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

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

Abstract

Leaf blotch is a globally important disease of barley crops and other grasses that is caused by at least five host-specialised species in the fungal genus *Rhynchosporium.* The pathogen *R. commune* (specialised to barley, brome-grass and Italian ryegrass) has long been considered to reproduce only by asexual means, but there has been accumulating evidence for recombination and gene flow from population genetic studies and the presence in the field of

complementary *MAT1-1* and *MAT1-2* isolates in an approximately 1:1 distribution. Here, we demonstrate that 28 isolates of the closely related species *R. agropyri* (on couch-grass) and *R. secalis* (on rye/triticale) collected from Europe were also either of *MAT1-1* or *MAT1-2* genotype and that the distribution of mating types did not deviate significantly from a 1:1 ratio. Evidence is then provided for *MAT1-1-1* and *MAT1-2-1* gene expression during mycelial growth for all three species. By contrast, 27 isolates of the more distantly related *R. orthosporum* (on cocksfoot) and *R. lolii* (on Italian and perennial ryegrasses) from Europe were exclusively of the *MAT1-1* genotype, and expression of the *MAT1-1-1* gene could not be detected during mycelial growth. These data suggest that cryptic sexual cycles are more likely to exist for *R. commune*, *R. agropyri* and *R. secalis* than for either *R. orthosporum* or *R. lolii*. A phylogenetic analysis of partial *MAT1-1* idiomorph sequence resolved these five species into two distinct groups (*R. commune*, *R. agropyri* and *R. secalis* versus *R. orthosporum* and *R. lolii*) but provided only limited resolution within each group.

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

Introduction

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

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

At present, all of these *Rhynchosporium* species are known to reproduce only by asexual means, with dispersal thought to be achieved by splash dispersal of conidia as observed for *R. commune* (Fitt *et al*. 1986) (NB. for the purposes of this study, previously published work that refers to isolates of '*R. secalis*' collected from barley will be considered to refer to *R. commune*). The mode of reproduction is a critical factor in understanding the population biology of plant pathogenic fungi because this impacts on the possibility for recombination and evolution of the pathogen (Milgroom 1996; McDonald & Linde 2002). In particular, a sexual cycle for *R. commune* has not yet been identified under either natural or laboratory conditions (Avrova & Knogge 2012). However, *R. commune* isolates have been shown to have a mating-type (*MAT*) locus resembling that of species with a heterothallic (obligate out-crossing) sexual mating system (Linde *et al*. 2003; Foster & Fitt 2003), with isolates of complementary mating type (referred to as *MAT1-1* and *MAT1-2*) present, which could enable sexual reproduction to occur. The *R. commune MAT* locus is flanked on both sides by nearly identical stretches of DNA in isolates of either mating type, but internally

contains highly divergent stretches of DNA termed 'idiomorphs' that differ between the mating types (Linde *et al*. 2003; Foster & Fitt 2003). The *MAT1-1* idiomorph contains a *MAT1-1-1* gene encoding a characteristic alpha-domain protein while the *MAT1-2* idiomorph contains a *MAT1-2-1* gene encoding a characteristic high mobility group (HMG)-domain protein (Debuchy *et al.* 2010).

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

Other evidence for sexuality for *R. commune* includes the detection of considerable genotypic diversity in populations, which is consistent with sexual recombination, and the production of microconidia that have been suggested to be a component of a so far undiscovered 'cryptic' sexual cycle (Salamati *et al*. 2000; Skoropad & Grinchenko 1957). In addition, sequencing of the internal transcribed spacer region (Goodwin 2002) revealed that *R. commune* is closely related to the discomycete species *Oculimacula yallundae* (synonym *Tapesia yallundae*; causal agent of eyespot disease of wheat) and *Pyrenopeziza brassicae* (light leaf spot of oilseed rape). Both of these pathogens, and the closely related *Oculimacula acuformis* (synonym *Tapesia acuformis*), have a heterothallic mating system, with known sexual cycles leading to the production of apothecia and air-borne ascospores on their

respective hosts (Dyer *et al.* 1996; Dyer *et al*. 2001; Gilles *et al*. 2001a). Based on the biology of these closely related crop pathogens, it is likely that if, a sexual cycle does exist for *R. commune*, it will involve the production of relatively small apothecia (<500 µm in diameter) that require rainfall for maturation on senescing host tissue (Dyer *et al*. 2001; Goodwin 2002; Gilles *et al*. 2001a; Welham *et al*. 2004).

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

Methods

Fungal isolate collection and DNA extraction

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

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

The *MAT* multiplex PCR diagnostic of Linde *et al*. (2003) was tested against 47 *Rhynchosporium* isolates (Table 1). This diagnostic amplifies DNA from regions of either the *MAT1-1-1* or *MAT1-2-1* genes, specific to *MAT1-1* or *MAT1-2* isolates, respectively. However, reaction components were modified to produce more distinct PCR amplicons. Each 25 µl reaction contained 12.5 µl of Jumpstart RedTaq ReadyMix (2 x concentrate, Sigma Aldrich, UK), 1 µl each of the four primers (0.4 µM concentration of each primer), 6.5 µl of sterile distilled water and 2 µl of template genomic DNA (20 ng total DNA). Unless otherwise specified, PCR throughout the present study used a PTC-100 Programmable Thermal Controller (MJ Research, USA), with PCR products (10 µl) routinely resolved by gel electrophoresis on 1% Tris-Borate-EDTA (TBE) agarose gels incorporated with ethidium bromide (0.5 µg / ml) and viewed under ultraviolet light. Occasionally, for high image quality, gels were instead stained after electrophoresis (2 µg / ml ethidium bromide in 1 x TBE solution). Isolates that produced either a 590-bp or a 360-bp amplicon were considered to be of *MAT1-1* or *MAT1-2* genotype, respectively (Linde *et al*. 2003). The hypothesis of a 1:1 ratio of mating types for the individual species *R. commune*, *R. agropyri* and *R. secalis* dataset was tested using a chi-squared (χ^2) test (GraphPad Software).

Sequence identity of these putative *MAT* amplicons was confirmed by PCR on seven isolates that had previously produced either putative *MAT1-1* (590-bp) or *MAT1-2* (360-bp) amplicons using the multiplex diagnostic of Linde *et al*. (2003). These isolates were: *R. commune* (*MAT1-1*: UK7; *MAT1-2*: 53hv09, 2lm11), *R. agropyri* (*MAT1-1*: 3ar10; *MAT1-2*: 10ar10) and *R. secalis* (*MAT1-1*: 1D4a; *MAT1-2*: I-Ia) (Table 1). Jumpstart high fidelity mix (Roche, Germany) was used with reaction components selected according to the manufacturer's instructions with the following modifications; DMSO was omitted and each reaction included 10 mM of each dNTP (Fermentas, UK) and 2.5 ng of template DNA. PCR

products were purified using a MinElute kit (Qiagen, UK) and sent to Eurofin MWG Operon for sequencing (590-bp and 360-bp amplicons were sequenced using primers RsMAT1F and RsMAT2F, respectively). Sequence data were edited in BioEdit Sequence Alignment Editor (version 7.0.9.0; Hall 1999), poor read quality data were removed, and final sequence data were analysed using BLASTN 2.2.29+ software (Zhang *et al*. 2000).

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

As the mating-type diagnostic of Linde *et al*. (2003) could not satisfactorily amplify *MAT* amplicons of the predicted size from isolates of *R. orthosporum* (see below), it was necessary to design new PCR-based diagnostic tests. Primers were first designed [throughout this study, all primers were designed using either the Geneious version 5.5.6. (Biomatters Ltd.) or MacVector 12 (MacVector Ltd.) software packages] to amplify a partial region of the *MAT1-1-1* gene based on sequence data obtained from the genome of *R. orthosporum* (RsCH04 Bär A.1.1.3; W. Knogge and *Rhynchosporium* genome consortium, unpublished results). Sequence data for both this isolate and an isolate of *R. commune* (GenBank accession: AY257472) were used to design primers predicted to produce a single amplicon of 598-bp specific for *MAT1-1* isolates of *Rhynchosporium* species; A-MAT1-F (5' AGCCATCCTGCATGCCGCC 3') and A-MAT1-R (5' CCGCGAGCACCACTGGACC 3'). Primer A-MAT1-R annealed to a region of the *MAT1-1-1* gene within the *MAT1-1* idiomorph (Fig. 1).

No sequence data were available for a *MAT1-2* isolate of *R. orthosporum* and primers were therefore designed, based on available *MAT1-2* idiomorph sequence data for an isolate of *R. commune* (GenBank accession: AJ537511), that were predicted to produce a single amplicon of 149-bp for *MAT1-2* isolates of *Rhynchosporium* species; C-MAT2-F (5'

TGGGGCTGAAGCAAGGAGACCA 3') and C-MAT2-R (5' ACACATCCTCCGGCCAAGCA 3'). Primer C-MAT2-R annealed to DNA sequence specific to the *MAT1-2* idiomorph (Fig. 1).

The primer pair A-MAT1-F/R was used for PCR, each 25 µl volume containing 12.5 µl of RedTaq ReadyMix (Sigma Aldrich, UK), 1 µl each of primers A-MAT1-F and A-MAT1-R (0.4 µM concentration), 9.5 µl of sterile distilled water and 1 µl of template fungal DNA (1 ng total DNA). Reaction conditions were as follows; an initial denaturation step of 94°C for 2 min, followed by 32 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 2 min. This was followed by a final extension step of 72°C for 5 min and a final hold at 4°C. Similar reaction conditions were used with primer pair C-MAT2-F/R, except that the annealing temperature was decreased to 66°C. The mating-type diagnostic tests using either primer pair A-MAT1-F/R or C-MAT2-F/R were then applied to 39 *Rhynchosporium* isolates (Table 1). The hypothesis that there was a 1:1 ratio of mating types for the datasets for individual species (*R. orthosporum* and *R. lolii*) was tested using a χ^2 analysis (larger samples of the other species had already been tested).

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

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

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

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

PCR primers were designed to amplify partial regions of the *MAT1-1-1*, *MAT1-2-1*, alpha-tubulin and beta-tubulin gene loci, based on alignments of publicly available GenBank data. Primers were designed to span a putative intron(s) to allow confirmation of RNA processing, based on previously published gene models for *R. commune* (Linde *et al*. 2003; Foster & Fitt 2003). Primer pair KM1RcF3 (5' AAGAAGGCTTTACCTCCCC 3') and KM1RcB11 (5' TGCTCGTGGTTTTCCGACTG 3') were targeted to amplify partial regions of the *MAT1-1-1* gene for isolates of *R. commune*, *R. agropyri* and *R. secalis*, with predicted amplicons of 425- and 377-bp for genomic DNA and processed RNA, respectively (Fig. 1). Primer pair KM2RcF1 (5' TCATCTCAACTCAGCCTGCC 3') and KM2RcB4 (5' TTCTCCAGCGACCTCAATAAAC 3') were targeted to amplify regions of the *MAT1-2-1* gene for these three species, with predicted amplicons of 407- and 360-bp for genomic DNA and processed RNA, respectively (Fig. 1). Due to sequence divergence at the *MAT1-1* locus, additional primers were designed (based on sequence data from the *R. orthosporum* genome; *Rhynchosporium* genome consortium, unpublished results) to amplify partial regions of this locus for isolates of the more distantly related *R. orthosporum* and *R. lolii*; primer pair KM1RoF2 (5' CCCGACGAGTATCTAATGAACC 3') and KM1RoB16 (5' AGAGCCACAGAAAAGCACG 3') were predicted to produce amplicons of 587- and 540-bp for genomic DNA and processed RNA, respectively.

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

GCCAATGTGGTAATCAAATCGG 3') targeted the beta-tubulin loci and were predicted to produce amplicons of 418- and 162-bp for genomic DNA and processed RNA, respectively.

All eight isolates were then tested using these primer sets with both RT-PCR (RNA template) and conventional PCR (genomic DNA template). In RT-PCR testing, a one-Step RT-PCR kit (Qiagen, UK) was used, with reaction components utilised according to the manufacturer's instructions (adding 150 ng total extracted RNA as a template). Reaction conditions for all RT-PCR reactions were as follows: an initial reverse transcription step of 50°C for 30 min, an initial PCR activation step of 95°C for 15 min, 35 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min and a final elongation step of 72°C for 10 min (although for primer pair KBRTcF1/KBTRcF20 an annealing temperature of 55°C was used). PCR products were run on 2% agarose gels and visualised. In parallel conventional PCR testing, RedTaq ReadyMix was used according to the manufacturer's instructions (0.5 µM concentration of each primer and 5-10 ng genomic template DNA), with reaction conditions identical to those described previously for RT-PCR testing except that the reverse transcription and initial PCR activation steps were omitted, and the final elongation step was decreased to 5 min. PCR products were visualised as described previously.

Phylogenetic analyses of partial *MAT1-1* idiomorph sequences

MAT1-1 idiomorph sequence data obtained from an isolate of *R. orthosporum* (RsCH04 Bär A.1.1.3) and *R. commune* (GenBank accession: AY257472) were aligned. Two primers, predicted to produce a single amplicon of 327-bp specific for only *MAT1-1* isolates of all *Rhynchosporium* species, were designed; MAT1-F central (5' CTGCTGTATAGCAACCCA 3') and MAT1-R central (5' GTGATGGGAGAATGTCGC 3'). These two primers annealed to apparently conserved DNA sequence within the *MAT1-1* idiomorph, upstream relative to the *MAT1-1-1* gene (Fig. 1). Preliminary testing of this primer pair against 12 isolates of *R.*

commune, *R. agropyri* or *R. secalis* of known *MAT1-1* or *MAT1-2* identity [as confirmed using the multiplex diagnostic of Linde *et al*. (2003)] confirmed that the expected 327-bp amplicon was produced using template DNA obtained from only *MAT1-1* isolates (data not shown).

DNA from a total of 17 isolates was amplified by PCR using primers MAT1-F/R central, comprising *R. commune* (19hv09, UK7), *R. agropyri* (6ar10, 10ar10), *R. secalis* (RS02CH4-4b1, RS02CH4-14a1), *R. orthosporum* (27dg09, 52dg09, 59dg09, RS04ITA D-4.1, RsCH04 Bär A.1.1.3) and *R. lolii* (4lm11, 15lp11, 18lp11, 20lp11, 21lm11, 22lm11) (Table 1). Jumpstart high fidelity mix was used in all reactions, with reaction components previously described. Reaction conditions were as follows; an initial denaturation step of 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 min. This was followed by a final extension step of 72°C for 5 min and a final hold at 4°C. PCR products were visualised on an agarose gel to confirm the presence of single 327-bp amplicons, and purified and sequenced (using primer MAT1-F central) as described above.

Sequences were imported into the Geneious version 7.0.6. software package (Biomatters Ltd.) and aligned using the MUSCLE algorithm (maximum number of iterations: 8). Sequences were manually edited to ensure an equal length of 235-bp including gaps. A neighbour joining phylogenetic tree was constructed using the Geneious Tree builder software, to which the Jukes-Cantor distance model was applied. The consensus tree was based on the bootstrap resampling method (100,000 replications), with the support threshold set at 80%. *MAT1-1* idiomorph sequence data from the closely related *P. brassicae* (GenBank accession: AJ006073) was used as an outgroup in the computation. The sequence alignment was deposited into TreeBASE (S15396).

TreeBASE Access link for reviewers: http://goo.gl/Gj3gZj

Results

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

The multiplex PCR diagnostic developed for *R. commune* by Linde *et al*. (2003) was successfully applied to 45 isolates of *R. commune*, *R. agropyri* or *R. secalis* (Table 1). Both mating types could readily be identified for all three species by the production of two differently sized PCR products; *MAT1-1* isolates produced an amplicon of 590-bp, while *MAT1-2* isolates produced an amplicon of 360-bp (Fig. 2). However, the test was not applicable to the two isolates of *R. orthosporum* assayed, which produced multiple non-specific PCR products (data not shown). For all three of the former species, both *MAT1-1* and *MAT1-2* isolates were identified from locations throughout Europe. The distribution of mating types for each individual species, namely *R. commune* (*MAT1-1* = 12, *MAT1-2* = 5; χ^2 = 2.882, 1 d.f., *P* = 0.0896), *R. agropyri* (*MAT1-1* = 6, *MAT1-2* = 6; χ^2 = 0, 1 d.f., *P* = 1.0000), and *R. secalis* (*MAT1-1* = 9, *MAT1-2* = 7; χ^2 = 0.250, 1 d.f., *P* = 0.6171) did not deviate significantly from a 1:1 ratio.

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

respectively) demonstrated 99-100% similarity to *Rhynchosporium MAT1-2-1* gene sequence (GenBank accession: AJ537511). These sequences for isolates 53hv09 (*R. commune*), 2lm11 (*R. commune*), 3ar10 (*R. agropyri*) and I-Ia (*R. secalis*) have been deposited on GenBank (Accessions: KF998185 - KF998188, respectively).

Comparative TBLASTN analyses of the *R. commune*, *R. agropyri* and *R. secalis* partial *MAT1-1-1* gene sequences (each aligned and trimmed to a length of 423-bp) obtained in the present study showed greater similarity between *R. commune* and *R. agropyri* (99.76%) than either *R. agropyri* and *R. secalis* (99.53%), or *R. commune* and *R. secalis* (99.29%). Analyses of partial *MAT1-2-1* gene sequences obtained from all three species (all 224-bp) also revealed greater similarity between *R. commune* and *R. agropyri* (100%) than between either of these two species and *R. secalis* (99.55%).

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

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

When these primer pairs were used in PCR with *R. orthosporum* and *R. lolii*, all 27 isolates (collected from England, Italy, Switzerland and Wales) produced only the 598-bp amplicon specific for *MAT1-1* isolates (using primer pair A-MAT1-F/R; Fig. 4a) and not the 149-bp product specific for *MAT1-2* isolates (using primer pair C-MAT2-F/R; Fig. 4b). The mating-type distributions of the individual species *R. orthosporum* (*MAT1-1* = 8, *MAT1-2* =0; $\chi^2 = 8.000$, 1 d.f., $P < 0.01$) and *R. lolii* (*MAT1-1* = 19, *MAT1-2* = 0; $\chi^2 = 19.000$, 1 d.f., $P < 0.01$) were statistically significantly different from that expected for a 1:1 ratio of *MAT1-*

1:*MAT1-2* mating types. BLASTN analyses of sequence data (query lengths: 508-bp) obtained from these putative *MAT1-1* amplicons (*R. orthosporum*: 27dg09; *R. lolii*: 15lp11) showed ~82% similarity with *Rhynchosporium MAT1-1-1* gene sequence (GenBank accession: AJ549759). Representative *MAT1-1-1* sequence data from isolates 27dg09 and 15lp11 have been deposited on GenBank (Accessions: KF998189 and KJ513481, respectively). To further confirm the absence of the *MAT1-2-1* gene from *R. orthosporum* and *R. lolii*, draft genome sequences of these species (RsCH04 Bär A.1.1.3 for *R. orthosporum*, W Knogge and *Rhynchosporium* genome consortium; 15lp11 for *R. lolii*, KM King, D Hughes, R Harrison, PS Dyer, BDL Fitt and JS West, unpublished results) were BLASTN searched with the *MAT1-1-1* and *MAT1-2-1* gene sequences from *R. commune.* This revealed clear matches for *MAT1-1-1* in both species (E-values of 1.8E-163 and 6.4E-61 for *R. orthosporum* and *R. lolii,* respectively). However, there were no matches for *MAT1-2-1* in either species (cut-off value of 1.0E-5), showing that the isolates used for genome sequencing only contained the *MAT1-1-1* idiomorph. This was consistent with a heterothallic organisation of *MAT* genes rather than a possible homothallic organisation, with both *MAT1-1-1* and *MAT1-2-1* within the same genome (Debuchy *et al*. 2010), and/or lack of annealing of primer pair C-MAT2-F/R due to low partial mismatch of *MAT1-2-1* sequence.

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

Sizes of the resulting PCR amplicons using both genomic DNA and cDNA template (RT-PCR) were used to confirm both the presence and processing, or otherwise, of introns within the *MAT1-1-1*, *MAT1-2-1*, alpha-tubulin and beta-tubulin genes. On this basis, evidence was obtained for expression at the mRNA level of the *MAT1-1-1* gene using primer pair KM1RcF3/KM1RcB11 for *MAT1-1* isolates of *R. agropyri* (Fig. 5a), *R. secalis* (Fig. 5b) and *R. commune* (data not shown), However, it is noted that RT-PCR with all *MAT1-1* isolates

produced only relatively faint bands corresponding to products with an intron removed, but also appeared to produce an identically sized amplicon (425-bp) to that of the genomic DNA template controls, despite efforts to ensure removal of genomic DNA from RNA extracts. By contrast, expression of the *MAT1-1* gene (in terms of processing of an intron) could not be confirmed using primer pair KM1RoF2/KM1RoB16 for the isolates of *R. orthosporum* and *R. lolii* examined, although again an amplicon identical in size to those of the genomic template DNA controls was produced (data not shown).

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

Phylogenetic analyses of partial *MAT1-1* idiomorph sequences

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

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

Discussion

It is important to understand the mode of reproduction of plant pathogenic fungi because this has a major influence on the evolutionary potential of pathogen populations. Knowing whether species have clonal or recombining population structures can provide an indication of the risk of breakdown of disease control strategies (McDonald & Linde 2002). This study has shown for the first time that isolates genetically confirmed as *R. agropyri* and *R. secalis*, and obtained from both the same and proximate geographical origins, are of either *MAT1-1* or *MAT1-2* genotype. These findings are similar to those of Linde *et al*. (2003) and Foster & Fitt (2003), who demonstrated that isolates of the closely related species *R. commune* were also of either *MAT1-1* or *MAT1-2* identity, i.e. they showed an organisation consistent with a heterothallic mating system. The sequence at the *MAT* loci for *R. commune*, *R. agropyri* and *R. secalis* appeared to be highly conserved, based on the ability of the multiplex PCR diagnostic test of Linde *et al*. (2003) to anneal and amplify similarly sized PCR products

from all of these species. Sequencing of putative *MAT1-1-1* and *MAT1-2-1* PCR amplicons revealed >99% sequence similarity between all three species across the sequence examined. In addition, the present study has provided the first evidence for expression (in terms of processing of an intron) of both the *MAT1-1-1* and *MAT1-2-1* genes for all three of these species during mycelial growth *in vitro*, although the *MAT1-1-1* gene was expressed at relatively low levels under the assay conditions.

These data, alongside previous reports of considerable genetic diversity in field populations (Linde *et al*. 2009) and the close genetic relationship to other sexually reproducing plant pathogenic fungi (Goodwin 2002), suggest that *R. commune, R. agropyri* and *R. secalis* might all have 'cryptic', so far unidentified, sexual cycles (Dyer & O'Gorman 2012); these could potentially allow these *Rhynchosporium* species to disperse widely by air-borne ascospores and respond rapidly to evolutionary selection factors such as introduction of resistant cultivars or fungicide treatments (Milgroom 1996; Dyer *et al.* 2000; Gilles *et al*. 2001b; McDonald & Linde 2002). Based on their close genetic relatedness to other sexually reproducing fungal species, such sexual cycles are predicted to involve the production of apothecia, from which air-borne ascospores are released (Goodwin 2002). However, UK air-borne spore trapping work identified only small amounts of *R. commune* DNA (in comparison to known ascospore-producing crop pathogens), and it has been suggested that these positive samples were likely to have originated as a result of asexual spores occasionally becoming airborne as opposed to ascospores (Fountaine *et al*. 2010). Moreover, mating-type genes have been found to be both present and expressed for a number of apparently asexual fungi for which it has not so far been possible to induce a sexual cycle (e.g. Wada *et al*. 2012; Bihon *et al*. 2014).

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

New mating-type diagnostic tests developed in the present study showed that the more distantly related *R. orthosporum* and *R. lolii* were exclusively of the *MAT1-1* genotype. Although only 27 such isolates were tested, they were collected from a diverse range of hosts and geographical locations throughout Europe. Therefore it is very unlikely, although not conclusive, that no *MAT1-2* isolates would have been detected if *MAT1-1*:*MAT1-2* isolates of these two species were present in a 1:1 distribution. This finding was confirmed by BLASTN

analyses of draft genome sequences of these species, which failed to detect the presence of any *MAT1-2-1* gene homologue. Such an absence of one mating type in the natural environment has been reported previously for certain other asexual plant pathogens (e.g. Christiansen *et al.* 1998; Groenewald *et al.* 2006). Moreover, preliminary work reported here could not confirm expression (in terms of processing of an intron) of the *MAT1-1-1* gene for the isolates of *R. orthosporum* and *R. lolii* examined. However, it is possible that more extensive testing (with different isolates and assay conditions) might subsequently confirm such expression. Given the limited expression of the confirmed *MAT1-1-1* gene described previously in the present study for three other *Rhynchosporium* species, it is possible that this gene may only have been weakly expressed for *R. orthosporum* and *R. lolii* but that this was not detected in PCR testing. Further investigations into the expression of the *MAT1-1-1* gene for all five *Rhynchosporium* species are required.

The combined *MAT* distribution and expression data generally suggest that *R. orthosporum* and *R. lolii* isolates might have an exclusively asexual life-style, if only due to the absence of a compatible mating partner. However, more robust population genetic analyses (e.g. neutral SNP or SSR data from well-defined field populations) are required to definitively address the possibility of cryptic sexual cycles for these two species. Nevertheless, the discovery of such a highly skewed mating-type distribution is consistent with the notion that asexual populations generally show strong deviations away from a 1:1 *MAT1-1*:*MAT1-2* ratio (Yun *et al*. 2000). However, some other fungal species with known sexual states can show highly biased *MAT* distributions in natural populations (e.g. Consolo *et al*. 2005; Rhaiem *et al*. 2008; Heitman *et al.* 2014); thus, the apparent absence of one mating type from the populations of *R. orthosporum* and *R. lolii* sampled does not preclude sexual reproduction.

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

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

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Figure legends

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

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

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

Figure 3. Development of new PCR diagnostic tests to identify *MAT1-1* or *MAT1-2* isolates of *R. commune*, *R. agropyri* or *R. secalis*. Isolates that produced (a) a 598-bp amplicon using primer pair A-MAT1-F/R were considered to be of *MAT1-1* genotype, and (b) isolates that produced a 149-bp amplicon using primer pair C-MAT2-F/R were considered to be of *MAT1-2* genotype. Isolates shown are *R. commune* (lanes 2–5; 19hv09, UK7, 53hv09, 2lm11), *R. agropyri* (lanes 6–9; 10ar10, 6ar10, 3ar10, RS04CG-RAC-A.6.1) and *R. secalis* (lanes 10-13; RS02CH4-4b1, RS02CH4-14a1, 6.2, 1E7a). Further information about these isolates is given in Table 1. Isolates had previously been identified as *MAT1-1* (2–3, 6–7, 10–11) or *MAT1-2* (4–5, 8–9, 12–13) genotype using the multiplex mating-type diagnostic of Linde *et al*. (2003). Lane 1 is a 100-bp ladder (Fermentas, UK); the no template (water) control was blank (data not shown).

Figure 4. Identification of only *MAT1-1* isolates of *R. orthosporum* and *R. lolii*. Isolates that produced (a) a 598-bp amplicon using primer pair A-MAT1-F/R were considered to be of *MAT1-1* genotype, and (b) isolates that produced a 149-bp amplicon using primer pair C-MAT2-F/R were considered to be of *MAT1-2* genotype. Representative isolates shown are: *R. agropyri* (lanes 2–3; isolates 1ar10, 2ar10), *R. lolii* (lanes 4–11; 12lp11, 13lp11, 14lp11, 17lp11, 18lp11, 21lm11, 22lm11, 15lp11) and *R. orthosporum* (lanes 12–15; 27dg09, RS04CG-BAR-A.1.1.4, RS04ITA D-6.2, RS04ITA D-4.1). Further information about these isolates is given in Table 1. Isolates of *R. agropyri* in lanes 2–3 were positive controls, and had been previously identified as *MAT1-1* and *MAT1-2*, respectively, using the multiplex mating-type diagnostic of Linde *et al*. (2003). Lanes 1 and 16 are a 100-bp ladder (Fermentas, UK) and a no template (water) control, respectively.

Figure 5. Expression of *MAT1-1-1* and *MAT1-2-1* genes for different isolates of *R. agropyri* and *R. secalis*. Data are shown for isolates of known *MAT1-1* or *MAT1-2* genotype of (a) *R. agropyri* (*MAT1-1*: 1ar10, lanes 1–6; *MAT1-2*: 3ar10, lanes 7–12) and (b) *R. secalis* (*MAT1-1*: B8, lanes 1–6; *MAT1-2*: I-Ia, lanes 7–12). Information under bars below gel indicate the gene loci amplified and the sizes of the predicted PCR products. Inverted arrows on gel image point to the predicted sized amplicons in each lane. Genomic DNA template controls (lanes 1, 3, 5, 7, 9, 11) were run alongside RNA template (lanes 2, 4, 6, 8, 10, 12), to confirm RNA processing through removal of putative introns that internally spanned the amplified sequence. Note that primers targeted to *MAT1-1-1* gene sequence amplified both processed RNA and contaminant DNA, based on the sizes of amplicons (lanes 2). Ladders (L) are 100-bp ladders (New England Biolabs). No template (water) controls were blank (data not shown).

Figure 6. Phylogeny of partial sequences of the *MAT1-1* idiomorph of isolates of five *Rhynchosporium* species. The consensus neighbour joining tree displayed was constructed using *MAT1-1* idiomorph sequence data from the closely related *Pyrenopeziza brassicae* (GenBank accession: AJ006073) as an outgroup. Species identity of isolates is provided in right-hand parentheses, with further information available in Table 1. Numbers at nodes indicate the bootstrap support (%) based on 100,000 replications of the tree; only consensus support values of >80% are shown, for clarity. Scale bar represents the number of nucleotide substitutions per site.

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 [a]	Year	Mating type (Linde *et al.* 2003) [b]	Present Study [c]
788	Barley	France	Rc	1997	*MAT1-1*	
QUB 30.10	Barley	Northern Ireland, UK	Rc	Unknown	*MAT1-1*	
QUB 30.13	Barley	Northern Ireland, UK	Rc	Unknown	*MAT1-1*	
OSA 28-2-2	Barley	Hertfordshire, UK	Rc	2002	*MAT1-1*	
FI12-63	Barley	Finland	Rc	1996	*MAT1-2*	
R.s. 2310 4.2	Barley	France	Rc	2008	*MAT1-1*	
R.s. 2318 4.2	Barley	France	Rc	2008	*MAT1-1*	
RS00CH H36	Barley	Switzerland	Rc	2000	*MAT1-1*	
19hv09	Barley	Hertfordshire, UK	Rc	2009	*MAT1-1*	*MAT1-1*
53hv09	Barley	Hertfordshire,	Rc	2009	*MAT1-*	*MAT1-*

		UK			*2*	*2*
62hv09	Barley	Hertfordshire, UK	Rc	2009	*MAT1-1*	
73hv09	Barley	Hertfordshire, UK	Rc	2009	*MAT1-2*	
UK7	Barley	Aberystwyth, UK	Rc	Unknown	*MAT1-1*	*MAT1-1*
D.1.1	Wall barley	Switzerland	Rc	2004	*MAT1-1*	
E.1.2	Wall barley	Switzerland	Rc	2004	*MAT1-1*	
2lm11	Italian ryegrass	Shropshire, UK	Rc	2011	*MAT1-2*	*MAT1-2*
5lm11	Italian ryegrass	Shropshire, UK	Rc	2011	*MAT1-2*	
RS04CG-RAC-A.4.3	Couch-grass	Switzerland	Ra	2004	*MAT1-2*	
RS04CG-RAC-A.5.2	Couch-grass	Switzerland	Ra	2004	*MAT1-2*	
RS04CG-RAC-A.6.1	Couch-grass	Switzerland	Ra	2004	*MAT1-2*	*MAT1-2*
1ar10	Couch-	Surrey, UK	Ra	2010	*MAT1-*	

	grass				*1*	
2ar10	Couch-grass	Surrey, UK	Ra	2010	*MAT1-2*	
3ar10	Couch-grass	Surrey, UK	Ra	2010	*MAT1-2*	*MAT1-2*
6ar10	Couch-grass	Cluj-Napoca, Romania	Ra	2010	*MAT1-1*	*MAT1-1*
7ar10	Couch-grass	Timisoara, Romania	Ra	2010	*MAT1-2*	
8ar10	Couch-grass	Nottinghamshire, UK	Ra	2010	*MAT1-1*	
9ar10	Couch-grass	Nottinghamshire, UK	Ra	2010	*MAT1-1*	
10ar10	Couch-grass	Nottinghamshire, UK	Ra	2010	*MAT1-1*	*MAT1-1*
11ar10	Couch-grass	Nottinghamshire, UK	Ra	2010	*MAT1-1*	
RS02CH4-2a1	Rye	Switzerland	Rs	2002	*MAT1-1*	
RS02CH4-4b1	Rye	Switzerland	Rs	2002	*MAT1-1*	*MAT1-1*
RS02CH4-5a1	Rye	Switzerland	Rs	2002	*MAT1-1*	
Rs02CH4-6a.1	Rye	Switzerland	Rs	2002	*MAT1-1*	

RS99CH1-H10B	Rye	Switzerland	Rs	1999	*MAT1-2*	
RS02CH4-13a1	Rye	Switzerland	Rs	2002	*MAT1-1*	
RS02CH4-14a1	Rye	Switzerland	Rs	2002	*MAT1-1*	*MAT1-1*
8.4	Rye	Russia	Rs	2003	*MAT1-2*	
6.2	Rye	Russia	Rs	2003	*MAT1-2*	*MAT1-2*
4.11.1	Rye	Russia	Rs	2003	*MAT1-1*	
1E7a	Rye	Switzerland	Rs	1999	*MAT1-2*	*MAT1-2*
1B8	Rye	Switzerland	Rs	1999	*MAT1-1*	
1D4a	Rye	Switzerland	Rs	1999	*MAT1-1*	
I-Ia	Triticale	Switzerland	Rs	2002	*MAT1-2*	
I-2a2	Triticale	Switzerland	Rs	2002	*MAT1-2*	
I-3a1	Triticale	Switzerland	Rs	2002	*MAT1-2*	
27dg09	Cocksfoot	Aberystwyth,	Ro	2009	n/d [d]	*MAT1-*

		UK				*1*
57dg09	Cocksfoot	Aberystwyth, UK	Ro	2009		*MAT1-1*
59dg09	Cocksfoot	Aberystwyth, UK	Ro	2009		*MAT1-1*
RS04CG-BAR-A.1.1.3	Cocksfoot	Switzerland	Ro	2004	n/d	*MAT1-1*
RS04CG-BAR-A.1.1.4	Cocksfoot	Switzerland	Ro	2004		*MAT1-1*
RS04ITAD-4.1	Cocksfoot	Italy	Ro	2004		*MAT1-1*
RS04ITAD-6.1	Cocksfoot	Italy	Ro	2004		*MAT1-1*
RS04ITAD-6.2	Cocksfoot	Italy	Ro	2004		*MAT1-1*
1lm11	Italian ryegrass	Shropshire, UK	Rl	2011		*MAT1-1*
3lm11	Italian ryegrass	Shropshire, UK	Rl	2011		*MAT1-1*
4lm11	Italian ryegrass	Shropshire, UK	Rl	2011		*MAT1-1*
6lm11	Italian ryegrass	Aberystwyth, UK	Rl	2011		*MAT1-1*

7lm11	Italian ryegrass	Aberystwyth, UK	Rl	2011	*MAT1-1*
8lm11	Italian ryegrass	Aberystwyth, UK	Rl	2011	*MAT1-1*
9lm11	Italian ryegrass	Aberystwyth, UK	Rl	2011	*MAT1-1*
10lm11	Italian ryegrass	Aberystwyth, UK	Rl	2011	*MAT1-1*
21lm11	Italian ryegrass	Shropshire, UK	Rl	2011	*MAT1-1*
22lm11	Italian ryegrass	Shropshire, UK	Rl	2011	*MAT1-1*
11lp11	Perennial ryegrass	Aberystwyth, UK	Rl	2011	*MAT1-1*
12lp11	Perennial ryegrass	Aberystwyth, UK	Rl	2011	*MAT1-1*
13lp11	Perennial ryegrass	Aberystwyth, UK	Rl	2011	*MAT1-1*
14lp11	Perennial ryegrass	Aberystwyth, UK	Rl	2011	*MAT1-1*
15lp11	Perennial ryegrass	Shropshire, UK	Rl	2011	*MAT1-1*
16lp11	Perennial ryegrass	Surrey, UK	Rl	2011	*MAT1-1*
17lp11	Perennial	Hertfordshire,	Rl	2011	*MAT1-*

	ryegrass	UK			*1*
18lp11	Perennial ryegrass	Hertfordshire, UK	Rl	2011	*MAT1-1*
20lp11	Perennial ryegrass	Hertfordshire, UK	Rl	2011	*MAT1-1*

[a.] *Rhynchosporium* species identity had been confirmed previously by King *et al*. (2013) using either species-specific PCR primers or repetitive extragenic palindromic PCR. Species identities are as follows: Rc: *R. commune*; Ra: *R. agropyri*; Rs: *R. secalis*; Ro: *R. orthosporum*; Rl: *R. lolii*.

[b.] Mating type determined using the multiplex PCR diagnostic of Linde *et al*. (2003); production of a 590-bp or 360-bp amplicon indicated *MAT1-1* or *MAT1-2* type isolates, respectively.

[c.] Mating type determined using diagnostics developed in the present study; production of a 598-bp amplicon using primer pair A-MAT1-F/R or a 149-bp amplicon using primer pair C-MAT2-F/R indicated *MAT1-1* or *MAT1-2* type isolates, respectively.

[d.] 'n/d': could not be determined.