombination and gene flow from population genetic studies and the presence in the field of  
22 complementary *MAT1-1* and *MAT1-2* isolates in an approximately 1:1 distribution. Here, we  
23 demonstrate that 28 isolates of the closely related species *R. agropyri* (on couch-grass) and *R.*  
24 *secalis* (on rye/triticale) collected from Europe were also either of *MAT1-1* or *MAT1-2*  
25 genotype and that the distribution of mating types did not deviate significantly from a 1:1  
26 ratio. Evidence is then provided for *MAT1-1-1* and *MAT1-2-1* gene expression during  
27 mycelial growth for all three species. By contrast, 27 isolates of the more distantly related *R.*  
28 *orthosporum* (on cocksfoot) and *R. lolii* (on Italian and perennial ryegrasses) from Europe  
29 were exclusively of the *MAT1-1* genotype, and expression of the *MAT1-1-1* gene could not be  
30 detected during mycelial growth. These data suggest that cryptic sexual cycles are more  
31 likely to exist for *R. commune*, *R. agropyri* and *R. secalis* than for either *R. orthosporum* or *R.*  
32 *lolii*. A phylogenetic analysis of partial *MAT1-1* idiomorph sequence resolved these five  
33 species into two distinct groups (*R. commune*, *R. agropyri* and *R. secalis* versus *R.*  
34 *orthosporum* and *R. lolii*) but provided only limited resolution within each group.

35

36 **Keywords:** barley leaf blotch, mating type, *Rhynchosporium*, sexual cycle

## 37 **Introduction**

38

39 Leaf blotch (scald), caused by fungal pathogens in the genus *Rhynchosporium*, is an  
40 important disease of barley (*Hordeum vulgare*), rye (*Secale cereale*) and other graminaceous  
41 species (Brooks 1928; Avrova & Knogge 2012). This globally distributed disease of barley  
42 crops (Brooks 1928) is estimated to cause annual losses of £10.8 million to United Kingdom  
43 (UK) barley crops alone, despite extensive use of fungicides (Zhan *et al.* 2008; Anonymous  
44 2011; King *et al.* 2013). *Rhynchosporium* leaf blotch disease also occurs on triticale (x  
45 *Triticosecale*), cocksfoot (*Dactylis glomerata*; Fernandez & Welty 1991) and ryegrass  
46 species (*Lolium* species; King *et al.* 2013).

47 Recent work, based on sequencing of multiple gene loci, DNA fingerprinting and host  
48 range testing, has demonstrated that the genus *Rhynchosporium* includes a complex of at least  
49 five closely related but host-specialised species that can cause leaf blotch disease (Zaffarano  
50 *et al.* 2008; Zaffarano *et al.* 2011; King *et al.* 2013). These include: (a) *R. commune* causing  
51 leaf blotch symptoms on barley, wall barley (*Hordeum murinum*), wild barley (*Hordeum*  
52 *spontaneum*), barley grass (*Hordeum glaucum*, *Hordeum leporinum*), brome-grass (*Bromus*  
53 *diandrus*) and Italian ryegrass (*Lolium multiflorum*); (b) *R. agropyri* on bearded couch-grass  
54 (*Agropyron caninum*) and couch-grass (*Agropyron repens*); (c) *R. secalis* on rye and triticale;  
55 (d) *R. orthosporum* on cocksfoot; and (e) *R. lolii* on Italian (*Lolium multiflorum*) and  
56 perennial (*Lolium perenne*) ryegrasses.

57 At present, all of these *Rhynchosporium* species are known to reproduce only by  
58 asexual means, with dispersal thought to be achieved by splash dispersal of conidia as  
59 observed for *R. commune* (Fitt *et al.* 1986) (NB. for the purposes of this study, previously  
60 published work that refers to isolates of '*R. secalis*' collected from barley will be considered  
61 to refer to *R. commune*). The mode of reproduction is a critical factor in understanding the

62 population biology of plant pathogenic fungi because this impacts on the possibility for  
63 recombination and evolution of the pathogen (Milgroom 1996; McDonald & Linde 2002). In  
64 particular, a sexual cycle for *R. commune* has not yet been identified under either natural or  
65 laboratory conditions (Avrova & Knogge 2012). However, *R. commune* isolates have been  
66 shown to have a mating-type (*MAT*) locus resembling that of species with a heterothallic  
67 (obligate out-crossing) sexual mating system (Linde *et al.* 2003; Foster & Fitt 2003), with  
68 isolates of complementary mating type (referred to as *MAT1-1* and *MAT1-2*) present, which  
69 could enable sexual reproduction to occur. The *R. commune* *MAT* locus is flanked on both  
70 sides by nearly identical stretches of DNA in isolates of either mating type, but internally  
71 contains highly divergent stretches of DNA termed ‘idiomorphs’ that differ between the  
72 mating types (Linde *et al.* 2003; Foster & Fitt 2003). The *MAT1-1* idiomorph contains a  
73 *MAT1-1-1* gene encoding a characteristic alpha-domain protein while the *MAT1-2* idiomorph  
74 contains a *MAT1-2-1* gene encoding a characteristic high mobility group (HMG)-domain  
75 protein (Debuchy *et al.* 2010).

76 PCR-based diagnostic tests have now been developed to discriminate between *MAT1-1*  
77 and *MAT1-2* isolates of *R. commune* (Linde *et al.* 2003; Foster & Fitt 2003), and a study of  
78 1101 *R. commune* isolates (collected from several different countries and continents) found  
79 that isolates of both mating types were present in near 1:1 distributions in most populations  
80 examined (Linde *et al.* 2003). This is consistent with frequency-dependent selection  
81 maintaining an even balance of mating types and Linde *et al.* (2003) proposed that *R.*  
82 *commune* should be considered a sexually reproducing fungus, even if the sexual stage occurs  
83 infrequently in some populations. However, they did not determine whether the putative  
84 *MAT1-1-1* and *MAT1-2-1* genes were expressed at the mRNA level by *R. commune*.

85 Other evidence for sexuality for *R. commune* includes the detection of considerable  
86 genotypic diversity in populations, which is consistent with sexual recombination, and the

87 production of microconidia that have been suggested to be a component of a so far  
88 undiscovered ‘cryptic’ sexual cycle (Salamati *et al.* 2000; Skoropad & Grinchenko 1957). In  
89 addition, sequencing of the internal transcribed spacer region (Goodwin 2002) revealed that  
90 *R. commune* is closely related to the discomycete species *Oculimacula yallundae* (synonym  
91 *Tapesia yallundae*; causal agent of eyespot disease of wheat) and *Pyrenopeziza brassicae*  
92 (light leaf spot of oilseed rape). Both of these pathogens, and the closely related *Oculimacula*  
93 *aciformis* (synonym *Tapesia aciformis*), have a heterothallic mating system, with known  
94 sexual cycles leading to the production of apothecia and air-borne ascospores on their  
95 respective hosts (Dyer *et al.* 1996; Dyer *et al.* 2001; Gilles *et al.* 2001a). Based on the  
96 biology of these closely related crop pathogens, it is likely that if, a sexual cycle does exist  
97 for *R. commune*, it will involve the production of relatively small apothecia (<500 µm in  
98 diameter) that require rainfall for maturation on senescing host tissue (Dyer *et al.* 2001;  
99 Goodwin 2002; Gilles *et al.* 2001a; Welham *et al.* 2004).

100 To date, investigations into the possibility of a cryptic sexual cycle have been made  
101 only in *R. commune*. In the present study, we first describe the isolation of *MAT* genes from  
102 other members of the genus *Rhynchosporium* pathogenic on graminaceous species. By  
103 investigating the patterns of *MAT* distribution in the field and *MAT* gene expression we then  
104 provide novel insights into the potential for sexuality of the five related *Rhynchosporium*  
105 species, and construct a phylogeny of the species based on partial *MAT1-1* idiomorph  
106 sequence data.

107

## 108 **Methods**

109

### 110 **Fungal isolate collection and DNA extraction**

111

112 Seventy-two *Rhynchosporium* isolates (Table 1), whose species identity had previously been  
113 confirmed using either species-specific PCR primers and/or repetitive extragenic palindromic  
114 PCR, were maintained for long-term storage at -80°C as silica stocks (King *et al.* 2013).  
115 Isolates were grown on potato dextrose agar (PDA, Oxoid, UK) plates overlaid with a single  
116 cellulose disk (A.A. Packaging Ltd., UK) and incubated at 18°C. After *ca.* 2 weeks,  
117 mycelium was scraped from the surface of the disc and DNA extracted using a DNeasy  
118 extraction kit (Qiagen, UK). DNA was quantified using a NanoDrop-1000 spectrophotometer  
119 (Labtech International, Ringmer, UK) and diluted to the required concentration using either  
120 Tris-EDTA buffer or sterile distilled water.

121

#### 122 **Mating-type identification for isolates of *R. commune*, *R. agropyri* and *R. secalis***

123

124 The *MAT* multiplex PCR diagnostic of Linde *et al.* (2003) was tested against 47  
125 *Rhynchosporium* isolates (Table 1). This diagnostic amplifies DNA from regions of either the  
126 *MAT1-1-1* or *MAT1-2-1* genes, specific to *MAT1-1* or *MAT1-2* isolates, respectively.  
127 However, reaction components were modified to produce more distinct PCR amplicons. Each  
128 25 µl reaction contained 12.5 µl of Jumpstart RedTaq ReadyMix (2 x concentrate, Sigma  
129 Aldrich, UK), 1 µl each of the four primers (0.4 µM concentration of each primer), 6.5 µl of  
130 sterile distilled water and 2 µl of template genomic DNA (20 ng total DNA). Unless  
131 otherwise specified, PCR throughout the present study used a PTC-100 Programmable  
132 Thermal Controller (MJ Research, USA), with PCR products (10 µl) routinely resolved by  
133 gel electrophoresis on 1% Tris-Borate-EDTA (TBE) agarose gels incorporated with ethidium  
134 bromide (0.5 µg / ml) and viewed under ultraviolet light. Occasionally, for high image  
135 quality, gels were instead stained after electrophoresis (2 µg / ml ethidium bromide in 1 x  
136 TBE solution). Isolates that produced either a 590-bp or a 360-bp amplicon were considered

137 to be of *MAT1-1* or *MAT1-2* genotype, respectively (Linde *et al.* 2003). The hypothesis of a  
138 1:1 ratio of mating types for the individual species *R. commune*, *R. agropyri* and *R. secalis*  
139 dataset was tested using a chi-squared ( $\chi^2$ ) test (GraphPad Software).

140 Sequence identity of these putative *MAT* amplicons was confirmed by PCR on seven  
141 isolates that had previously produced either putative *MAT1-1* (590-bp) or *MAT1-2* (360-bp)  
142 amplicons using the multiplex diagnostic of Linde *et al.* (2003). These isolates were: *R.*  
143 *commune* (*MAT1-1*: UK7; *MAT1-2*: 53hv09, 2lm11), *R. agropyri* (*MAT1-1*: 3ar10; *MAT1-2*:  
144 10ar10) and *R. secalis* (*MAT1-1*: 1D4a; *MAT1-2*: I-Ia) (Table 1). Jumpstart high fidelity mix  
145 (Roche, Germany) was used with reaction components selected according to the  
146 manufacturer's instructions with the following modifications; DMSO was omitted and each  
147 reaction included 10 mM of each dNTP (Fermentas, UK) and 2.5 ng of template DNA. PCR  
148 products were purified using a MinElute kit (Qiagen, UK) and sent to Eurofin MWG Operon  
149 for sequencing (590-bp and 360-bp amplicons were sequenced using primers RsMAT1F and  
150 RsMAT2F, respectively). Sequence data were edited in BioEdit Sequence Alignment Editor  
151 (version 7.0.9.0; Hall 1999), poor read quality data were removed, and final sequence data  
152 were analysed using BLASTN 2.2.29+ software (Zhang *et al.* 2000).

153

#### 154 **Mating-type identification for isolates of *R. orthosporum* and *R. lolii***

155

156 As the mating-type diagnostic of Linde *et al.* (2003) could not satisfactorily amplify *MAT*  
157 amplicons of the predicted size from isolates of *R. orthosporum* (see below), it was necessary  
158 to design new PCR-based diagnostic tests. Primers were first designed [throughout this study,  
159 all primers were designed using either the Geneious version 5.5.6. (Biomatters Ltd.) or  
160 MacVector 12 (MacVector Ltd.) software packages] to amplify a partial region of the *MAT1-*  
161 *I-1* gene based on sequence data obtained from the genome of *R. orthosporum* (RsCH04 Bär

162 A.1.1.3; W. Knogge and *Rhynchosporium* genome consortium, unpublished results).  
163 Sequence data for both this isolate and an isolate of *R. commune* (GenBank accession:  
164 AY257472) were used to design primers predicted to produce a single amplicon of 598-bp  
165 specific for *MAT1-1* isolates of *Rhynchosporium* species; A-MAT1-F (5'  
166 AGCCATCCTGCATGCCGCC 3') and A-MAT1-R (5' CCGCGAGCACCACTGGACC 3').  
167 Primer A-MAT1-R annealed to a region of the *MAT1-1-1* gene within the *MAT1-1* idiomorph  
168 (Fig. 1).

169  
170 Fig. 1 near here

171  
172 No sequence data were available for a *MAT1-2* isolate of *R. orthosporum* and primers  
173 were therefore designed, based on available *MAT1-2* idiomorph sequence data for an isolate  
174 of *R. commune* (GenBank accession: AJ537511), that were predicted to produce a single  
175 amplicon of 149-bp for *MAT1-2* isolates of *Rhynchosporium* species; C-MAT2-F (5'  
176 TGGGGCTGAAGCAAGGAGACCA 3') and C-MAT2-R (5'  
177 ACACATCCTCCGGCCAAGCA 3'). Primer C-MAT2-R annealed to DNA sequence  
178 specific to the *MAT1-2* idiomorph (Fig. 1).

179 The primer pair A-MAT1-F/R was used for PCR, each 25 µl volume containing 12.5 µl  
180 of RedTaq ReadyMix (Sigma Aldrich, UK), 1 µl each of primers A-MAT1-F and A-MAT1-  
181 R (0.4 µM concentration), 9.5 µl of sterile distilled water and 1 µl of template fungal DNA (1  
182 ng total DNA). Reaction conditions were as follows; an initial denaturation step of 94°C for 2  
183 min, followed by 32 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 2 min. This was  
184 followed by a final extension step of 72°C for 5 min and a final hold at 4°C. Similar reaction  
185 conditions were used with primer pair C-MAT2-F/R, except that the annealing temperature  
186 was decreased to 66°C. The mating-type diagnostic tests using either primer pair A-MAT1-



187 F/R or C-MAT2-F/R were then applied to 39 *Rhynchosporium* isolates (Table 1). The  
188 hypothesis that there was a 1:1 ratio of mating types for the datasets for individual species (*R.*  
189 *orthosporum* and *R. lolii*) was tested using a  $\chi^2$  analysis (larger samples of the other species  
190 had already been tested).

191 To confirm that putative *MAT1-1* amplicons of *R. orthosporum* and *R. lolii* shared  
192 sequence homology with known *MAT1-1* idiomorph sequence data, primers A-MAT1-F/R  
193 were used in PCR to amplify DNA from six representative isolates of *R. orthosporum*  
194 (27dg09, RsCH04 Bär A.1.1.3, RS04ITA D-6.2) or *R. lolii* (14lp11, 15lp11, 20lp11) (Table  
195 1). Jumpstart high fidelity mix was used in all reactions, with reaction components selected as  
196 described previously. PCR products were visualised on an agarose gel to confirm the  
197 presence of a single 598-bp amplicon, and purified and sequenced (using primer A-MAT1-F)  
198 as described previously.

199

## 200 **Expression of mating-type genes for the different *Rhynchosporium* species**

201

202 Reverse transcription PCR (RT-PCR) was used to assess whether the mating-type genes of  
203 the different *Rhynchosporium* species were expressed at the mRNA level. Eight  
204 representative isolates selected were of *R. commune* (*MAT1-1*: E.1.2; *MAT1-2*: 53hv09), *R.*  
205 *agropyri* (*MAT1-1*: 1ar10; *MAT1-2*: 3ar10), *R. secalis* (*MAT1-1*: B8; *MAT1-2*: I-Ia), *R.*  
206 *orthosporum* (*MAT1-1*: RsCH04 Bär A.1.1.3) or *R. lolii* (*MAT1-1*: 13lp11) (Table 1). They  
207 were grown from silica stocks onto 40% strength PDA plates (supplemented with 50 mg ml<sup>-1</sup>  
208 penicillin G and streptomycin sulphate) overlaid with a single cellulose disc. Plates were  
209 sealed with a double layer of parafilm (Pechiney Plastic Packaging, USA) and incubated in  
210 the dark at 15°C for 20 days.

211 For extraction of RNA, mycelium was scraped from the surface of discs, ground under  
212 liquid nitrogen, and then 100 mg was transferred to 1 ml of TRIzol (Invitrogen, UK).  
213 Samples were mixed by inversion and incubated at *ca.* 20°C for 15 min, followed by  
214 centrifugation (11,688 g) for 15 min. The supernatant was added to 0.25 ml chloroform,  
215 vortexed, incubated at 20°C for 5 min and centrifuged for a further 5 min. The aqueous phase  
216 was subsequently added to an equal volume of 2-propanol, inverted thoroughly, incubated for  
217 10 min at 20°C and then centrifuged at 4°C for 10 min. The supernatant was removed, and  
218 pellet washed with 70% ethanol before the RNA was re-suspended in 100 µl of DEPC water.  
219 A Nucleospin RNA II kit (Machery-Nagel) was used for subsequent DNase treatment and  
220 RNA purification steps according to the manufacturer's instructions. Finally, eluted RNA  
221 was further DNase-treated with RNA-free RQ1 DNase (Promega) prior to RT-PCR. RNA  
222 was visualised on an agarose gel to ensure quality, quantified using a NanoDrop-1000  
223 spectrophotometer and diluted to the required concentration using DEPC water.

224 PCR primers were designed to amplify partial regions of the *MAT1-1-1*, *MAT1-2-1*,  
225 alpha-tubulin and beta-tubulin gene loci, based on alignments of publicly available GenBank  
226 data. Primers were designed to span a putative intron(s) to allow confirmation of RNA  
227 processing, based on previously published gene models for *R. commune* (Linde *et al.* 2003;  
228 Foster & Fitt 2003). Primer pair KM1RcF3 (5' AAGAAGGCTTTACCTCCCC 3') and  
229 KM1RcB11 (5' TGCTCGTGGTTTTCCGACTG 3') were targeted to amplify partial regions of  
230 the *MAT1-1-1* gene for isolates of *R. commune*, *R. agropyri* and *R. secalis*, with predicted  
231 amplicons of 425- and 377-bp for genomic DNA and processed RNA, respectively (Fig. 1).  
232 Primer pair KM2RcF1 (5' TCATCTCAACTCAGCCTGCC 3') and KM2RcB4 (5'  
233 TTCTCCAGCGACCTCAATAAAC 3') were targeted to amplify regions of the *MAT1-2-1* gene  
234 for these three species, with predicted amplicons of 407- and 360-bp for genomic DNA and  
235 processed RNA, respectively (Fig. 1). Due to sequence divergence at the *MAT1-1* locus,

236 additional primers were designed (based on sequence data from the *R. orthosporum* genome;  
237 *Rhynchosporium* genome consortium, unpublished results) to amplify partial regions of this  
238 locus for isolates of the more distantly related *R. orthosporum* and *R. lolii*; primer pair  
239 KM1RoF2 (5' CCCGACGAGTATCTAATGAACC 3') and KM1RoB16 (5'  
240 AGAGCCACAGAAAAGCACG 3') were predicted to produce amplicons of 587- and 540-bp  
241 for genomic DNA and processed RNA, respectively.

242 Finally, two additional pairs of primers were designed to amplify partial sequences of  
243 two different housekeeping genes for possible use with all five *Rhynchosporium* species.  
244 Primer pair KATRcF15 (5' CGACGAGAGGGAAATGGATACG 3') and KATRcB11 (5'  
245 ACACCACACTTGAGCACTCC 3') targeted the alpha-tubulin loci and were predicted to  
246 produce amplicons of 289- and 237-bp for genomic DNA and processed RNA, respectively;  
247 primer pair KBTRcF1 (5' CGGCACGAGGAACATACTTATTAC 3') and KBTRcF20 (5'  
248 GCCAATGTGGTAATCAAATCGG 3') targeted the beta-tubulin loci and were predicted to  
249 produce amplicons of 418- and 162-bp for genomic DNA and processed RNA, respectively.

250 All eight isolates were then tested using these primer sets with both RT-PCR (RNA  
251 template) and conventional PCR (genomic DNA template). In RT-PCR testing, a one-Step  
252 RT-PCR kit (Qiagen, UK) was used, with reaction components utilised according to the  
253 manufacturer's instructions (adding 150 ng total extracted RNA as a template). Reaction  
254 conditions for all RT-PCR reactions were as follows: an initial reverse transcription step of  
255 50°C for 30 min, an initial PCR activation step of 95°C for 15 min, 35 cycles of 94°C for 1  
256 min, 57°C for 1 min and 72°C for 1 min and a final elongation step of 72°C for 10 min  
257 (although for primer pair KBTRcF1/KBTRcF20 an annealing temperature of 55°C was used).  
258 PCR products were run on 2% agarose gels and visualised. In parallel conventional PCR  
259 testing, RedTaq ReadyMix was used according to the manufacturer's instructions (0.5 µM  
260 concentration of each primer and 5-10 ng genomic template DNA), with reaction conditions

261 identical to those described previously for RT-PCR testing except that the reverse  
262 transcription and initial PCR activation steps were omitted, and the final elongation step was  
263 decreased to 5 min. PCR products were visualised as described previously.

264

### 265 **Phylogenetic analyses of partial *MAT1-1* idiomorph sequences**

266

267 *MAT1-1* idiomorph sequence data obtained from an isolate of *R. orthosporum* (RsCH04 Bär  
268 A.1.1.3) and *R. commune* (GenBank accession: AY257472) were aligned. Two primers,  
269 predicted to produce a single amplicon of 327-bp specific for only *MAT1-1* isolates of all  
270 *Rhynchosporium* species, were designed; MAT1-F central (5' CTGCTGTATAGCAACCCA  
271 3') and MAT1-R central (5' GTGATGGGAGAATGTTCGC 3'). These two primers annealed  
272 to apparently conserved DNA sequence within the *MAT1-1* idiomorph, upstream relative to  
273 the *MAT1-1-1* gene (Fig. 1). Preliminary testing of this primer pair against 12 isolates of *R.*  
274 *commune*, *R. agropyri* or *R. secalis* of known *MAT1-1* or *MAT1-2* identity [as confirmed  
275 using the multiplex diagnostic of Linde *et al.* (2003)] confirmed that the expected 327-bp  
276 amplicon was produced using template DNA obtained from only *MAT1-1* isolates (data not  
277 shown).

278 DNA from a total of 17 isolates was amplified by PCR using primers MAT1-F/R  
279 central, comprising *R. commune* (19hv09, UK7), *R. agropyri* (6ar10, 10ar10), *R. secalis*  
280 (RS02CH4-4b1, RS02CH4-14a1), *R. orthosporum* (27dg09, 52dg09, 59dg09, RS04ITA D-  
281 4.1, RsCH04 Bär A.1.1.3) and *R. lolii* (4lm11, 15lp11, 18lp11, 20lp11, 21lm11, 22lm11)  
282 (Table 1). Jumpstart high fidelity mix was used in all reactions, with reaction components  
283 previously described. Reaction conditions were as follows; an initial denaturation step of  
284 94°C for 2 min, followed by 34 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 2  
285 min. This was followed by a final extension step of 72°C for 5 min and a final hold at 4°C.

286 PCR products were visualised on an agarose gel to confirm the presence of single 327-bp  
287 amplicons, and purified and sequenced (using primer MAT1-F central) as described above.

288 Sequences were imported into the Geneious version 7.0.6. software package  
289 (Biomatters Ltd.) and aligned using the MUSCLE algorithm (maximum number of iterations:  
290 8). Sequences were manually edited to ensure an equal length of 235-bp including gaps. A  
291 neighbour joining phylogenetic tree was constructed using the Geneious Tree builder  
292 software, to which the Jukes-Cantor distance model was applied. The consensus tree was  
293 based on the bootstrap resampling method (100,000 replications), with the support threshold  
294 set at 80%. *MAT1-1* idiomorph sequence data from the closely related *P. brassicae* (GenBank  
295 accession: AJ006073) was used as an outgroup in the computation. The sequence alignment  
296 was deposited into TreeBASE and is available at:

297 TreeBASE: [http://purl.org/phylo/treebase/phyloids/study/TB2:S15396?x-access-](http://purl.org/phylo/treebase/phyloids/study/TB2:S15396?x-access-code=8462cba1c267aa1074f747b15dd5b3&format=html)  
298 [code=8462cba1c267aa1074f747b15dd5b3&format=html](http://purl.org/phylo/treebase/phyloids/study/TB2:S15396?x-access-code=8462cba1c267aa1074f747b15dd5b3&format=html)

299 Nexus file: [http://purl.org/phylo/treebase/phyloids/study/TB2:S15396?format=nexus&x-](http://purl.org/phylo/treebase/phyloids/study/TB2:S15396?format=nexus&x-access-code=8462cba1c267aa1074f747b15dd5b3)  
300 [access-code=8462cba1c267aa1074f747b15dd5b3](http://purl.org/phylo/treebase/phyloids/study/TB2:S15396?format=nexus&x-access-code=8462cba1c267aa1074f747b15dd5b3)

301

## 302 **Results**

303

### 304 **Mating-type identification for isolates of *R. commune*, *R. agropyri* and *R. secalis***

305

306 The multiplex PCR diagnostic developed for *R. commune* by Linde *et al.* (2003) was  
307 successfully applied to 45 isolates of *R. commune*, *R. agropyri* or *R. secalis* (Table 1). Both  
308 mating types could readily be identified for all three species by the production of two  
309 differently sized PCR products; *MAT1-1* isolates produced an amplicon of 590-bp, while

310 *MAT1-2* isolates produced an amplicon of 360-bp (Fig. 2). However, the test was not  
311 applicable to the two isolates of *R. orthosporum* assayed, which produced multiple non-  
312 specific PCR products (data not shown). For all three of the former species, both *MAT1-1* and  
313 *MAT1-2* isolates were identified from locations throughout Europe. The distribution of  
314 mating types for each individual species, namely *R. commune* (*MAT1-1* = 12, *MAT1-2* = 5;  $\chi^2$   
315 = 2.882, 1 d.f.,  $P = 0.0896$ ), *R. agropyri* (*MAT1-1* = 6, *MAT1-2* = 6;  $\chi^2 = 0$ , 1 d.f.,  $P =$   
316 1.0000), and *R. secalis* (*MAT1-1* = 9, *MAT1-2* = 7;  $\chi^2 = 0.250$ , 1 d.f.,  $P = 0.6171$ ) did not  
317 deviate significantly from a 1:1 ratio.

318

319 Table 1 and Fig. 2 near here

320

321 BLASTN analysis of partial sequences of putative 590-bp (*MAT1-1*) amplicons  
322 produced by isolates UK7, 10ar10 and 1D4a (query lengths: 519-, 518-, 423-bp, respectively)  
323 demonstrated 99-100% similarity to *Rhynchosporium MAT1-1-1* gene sequence (GenBank  
324 accession: AJ549759). These sequences for isolates UK7 (*R. commune*), 10ar10 (*R. agropyri*)  
325 and 1D4a (*R. secalis*) have been deposited on GenBank (Accessions: KF998182 - KF998184,  
326 respectively). BLASTN analysis of partial sequences of putative 360-bp (*MAT1-2*) amplicons  
327 produced by isolates 53hv09, 2lm11, 3ar10 and I-Ia (query lengths: 277-, 231-, 235-, 236-bp,  
328 respectively) demonstrated 99-100% similarity to *Rhynchosporium MAT1-2-1* gene sequence  
329 (GenBank accession: AJ537511). These sequences for isolates 53hv09 (*R. commune*), 2lm11  
330 (*R. commune*), 3ar10 (*R. agropyri*) and I-Ia (*R. secalis*) have been deposited on GenBank  
331 (Accessions: KF998185 - KF998188, respectively).

332 Comparative TBLASTN analyses of the *R. commune*, *R. agropyri* and *R. secalis* partial  
333 *MAT1-1-1* gene sequences (each aligned and trimmed to a length of 423-bp) obtained in the  
334 present study showed greater similarity between *R. commune* and *R. agropyri* (99.76%) than

335 either *R. agropyri* and *R. secalis* (99.53%), or *R. commune* and *R. secalis* (99.29%). Analyses  
336 of partial *MAT1-2-1* gene sequences obtained from all three species (all 224-bp) also revealed  
337 greater similarity between *R. commune* and *R. agropyri* (100%) than between either of these  
338 two species and *R. secalis* (99.55%).

339

#### 340 **Mating-type identification for isolates of *R. orthosporum* and *R. lolii***

341

342 The PCR diagnostics developed using primer pairs A-MAT1-F/R and C-MAT2-F/R were  
343 specific for *MAT1-1* and *MAT1-2* type isolates, respectively, of *R. commune*, *R. agropyri* and  
344 *R. secalis* (Table 1). Primer pair A-MAT1-F/R produced the predicted 598-bp amplicon  
345 specifically for *MAT1-1* isolates (Fig. 3a), while primer pair C-MAT2-F/R produced the  
346 predicted 149-bp amplicon specifically for *MAT1-2* isolates (Fig. 3b).

347 When these primer pairs were used in PCR with *R. orthosporum* and *R. lolii*, all 27  
348 isolates (collected from England, Italy, Switzerland and Wales) produced only the 598-bp  
349 amplicon specific for *MAT1-1* isolates (using primer pair A-MAT1-F/R; Fig. 4a) and not the  
350 149-bp product specific for *MAT1-2* isolates (using primer pair C-MAT2-F/R; Fig. 4b). The  
351 mating-type distributions of the individual species *R. orthosporum* (*MAT1-1* = 8, *MAT1-2* = 0;  
352  $\chi^2 = 8.000$ , 1 d.f.,  $P < 0.01$ ) and *R. lolii* (*MAT1-1* = 19, *MAT1-2* = 0;  $\chi^2 = 19.000$ , 1 d.f.,  $P <$   
353 0.01) were statistically significantly different from that expected for a 1:1 ratio of *MAT1-*  
354 *1*:*MAT1-2* mating types. BLASTN analyses of sequence data (query lengths: 508-bp)  
355 obtained from these putative *MAT1-1* amplicons (*R. orthosporum*: 27dg09; *R. lolii*: 15lp11)  
356 showed ~82% similarity with *Rhynchosporium MAT1-1-1* gene sequence (GenBank  
357 accession: AJ549759). Representative *MAT1-1-1* sequence data from isolates 27dg09 and  
358 15lp11 have been deposited on GenBank (Accessions: KF998189 and KJ513481,  
359 respectively). To further confirm the absence of the *MAT1-2-1* gene from *R. orthosporum* and

360 *R. lolii*, draft genome sequences of these species (RsCH04 Bär A.1.1.3 for *R. orthosporum*,  
361 W Knogge and *Rhynchosporium* genome consortium; 15lp11 for *R. lolii*, KM King, D  
362 Hughes, R Harrison, PS Dyer, BDL Fitt and JS West, unpublished results) were BLASTN  
363 searched with the *MAT1-1-1* and *MAT1-2-1* gene sequences from *R. commune*. This revealed  
364 clear matches for *MAT1-1-1* in both species (E-values of 1.8E-163 and 6.4E-61 for *R.*  
365 *orthosporum* and *R. lolii*, respectively). However, there were no matches for *MAT1-2-1* in  
366 either species (cut-off value of 1.0E-5), showing that the isolates used for genome sequencing  
367 only contained the *MAT1-1-1* idiomorph. This was consistent with a heterothallic  
368 organisation of *MAT* genes rather than a possible homothallic organisation, with both *MAT1-*  
369 *1-1* and *MAT1-2-1* within the same genome (Debuchy *et al.* 2010), and/or lack of annealing  
370 of primer pair C-MAT2-F/R due to low partial mismatch of *MAT1-2-1* sequence.

371

372 Fig. 3 and Fig. 4 near here

373

#### 374 **Expression of mating-type genes for some *Rhynchosporium* species**

375

376 Sizes of the resulting PCR amplicons using both genomic DNA and cDNA template (RT-  
377 PCR) were used to confirm both the presence and processing, or otherwise, of introns within  
378 the *MAT1-1-1*, *MAT1-2-1*, alpha-tubulin and beta-tubulin genes. On this basis, evidence was  
379 obtained for expression at the mRNA level of the *MAT1-1-1* gene using primer pair  
380 KM1RcF3/KM1RcB11 for *MAT1-1* isolates of *R. agropyri* (Fig. 5a), *R. secalis* (Fig. 5b) and  
381 *R. commune* (data not shown). However, it is noted that RT-PCR with all *MAT1-1* isolates  
382 produced only relatively faint bands corresponding to products with an intron removed, but  
383 also appeared to produce an identically sized amplicon (425-bp) to that of the genomic DNA  
384 template controls, despite efforts to ensure removal of genomic DNA from RNA extracts. By



385 contrast, expression of the *MAT1-1* gene (in terms of processing of an intron) could not be  
386 confirmed using primer pair KM1RoF2/KM1RoB16 for the isolates of *R. orthosporum* and *R.*  
387 *lolii* examined, although again an amplicon identical in size to those of the genomic template  
388 DNA controls was produced (data not shown).

389         Meanwhile, evidence for *MAT1-2-1* gene expression (in terms of processing of an  
390 intron) was obtained using primer pair KM2RcF1/KM2RcB4 for *MAT1-2* isolates of *R.*  
391 *agropyri* (Fig. 5a), *R. secalis* (Fig. 5b) and *R. commune* (data not shown). Moreover for all  
392 eight isolates of the five different species, expression of both the alpha-tubulin  
393 (KATRcF15/KATRcB11) and beta-tubulin (KBTRcF1/KBTRcF20) housekeeping control  
394 genes was confirmed by the presence of amplicons of smaller size following processing of  
395 introns [representative data for *R. agropyri* (Fig. 5a) and *R. secalis* (Fig. 5b) is shown]. The  
396 water (no template) controls were blank in all PCR assays (data not shown).

397

398 Fig. 5 near here

399

#### 400 **Phylogenetic analyses of partial *MAT1-1* idiomorph sequences**

401

402 The PCR assay using primer pair MAT1-F/R central, designed to amplify partial sequences  
403 of the *MAT1-1* idiomorph, was successfully applied to 17 isolates of *R. commune*, *R.*  
404 *agropyri*, *R. secalis*, *R. orthosporum* or *R. lolii*. All of the isolates tested with this primer pair  
405 produced the expected 327-bp PCR amplicon. BLASTN analyses of putative *MAT1-1*  
406 idiomorph sequence data (query length: 247-bp) obtained from *R. orthosporum* isolate  
407 27dg09 revealed 81% similarity to previously deposited *Rhynchosporium MAT1-1* idiomorph  
408 sequence data (GenBank accession: AJ549759).

409 Subsequent phylogenetic analyses identified two main *Rhynchosporium* groupings,  
410 with branching supported by bootstrap values of 100% (Fig. 6). Isolates of *R. commune*, *R.*  
411 *agropyri* and *R. secalis* grouped distinct from those of isolates of *R. orthosporum* and *R. lolii*.  
412 Phylogenetic analyses did not resolve between isolates of *R. commune*, *R. agropyri* and *R.*  
413 *secalis*. However, visual inspection of the data revealed one single nucleotide polymorphism  
414 (G; position 59/235 in the TreeBASE alignment) that was fixed in *R. secalis* and could be  
415 used to discriminate it from the other two species. By contrast, isolates of *R. orthosporum* and  
416 *R. lolii* could be visibly resolved from each other (bootstrap support of 91%), although there  
417 was relatively little genetic divergence between the species in this region. Representative  
418 *MAT1-1* sequence data obtained for isolates 27dg09 (*R. orthosporum*) and 15lp11 (*R. lolii*)  
419 have also been deposited at GenBank (Accessions: KF998190 and KF998191, respectively).

420

421 Fig. 6 near here

422

## 423 Discussion

424

425 It is important to understand the mode of reproduction of plant pathogenic fungi because this  
426 has a major influence on the evolutionary potential of pathogen populations. Knowing  
427 whether species have clonal or recombining population structures can provide an indication  
428 of the risk of breakdown of disease control strategies (McDonald & Linde 2002). This study  
429 has shown for the first time that isolates genetically confirmed as *R. agropyri* and *R. secalis*,  
430 and obtained from both the same and proximate geographical origins, are of either *MAT1-1* or  
431 *MAT1-2* genotype. These findings are similar to those of Linde *et al.* (2003) and Foster & Fitt  
432 (2003), who demonstrated that isolates of the closely related species *R. commune* were also of  
433 either *MAT1-1* or *MAT1-2* identity, i.e. they showed an organisation consistent with a

434 heterothallic mating system. The sequence at the *MAT* loci for *R. commune*, *R. agropyri* and  
435 *R. secalis* appeared to be highly conserved, based on the ability of the multiplex PCR  
436 diagnostic test of Linde *et al.* (2003) to anneal and amplify similarly sized PCR products  
437 from all of these species. Sequencing of putative *MAT1-1-1* and *MAT1-2-1* PCR amplicons  
438 revealed >99% sequence similarity between all three species across the sequence examined.  
439 In addition, the present study has provided the first evidence for expression (in terms of  
440 processing of an intron) of both the *MAT1-1-1* and *MAT1-2-1* genes for all three of these  
441 species during mycelial growth *in vitro*, although the *MAT1-1-1* gene was expressed at  
442 relatively low levels under the assay conditions.

443         These data, alongside previous reports of considerable genetic diversity in field  
444 populations (Linde *et al.* 2009) and the close genetic relationship to other sexually  
445 reproducing plant pathogenic fungi (Goodwin 2002), suggest that *R. commune*, *R. agropyri*  
446 and *R. secalis* might all have ‘cryptic’, so far unidentified, sexual cycles (Dyer & O’Gorman  
447 2012); these could potentially allow these *Rhynchosporium* species to disperse widely by air-  
448 borne ascospores and respond rapidly to evolutionary selection factors such as introduction of  
449 resistant cultivars or fungicide treatments (Milgroom 1996; Dyer *et al.* 2000; Gilles *et al.*  
450 2001b; McDonald & Linde 2002). Based on their close genetic relatedness to other sexually  
451 reproducing fungal species, such sexual cycles are predicted to involve the production of  
452 apothecia, from which air-borne ascospores are released (Goodwin 2002). However, UK air-  
453 borne spore trapping work identified only small amounts of *R. commune* DNA (in  
454 comparison to known ascospore-producing crop pathogens), and it has been suggested that  
455 these positive samples were likely to have originated as a result of asexual spores  
456 occasionally becoming airborne as opposed to ascospores (Fountaine *et al.* 2010). Moreover,  
457 mating-type genes have been found to be both present and expressed for a number of

458 apparently asexual fungi for which it has not so far been possible to induce a sexual cycle  
459 (e.g. Wada *et al.* 2012; Bihon *et al.* 2014).

460         The question therefore arises as to why any such sexual cycles, if they exist, have not  
461 yet been identified for these three species. The data presented suggest that a lack of isolates  
462 of compatible mating types in natural populations are unlikely to be the reason, as had been  
463 suggested for some other fungal species (Dyer & Paoletti 2005; Rhaiem *et al.* 2008).  
464 Alternatively, it is possible that field isolates may require very specific environmental  
465 conditions to induce sexuality that may occur infrequently in the wild, as has been suggested  
466 for the closely related *O. aciformis* (Dyer *et al.* 2001). It should be noted that an exclusively  
467 asexual life-cycle might contribute to the considerable success of *Rhynchosporium* as a plant  
468 pathogen; it allows the production of large numbers of conidia for dispersal in a shorter time  
469 than that required for ascospore production, there are lower metabolic costs associated with  
470 asexual than sexual sporulation, asexual reproduction can normally occur over a wider range  
471 of environmental conditions, and sexual recombination might break up favourable sets of  
472 genes (Dyer & O’Gorman 2012; Lehtonen *et al.* 2012). However, given that gametic  
473 equilibrium has been found in most *R. commune* populations throughout the world, it has  
474 been previously suggested that it should be considered a sexual pathogen although such  
475 sexual reproduction may occur infrequently in some populations (Linde *et al.* 2003). Indeed,  
476 a mixed reproductive system could provide many of the benefits of sexual reproduction, with  
477 rare sexual recombination producing new combinations of alleles that are than rapidly and  
478 widely dispersed by prolific asexual reproduction.

479         New mating-type diagnostic tests developed in the present study showed that the more  
480 distantly related *R. orthosporum* and *R. lolii* were exclusively of the *MAT1-1* genotype.  
481 Although only 27 such isolates were tested, they were collected from a diverse range of hosts  
482 and geographical locations throughout Europe. Therefore it is very unlikely, although not

483 conclusive, that no *MATI-2* isolates would have been detected if *MATI-1:MATI-2* isolates of  
484 these two species were present in a 1:1 distribution. This finding was confirmed by BLASTN  
485 analyses of draft genome sequences of these species, which failed to detect the presence of  
486 any *MATI-2-1* gene homologue. Such an absence of one mating type in the natural  
487 environment has been reported previously for certain other asexual plant pathogens (e.g.  
488 Christiansen *et al.* 1998; Groenewald *et al.* 2006). Moreover, preliminary work reported here  
489 could not confirm expression (in terms of processing of an intron) of the *MATI-1-1* gene for  
490 the isolates of *R. orthosporum* and *R. lolii* examined. However, it is possible that more  
491 extensive testing (with different isolates and assay conditions) might subsequently confirm  
492 such expression. Given the limited expression of the confirmed *MATI-1-1* gene described  
493 previously in the present study for three other *Rhynchosporium* species, it is possible that this  
494 gene may only have been weakly expressed for *R. orthosporum* and *R. lolii* but that this was  
495 not detected in PCR testing. Further investigations into the expression of the *MATI-1-1* gene  
496 for all five *Rhynchosporium* species are required.

497         The combined *MAT* distribution and expression data generally suggest that *R.*  
498 *orthosporum* and *R. lolii* isolates might have an exclusively asexual life-style, if only due to  
499 the absence of a compatible mating partner. However, more robust population genetic  
500 analyses (e.g. neutral SNP or SSR data from well-defined field populations) are required to  
501 definitively address the possibility of cryptic sexual cycles for these two species.  
502 Nevertheless, the discovery of such a highly skewed mating-type distribution is consistent  
503 with the notion that asexual populations generally show strong deviations away from a 1:1  
504 *MATI-1:MATI-2* ratio (Yun *et al.* 2000). However, some other fungal species with known  
505 sexual states can show highly biased *MAT* distributions in natural populations (e.g. Consolo  
506 *et al.* 2005; Rhaïem *et al.* 2008; Heitman *et al.* 2014); thus, the apparent absence of one

507 mating type from the populations of *R. orthosporum* and *R. lolii* sampled does not preclude  
508 sexual reproduction.

509 Phylogenetic analyses of partial *MAT1-1* idiomorph sequence data identified two  
510 genetically distinct *Rhynchosporium* clusters, namely *R. commune*, *R. agropyri* and *R. secalis*  
511 as distinct from *R. orthosporum* and *R. lolii*. This subdivision is consistent with the  
512 substantial morphological and phylogenetic divide detected by King *et al.* (2013). Only two  
513 of the species (*R. orthosporum* versus *R. lolii*) were visibly resolved using the *MAT1-1*  
514 idiomorph sequence data, and it is cautioned that the genetic distance between them was very  
515 low. Therefore, this particular region of the *MAT1-1* idiomorph is only of limited use for  
516 resolving individual *Rhynchosporium* species (Zaffarano *et al.* 2008; Zaffarano *et al.* 2011;  
517 King *et al.* 2013), although it is noted that other regions of the *MAT* idiomorphs might be  
518 better suited for such a purpose.

519 Data presented here on both the patterns of distribution and expression of *MAT* genes  
520 generally suggest that sexual cycles are more likely to exist for some *Rhynchosporium*  
521 species (*R. commune*, *R. agropyri* and *R. secalis*) than others (*R. orthosporum* and *R. lolii*).  
522 These findings should therefore be of practical interest to both farmers and forage grass  
523 breeders. Moreover, the presence of apparently only one mating type for *R. orthosporum* and  
524 *R. lolii* suggests that at least some species in the genus may be undergoing a shift towards  
525 asexuality, and that a sexual cycle may not be required to permit the continued success of the  
526 pathogens. This hypothesis is consistent with extensive efforts over many years that have to  
527 date failed to demonstrate the existence of a sexual stage for any *Rhynchosporium* species.

528

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530

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543

544

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654 **Figure legends**

655

656 **Figure 1. Diagram showing location of primers designed in the present study used for**  
657 **mating-type determination, assessment of *MAT* gene expression, and phylogenetic**  
658 **analysis of *Rhynchosporium* species.** Primer locations are shown by arrowheads within  
659 either the *MAT1-1* or *MAT1-2* idiomorph or flanking regions (diagram to approximate scale,  
660 some annotated parts extended for clarity). Dark grey boxes indicated conserved flanking  
661 sequence common to *MAT1-1* and *MAT1-2* isolates; light grey arrows indicate *MAT1-1-1* or  
662 *MAT1-2-1* genes (pointing in direction of transcription, gene names labelled above); diagonal  
663 marked regions indicate putative introns; thick black lines indicate *MAT1-1* or *MAT1-2*  
664 specific idiomorph sequence. Note that a portion of the *MAT1-1-1* gene extends into the  
665 flanking region common to both *MAT1-1* and *MAT1-2* isolates, but the coding region in  
666 *MAT1-2* isolates is apparently non-functional and therefore designated as disabled sequence  
667 [*dMAT1-1-1*, in accordance with Rydholm *et al.* (2007)]. Primers for *MAT* gene expression  
668 assessment of *R. commune*, *R. agropyri* and *R. secalis* are shown, but those for *R.*  
669 *orthosporum* and *R. lolii* are omitted for clarity (but were also located either side of the intron  
670 in the *MAT1-1-1* gene). Note that, for clarity, the names of primers MAT1-F central and  
671 MAT1-R central have been shortened (MAT1-F, MAT1-R, respectively) in the diagram.

672

673 **Figure 2. Isolates of *R. commune*, *R. agropyri* and *R. secalis* are of either *MAT1-1* or**  
674 ***MAT1-2* genotype.** Isolates that produced 590-bp or 360-bp amplicons using the multiplex  
675 mating type diagnostic of Linde *et al.* (2003) were considered of *MAT1-1* or *MAT1-2*  
676 genotype, respectively. Representative isolates shown are *R. commune* (lanes 2–5; isolates  
677 62hv09, RS00CH H36, 53hv09, 73hv09), *R. agropyri* (lanes 6–9; 1ar10, 6ar10, 2ar10, 7ar10)  
678 and *R. secalis* (lanes 10–13; 4.11.1, 1D4a, 6.2, 1E7a). Further information about these

679 isolates is given in Table 1. Lane 1 is a 100-bp ladder (Fermentas, UK); the no template  
680 (water) control was blank (data not shown).

681

682 **Figure 3. Development of new PCR diagnostic tests to identify *MAT1-1* or *MAT1-2***

683 **isolates of *R. commune*, *R. agropyri* or *R. secalis*.** Isolates that produced (a) a 598-bp

684 amplicon using primer pair A-MAT1-F/R were considered to be of *MAT1-1* genotype, and

685 (b) isolates that produced a 149-bp amplicon using primer pair C-MAT2-F/R were considered

686 to be of *MAT1-2* genotype. Isolates shown are *R. commune* (lanes 2–5; 19hv09, UK7,

687 53hv09, 2lm11), *R. agropyri* (lanes 6–9; 10ar10, 6ar10, 3ar10, RS04CG-RAC-A.6.1) and *R.*

688 *secalis* (lanes 10-13; RS02CH4-4b1, RS02CH4-14a1, 6.2, 1E7a). Further information about

689 these isolates is given in Table 1. Isolates had been previously identified as *MAT1-1* (2–3, 6–

690 7, 10–11) or *MAT1-2* (4–5, 8–9, 12–13) genotype using the multiplex mating-type diagnostic

691 of Linde *et al.* (2003). Lane 1 is a 100-bp ladder (Fermentas, UK); the no template (water)

692 control was blank (data not shown).

693

694 **Figure 4. Identification of only *MAT1-1* isolates of *R. orthosporum* and *R. lolii*.** Isolates

695 that produced (a) a 598-bp amplicon using primer pair A-MAT1-F/R were considered to be

696 of *MAT1-1* genotype, and (b) isolates that produced a 149-bp amplicon using primer pair C-

697 MAT2-F/R were considered to be of *MAT1-2* genotype. Representative isolates shown are: *R.*

698 *agropyri* (lanes 2–3; isolates 1ar10, 2ar10), *R. lolii* (lanes 4–11; 12lp11, 13lp11, 14lp11,

699 17lp11, 18lp11, 21lm11, 22lm11, 15lp11) and *R. orthosporum* (lanes 12–15; 27dg09,

700 RS04CG-BAR-A.1.1.4, RS04ITA D-6.2, RS04ITA D-4.1). Further information about these

701 isolates is given in Table 1. Isolates of *R. agropyri* in lanes 2–3 were positive controls, and

702 had been previously identified as *MAT1-1* and *MAT1-2*, respectively, using the multiplex

703 mating-type diagnostic of Linde *et al.* (2003). Lanes 1 and 16 are a 100-bp ladder (Fermentas,  
704 UK) and a no template (water) control, respectively.

705

706 **Figure 5. Expression of *MATI-1-1* and *MATI-2-1* genes for different isolates of *R.***

707 ***agropyri* and *R. secalis*.** Data are shown for isolates of known *MATI-1* or *MATI-2* genotype

708 of (a) *R. agropyri* (*MATI-1*: 1ar10, lanes 1–6; *MATI-2*: 3ar10, lanes 7–12) and (b) *R. secalis*

709 (*MATI-1*: B8, lanes 1–6; *MATI-2*: I-Ia, lanes 7–12). Information under bars below gel

710 indicate the gene loci amplified and the sizes of the predicted PCR products. Inverted arrows

711 on gel image point to the predicted sized amplicons in each lane. Genomic DNA template

712 controls (lanes 1, 3, 5, 7, 9, 11) were run alongside RNA template (lanes 2, 4, 6, 8, 10, 12), to

713 confirm RNA processing through removal of putative introns that internally spanned the

714 amplified sequence. Note that primers targeted to *MATI-1-1* gene sequence amplified both

715 processed RNA and contaminant DNA, based on the sizes of amplicons (lanes 2). Ladders

716 (L) are 100-bp ladders (New England Biolabs). No template (water) controls were blank (data

717 not shown).

718

719 **Figure 6. Phylogeny of partial sequences of the *MATI-1* idiomorph of isolates of five**

720 ***Rhynchosporium* species.** The consensus neighbour joining tree displayed was constructed

721 using *MATI-1* idiomorph sequence data from the closely related *Pyrenopeziza brassicae*

722 (GenBank accession: AJ006073) as an outgroup. Species identity of isolates is provided in

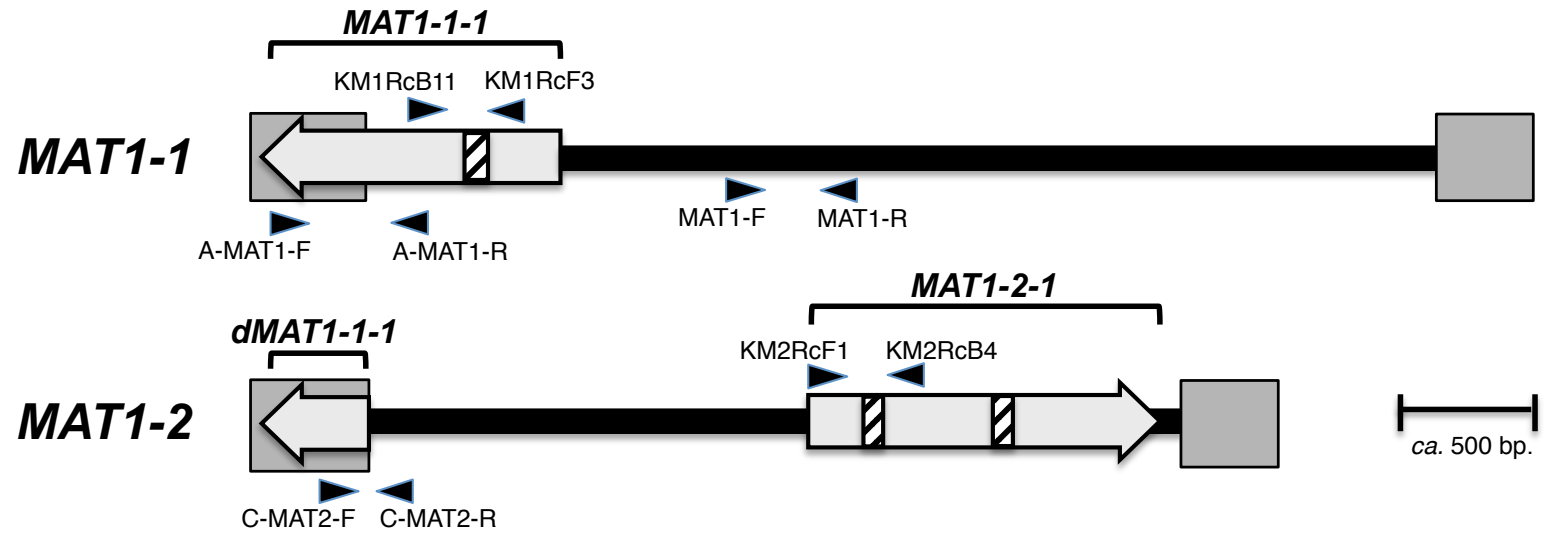
723 right-hand parentheses, with further information available in Table 1. Numbers at nodes

724 indicate the bootstrap support (%) based on 100,000 replications of the tree; only consensus

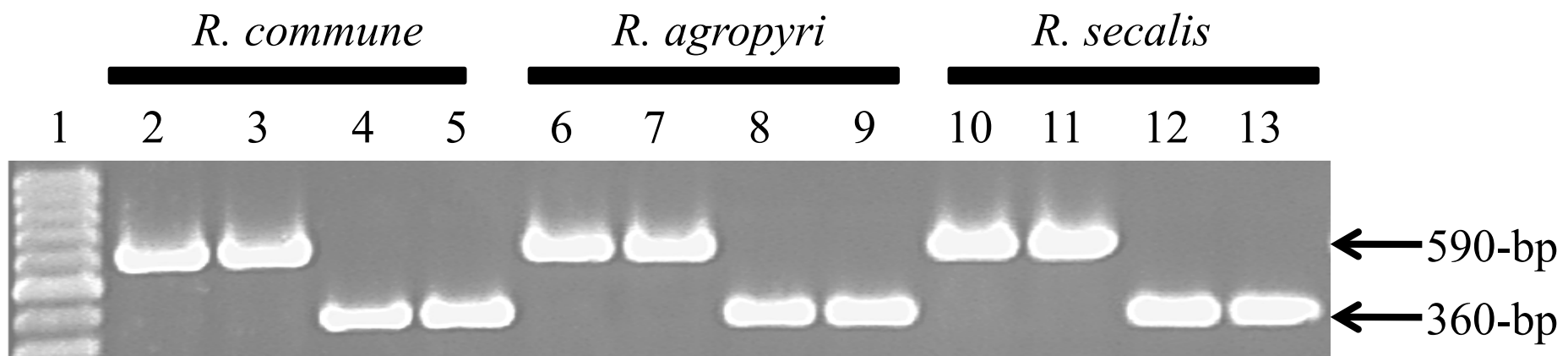
725 support values of  $\geq 80\%$  are shown, for clarity. Scale bar represents the number of nucleotide

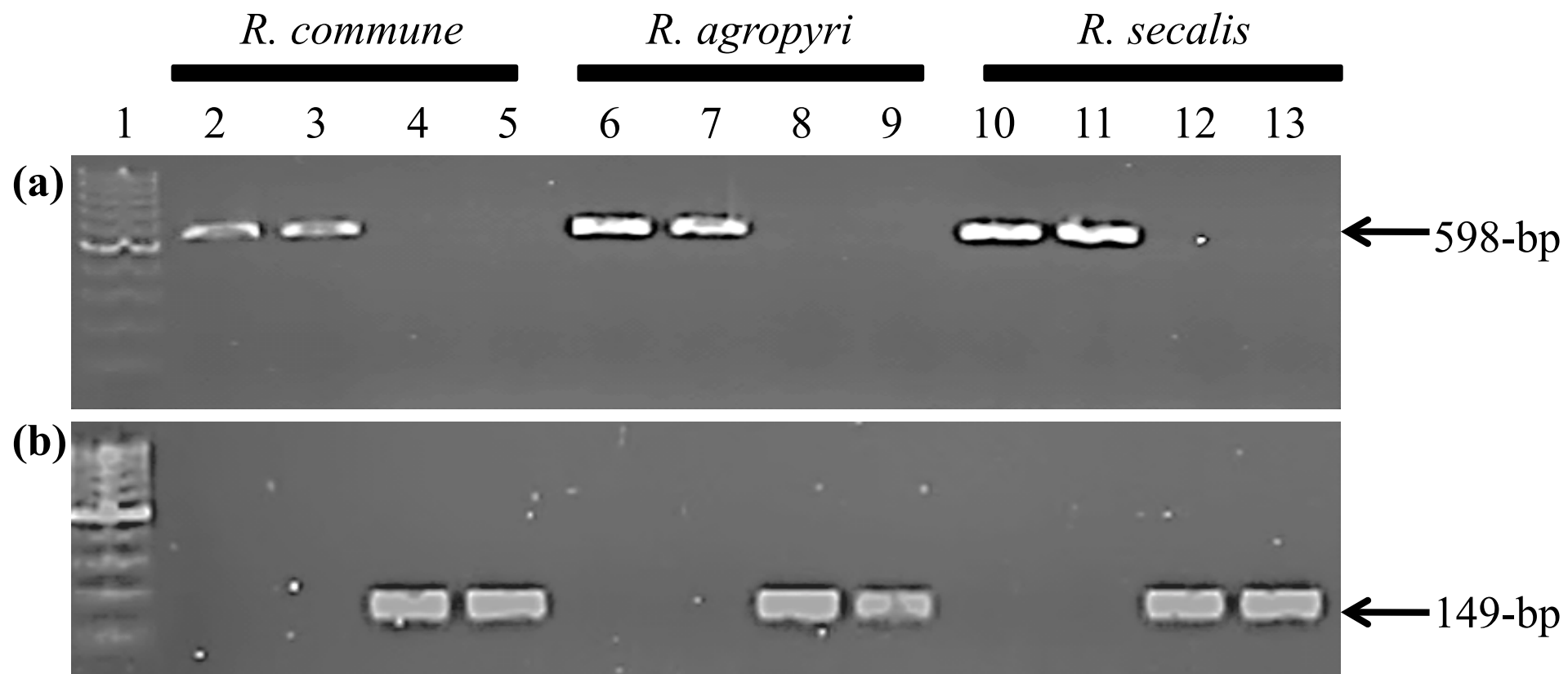
726 substitutions per site.

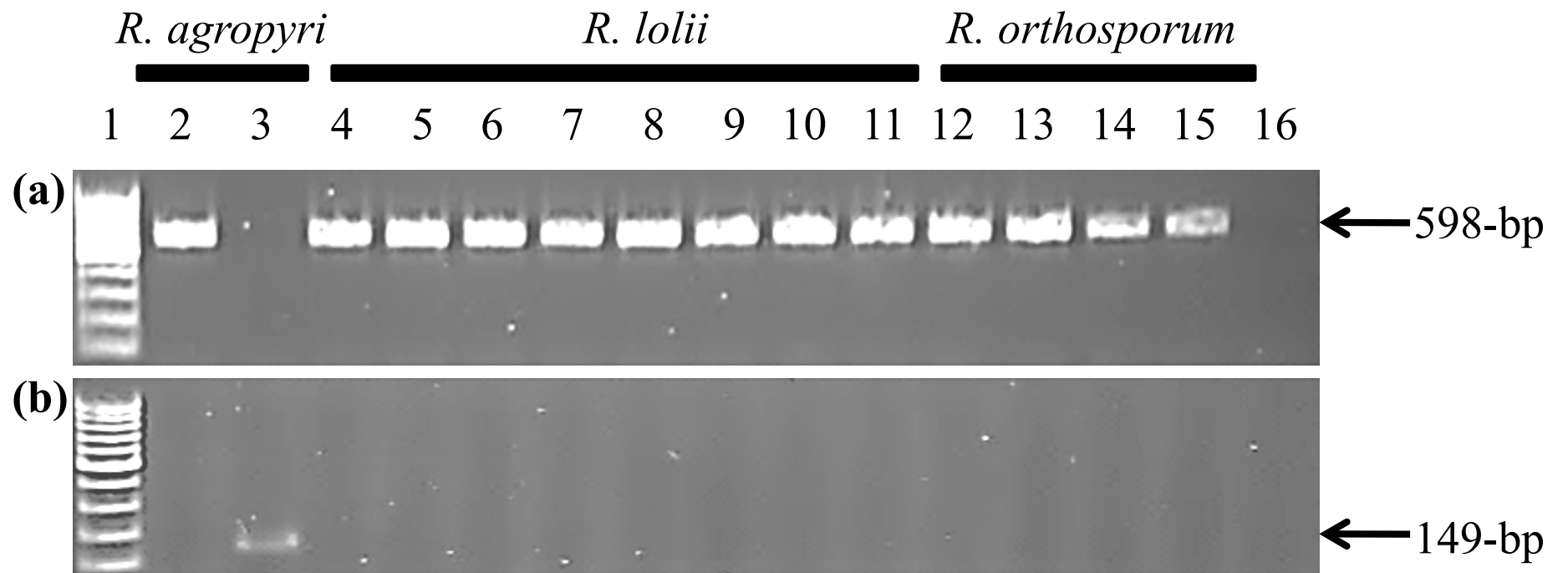
727

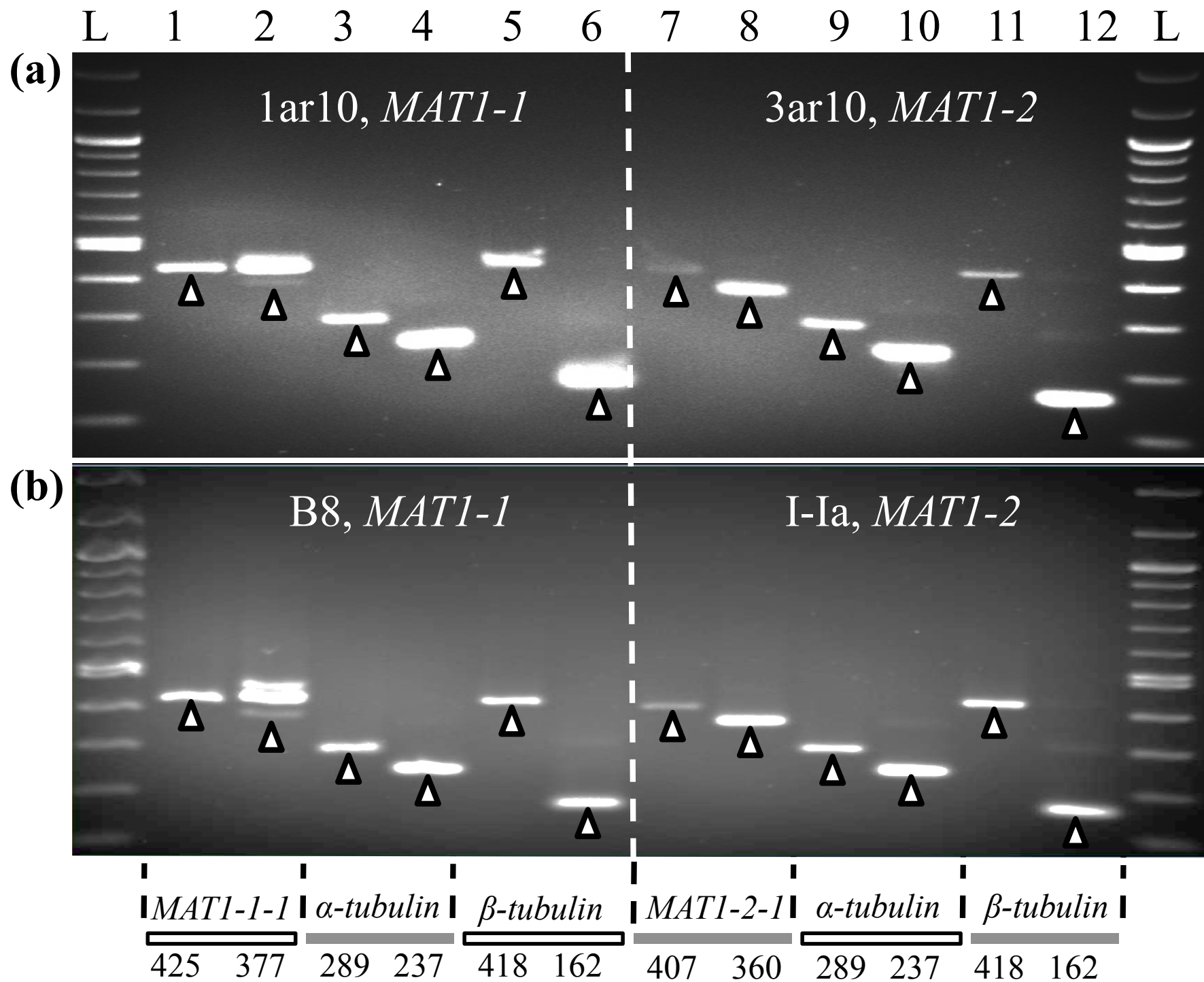


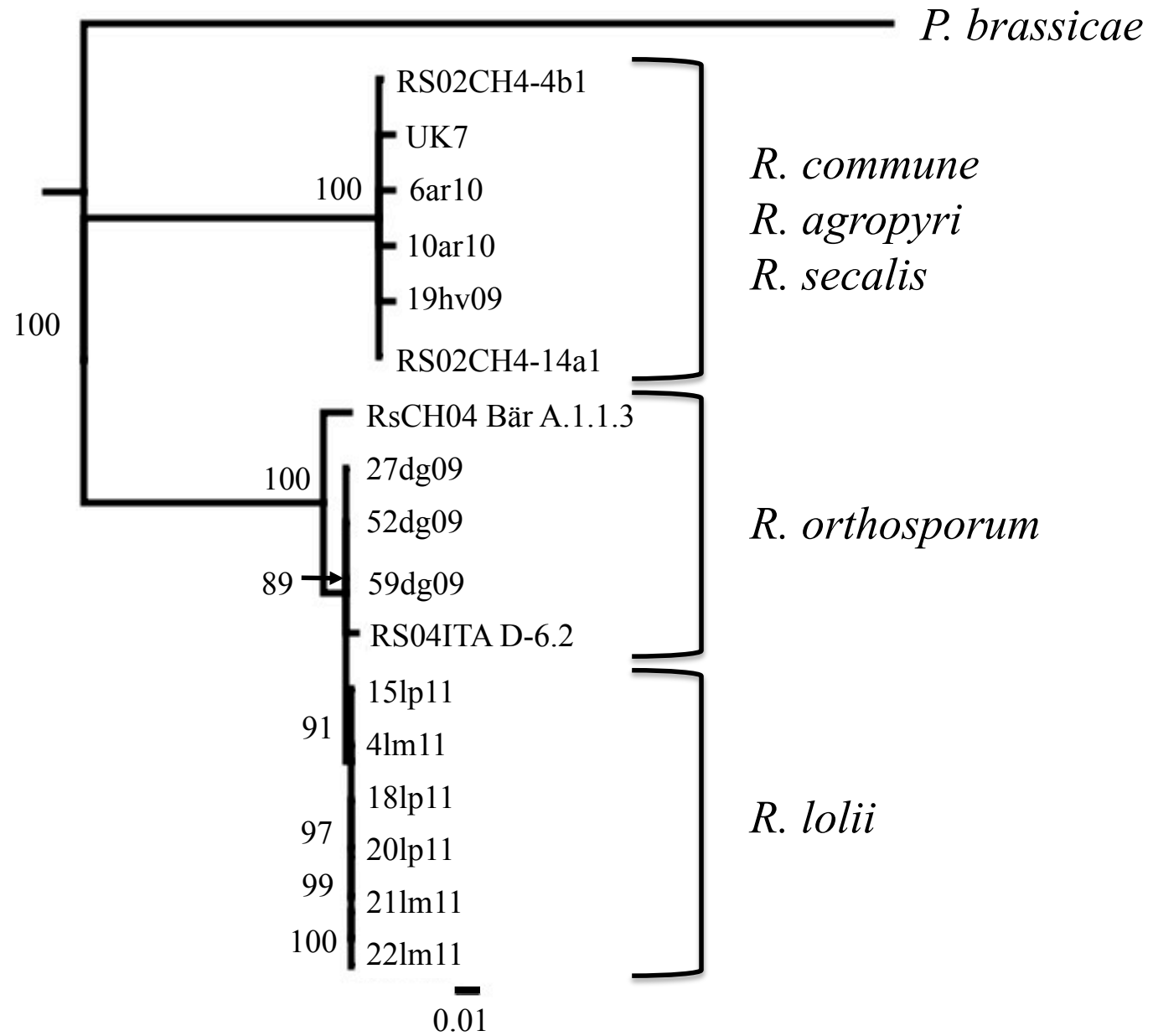












1 Table 1. Determination of mating types of 72 isolates of *Rhynchosporium commune*, *R. agropyri*, *R. secalis*, *R. orthosporum* or *R. lolii*.

Isolate code	Host	Origin	Species <sup>a</sup>	Year	<u>Mating type</u>	
					(Linde <i>et al.</i> 2003) <sup>b</sup>	Present Study <sup>c</sup>
788	Barley	France	Rc	1997	<i>MAT1-1</i>	
QUB 30.10	Barley	Northern Ireland, UK	Rc	Unknown	<i>MAT1-1</i>	
QUB 30.13	Barley	Northern Ireland, UK	Rc	Unknown	<i>MAT1-1</i>	
OSA 28-2-2	Barley	Hertfordshire, UK	Rc	2002	<i>MAT1-1</i>	
FI12-63	Barley	Finland	Rc	1996	<i>MAT1-2</i>	
R.s. 2310 4.2	Barley	France	Rc	2008	<i>MAT1-1</i>	
R.s. 2318 4.2	Barley	France	Rc	2008	<i>MAT1-1</i>	
RS00CH H36	Barley	Switzerland	Rc	2000	<i>MAT1-1</i>	
19hv09	Barley	Hertfordshire, UK	Rc	2009	<i>MAT1-1</i>	<i>MAT1-1</i>
53hv09	Barley	Hertfordshire, UK	Rc	2009	<i>MAT1-2</i>	<i>MAT1-2</i>
62hv09	Barley	Hertfordshire, UK	Rc	2009	<i>MAT1-1</i>	

73hv09	Barley	Hertfordshire, UK	Rc	2009	<i>MATI-2</i>	
UK7	Barley	Aberystwyth, UK	Rc	Unknown	<i>MATI-1</i>	<i>MATI-1</i>
D.1.1	Wall barley	Switzerland	Rc	2004	<i>MATI-1</i>	
E.1.2	Wall barley	Switzerland	Rc	2004	<i>MATI-1</i>	
2lm11	Italian ryegrass	Shropshire, UK	Rc	2011	<i>MATI-2</i>	<i>MATI-2</i>
5lm11	Italian ryegrass	Shropshire, UK	Rc	2011	<i>MATI-2</i>	
RS04CG-RAC-A.4.3	Couch-grass	Switzerland	Ra	2004	<i>MATI-2</i>	
RS04CG-RAC-A.5.2	Couch-grass	Switzerland	Ra	2004	<i>MATI-2</i>	
RS04CG-RAC-A.6.1	Couch-grass	Switzerland	Ra	2004	<i>MATI-2</i>	<i>MATI-2</i>
1ar10	Couch-grass	Surrey, UK	Ra	2010	<i>MATI-1</i>	
2ar10	Couch-grass	Surrey, UK	Ra	2010	<i>MATI-2</i>	
3ar10	Couch-grass	Surrey, UK	Ra	2010	<i>MATI-2</i>	<i>MATI-2</i>
6ar10	Couch-grass	Cluj-Napoca, Romania	Ra	2010	<i>MATI-1</i>	<i>MATI-1</i>
7ar10	Couch-grass	Timisoara, Romania	Ra	2010	<i>MATI-2</i>	
8ar10	Couch-grass	Nottinghamshire, UK	Ra	2010	<i>MATI-1</i>	
9ar10	Couch-grass	Nottinghamshire, UK	Ra	2010	<i>MATI-1</i>	

10ar10	Couch-grass	Nottinghamshire, UK	Ra	2010	<i>MAT1-1</i>	<i>MAT1-1</i>
11ar10	Couch-grass	Nottinghamshire, UK	Ra	2010	<i>MAT1-1</i>	
RS02CH4-2a1	Rye	Switzerland	Rs	2002	<i>MAT1-1</i>	
RS02CH4-4b1	Rye	Switzerland	Rs	2002	<i>MAT1-1</i>	<i>MAT1-1</i>
RS02CH4-5a1	Rye	Switzerland	Rs	2002	<i>MAT1-1</i>	
Rs02CH4-6a.1	Rye	Switzerland	Rs	2002	<i>MAT1-1</i>	
RS99CH1-H10B	Rye	Switzerland	Rs	1999	<i>MAT1-2</i>	
RS02CH4-13a1	Rye	Switzerland	Rs	2002	<i>MAT1-1</i>	
RS02CH4-14a1	Rye	Switzerland	Rs	2002	<i>MAT1-1</i>	<i>MAT1-1</i>
8.4	Rye	Russia	Rs	2003	<i>MAT1-2</i>	
6.2	Rye	Russia	Rs	2003	<i>MAT1-2</i>	<i>MAT1-2</i>
4.11.1	Rye	Russia	Rs	2003	<i>MAT1-1</i>	
1E7a	Rye	Switzerland	Rs	1999	<i>MAT1-2</i>	<i>MAT1-2</i>
1B8	Rye	Switzerland	Rs	1999	<i>MAT1-1</i>	
1D4a	Rye	Switzerland	Rs	1999	<i>MAT1-1</i>	
I-Ia	Triticale	Switzerland	Rs	2002	<i>MAT1-2</i>	



I-2a2	Triticale	Switzerland	Rs	2002	<i>MAT1-2</i>
I-3a1	Triticale	Switzerland	Rs	2002	<i>MAT1-2</i>
27dg09	Cocksfoot	Aberystwyth, UK	Ro	2009	n/d <sup>d</sup> <i>MAT1-1</i>
57dg09	Cocksfoot	Aberystwyth, UK	Ro	2009	<i>MAT1-1</i>
59dg09	Cocksfoot	Aberystwyth, UK	Ro	2009	<i>MAT1-1</i>
RS04CG-BAR-A.1.1.3	Cocksfoot	Switzerland	Ro	2004	n/d <i>MAT1-1</i>
RS04CG-BAR-A.1.1.4	Cocksfoot	Switzerland	Ro	2004	<i>MAT1-1</i>
RS04ITA D-4.1	Cocksfoot	Italy	Ro	2004	<i>MAT1-1</i>
RS04ITA D-6.1	Cocksfoot	Italy	Ro	2004	<i>MAT1-1</i>
RS04ITA D-6.2	Cocksfoot	Italy	Ro	2004	<i>MAT1-1</i>
1lm11	Italian ryegrass	Shropshire, UK	Rl	2011	<i>MAT1-1</i>
3lm11	Italian ryegrass	Shropshire, UK	Rl	2011	<i>MAT1-1</i>
4lm11	Italian ryegrass	Shropshire, UK	Rl	2011	<i>MAT1-1</i>
6lm11	Italian ryegrass	Aberystwyth, UK	Rl	2011	<i>MAT1-1</i>
7lm11	Italian ryegrass	Aberystwyth, UK	Rl	2011	<i>MAT1-1</i>
8lm11	Italian ryegrass	Aberystwyth, UK	Rl	2011	<i>MAT1-1</i>

2	9lm11	Italian ryegrass	Aberystwyth, UK	R1	2011	<i>MAT1-1</i>
3	10lm11	Italian ryegrass	Aberystwyth, UK	R1	2011	<i>MAT1-1</i>
4	21lm11	Italian ryegrass	Shropshire, UK	R1	2011	<i>MAT1-1</i>
5	22lm11	Italian ryegrass	Shropshire, UK	R1	2011	<i>MAT1-1</i>
6	11lp11	Perennial ryegrass	Aberystwyth, UK	R1	2011	<i>MAT1-1</i>
7	12lp11	Perennial ryegrass	Aberystwyth, UK	R1	2011	<i>MAT1-1</i>
8	13lp11	Perennial ryegrass	Aberystwyth, UK	R1	2011	<i>MAT1-1</i>
9	14lp11	Perennial ryegrass	Aberystwyth, UK	R1	2011	<i>MAT1-1</i>
10	15lp11	Perennial ryegrass	Shropshire, UK	R1	2011	<i>MAT1-1</i>
11	16lp11	Perennial ryegrass	Surrey, UK	R1	2011	<i>MAT1-1</i>
12	17lp11	Perennial ryegrass	Hertfordshire, UK	R1	2011	<i>MAT1-1</i>
13	18lp11	Perennial ryegrass	Hertfordshire, UK	R1	2011	<i>MAT1-1</i>
14	20lp11	Perennial ryegrass	Hertfordshire, UK	R1	2011	<i>MAT1-1</i>

15

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17

18 <sup>a</sup>. *Rhynchosporium* species identity had been confirmed previously by King *et al.* (2013) using either species-specific PCR primers or repetitive  
19 extragenic palindromic PCR. Species identities are as follows: Rc: *R. commune*; Ra: *R. agropyri*; Rs: *R. secalis*; Ro: *R. orthosporum*; Rl: *R. lolii*.  
20 <sup>b</sup>. Mating type determined using the multiplex PCR diagnostic of Linde *et al.* (2003); production of a 590-bp or 360-bp amplicon indicated  
21 *MAT1-1* or *MAT1-2* type isolates, respectively.  
22 <sup>c</sup>. Mating type determined using diagnostics developed in the present study; production of a 598-bp amplicon using primer pair A-MAT1-F/R or  
23 a 149-bp amplicon using primer pair C-MAT2-F/R indicated *MAT1-1* or *MAT1-2* type isolates, respectively.  
24 <sup>d</sup>. ‘n/d’: could not be determined.  
25