Zymoseptoria gen. nov.: a new genus to accommodate Septoria-like species occurring on graminicolous hosts

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INTRODUCTION

More than 10 000 names have been described in the genus Mycosphaerella (Capnodiales, Dothideomycetes) and its associated anamorph genera (Cercospora, Pseudocercospora, Septoria, Ramularia, etc.) (Crous et al. 2009a), making it one of the largest genera of plant pathogenic Ascomycetes known to date (Crous 2009). However, in contrast to earlier phylogenetic studies based on the ITS region (Stewart et al. 1999, Crous et al. 1999, 2000, 2001, Goodwin et al. 2001), more robust multi-gene phylogenies have revealed Mycosphaerella to be polyphyletic (Crous et al. 2007, 2009b, Schoch et al. 2009a, b), suggesting that Mycosphaerella s.l. should be subdivided to reflect natural groups (genera) as defined by their anamorphs.

The genus Mycosphaerella is typified by M. punctiformis, which has a Ramularia anamorph, R. endophylla (Verkley et al. 2004a). Even since it was established, the name Mycosphaerella has ever been used to describe related and unrelated, small loculoascomycetes (in some cases even asexual coelomycetes) (Aptroot 2006), prompting Crous et al. (2009b), to suggest that the older generic name Ramularia (1833), rather than the confused name Mycosphaerella (1884) should be used for this well-defined morphologic (Braun 1998) and phylogenetic clade of fungi (Crous et al. 2009b, Kirschner 2009).

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Abstract The Mycosphaerella complex is both poly- and paraphyletic, containing several different families and genera. The genus Mycosphaerella is restricted to species with Ramularia anamorphs, while Septoria is restricted to taxa that cluster with the type species of Septoria, S. cytisi, being closely related to Cercospora in the Mycosphaerellaceae. Species that occur on graminicolous hosts represent an as yet undescribed genus, for which the name Zymoseptoria is proposed. Based on the 28S nrDNA phylogeny derived in this study, Zymoseptoria is shown to cluster apart from Septoria. Morphologically species of Zymoseptoria can also be distinguished by their yeast-like growth in culture, and the formation of different conidal types that are absent in Septoria s.str. Other than the well-known pathogens such as Z. tritici, the causal agent of septoria tritici blotch on wheat, and Z. passerinii, the causal agent of septoria speckled leaf blotch of barley, both for which epitotypes are designated, two leaf blotch pathogens are also described on graminicolous hosts from Iran. Zymoseptoria brevis sp. nov. is described from Phalaris minor, and Z. halophila comb. nov. from leaves of Hordeum glaucum. Further collections are now required to elucidate the relative importance, host range and distribution of these species.

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Table 1  Details of cultures subjected to DNA sequencing.

| Species               | Isolate no     | Host          | Location | GenBank Accession no |
|-----------------------|----------------|---------------|----------|----------------------|
| Cercospora apii       | –              | Hedysarum coronarium | Italy    | M. Ribaldi |
|                       | –              | Beta vulgaris | Romania  | –                     |
| C. beticola           | CBS 115683     | Douglas-fir pole | USA      | W. Himaman |
|                       | –              | Dissoconium australiensis | Australia | P.W. Crous |
|                       | –              | Eucalyptus camaldulensis | Thailand | W. Himaman |
|                       | –              | Eucalyptus camaldulensis | Thailand | W. Himaman |
|                       | –              | Eucalyptus globulus | Australia | I.W. Smith |
|                       | –              | Pinus nigra   | USA      | G. Adams |
|                       | –              | Pinus radiata | France   | M. Morelet |
|                       | –              | Pinus oocarpa | Mexico   | M. de Jesus Yanez Morales |
|                       | –              | Eucalyptus cladocalyx | South Africa | A.R. Wood |
| M. elongata           | –              | Eucalyptus cladocalyx | South Africa | P.W. Crous |
| M. marksii            | CBS 119974     | Eucalyptus grandis | South Africa | P.W. Crous |
|                       | –              | Eucalyptus grandis | South Africa | P.W. Crous |
|                       | –              | Eucalyptus saligna | South Africa | P.W. Crous |
|                       | –              | Eucalyptus heimii | South Africa | P.W. Crous |
|                       | –              | Eucalyptus pseudoeucalyptorum | South Africa | P.W. Crous |
|                       | –              | Eucalyptus irregulariramosa | South Africa | P.W. Crous |
|                       | –              | Eucalyptus punctata | South Africa | P.W. Crous |
|                       | –              | Eucalyptus heimii | South Africa | P.W. Crous |
|                       | –              | Eucalyptus robusta | Malaysia  | M.J. Wingfield |
|                       | –              | Protea sp.    | South Africa | S. Denman |
|                       | –              | Quercus robur | The Netherlands | G. Verkley |
|                       | –              | Corymbia grandifolia | Italy  | W. Gams |
|                       | –              | Leonurus sibiricus | The Netherlands | W. Quaedvlieg |
|                       | –              | Sorghum sp.   | South Africa | D. Nowell |
| R. lamii              | CBS 110578     | Betula pubescens | Scotland | S. Green |
|                       | –              | Laburnum anagyroides | – | – |
|                       | –              | Gerbera jamesonii | Italy  | W. Gerlach |
|                       | –              | Septoria betulae | Scotland | G. Verkley |
|                       | –              | Septoria cytisi | Italy  | W. Gerlach |
|                       | –              | Septoria menthae | Belgium  | J. van Holder |
|                       | –              | Septoria rosae | The Netherlands | G. Verkley |
|                       | –              | Septoria verbenae | New Zealand | G. Verkley |
|                       | –              | Trematosphaeria fibrillosa | South Africa | P.W. Crous & L. Mostert |
|                       | –              | Zasmidium anthuriicola | Thailand  | C.F. Hill |
|                       | –              | Zymoseptoria brevis | South Africa | A.J. Cargenie |
|                       | –              | Zymoseptoria xenoparkii | Colombia | T. Herrmann |
| IRAN1485C (= CPC 18102) | –              | Phalaris paradoxa | Iran  | – |
| IRAN1486C (= CPC 18107) | –              | Phalaris paradoxa | Iran  | – |
| CPC 18109 (= 81)      | –              | Phalaris paradoxa | Iran  | – |
| CPC 18109 (= 83)      | –              | Phalaris paradoxa | Iran  | – |
### Zymoseptoria halophila

| Strain Information | Description | Location |
|--------------------|-------------|----------|
| IRAN1483C (= CPC 18105) = CBS 128854 | Hordeum glaucum | Iran – JF701040 JF701111 JF700874 JF700976 JF700807 |
| IRAN1489C (= CPC 18099) | Hordeum vulgare | P83A, USA S. Ware |
| IRAN1484C (= CPC 18103) | Avena sp. | Iran – JF701053 JF701121 JF700885 JF700986 JF700816 |
| Triticum aestivum | Switzerland E. Muller JF700889 JF700991 JF700820 |
| Triticum durum | Tunisia – JF701070 JF701137 JF700899 JF701002 JF700833 |
| Triticum aestivum | Tunisia – JF701072 JF701140 JF700901 JF701004 JF700835 |
| Triticum aestivum | Morocco – JF701073 JF701141 JF700902 JF701005 JF700836 |
| Triticum aestivum | Portugal – JF701076 JF701144 JF700905 JF701008 JF700839 |
| Triticum aestivum | Portugal – JF701077 JF701145 JF700906 JF701009 JF700840 |
| Triticum aestivum | Portugal – JF701078 JF701146 JF700907 JF701010 JF700841 |
| Triticum aestivum | Kenya – JF701079 JF701147 JF700908 JF701011 JF700842 |
| Triticum aestivum | USA – JF701080 JF701148 JF700909 JF701012 JF700843 |
| Triticum aestivum | USA – JF701081 JF701149 JF700910 JF701013 JF700844 |
| Triticum aestivum | Algeria – JF701096 JF701153 JF700915 JF701019 JF700851 |
| Triticum aestivum | Algeria – JF701099 JF701156 JF700918 JF701022 JF700853 |
| Triticum aestivum | France – JF701102 JF701170 JF700934 JF701034 JF700865 |

1 CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Pedro Crous working collection housed at CBS; IMI: International Mycological Institute; USO: United States Department of Agriculture, National Fungus Collections (BPI); IPO: Research Institute for Plant Protection, Wageningen (IRAN); Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection. ACT = Actin, TUB = β-tubulin, CAL = Calmodulin, LSU = 28S large subunit of the nrRNA-gene, RPB2 = RNA polymerase II second largest subunit.
MATERIALS AND METHODS

Isolates
Symptomatic leaves were collected from several localities (Table 1), and leaves with visible asexual fruiting bodies were immediately subjected to direct fungal isolation, or alternatively were first incubated in moist chambers to stimulate sporulation. Single-conidial isolates were established on malt extract agar (MEA; 20 g/L Biolab malt extract, 15 g/L Biolab agar) using the previously described procedure (Crous et al. 2009c). Cultures were later plated on fresh MEA, 2 % tap water agar supplemented with green, sterile barley leaves (WAB), 2 % potato-dextrose agar (PDA), and oatmeal agar (OA) (Crous et al. 2009c), and subsequently incubated at 25 °C under near-ultraviolet light to promote sporulation. Reference strains are maintained in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands, the Plant Research Institute, Wageningen, the Netherlands, and the Iranian Research Institute of Plant Protection, Tehran, Iran (Table 1), and supplemented with other relevant isolates present in the CBS collection. Descriptions, nomenclature, and illustrations were deposited in MycoBank (www.mycobank.org, Crous et al. 2004).

DNA extraction, amplification and sequencing

Herbarium specimens
Ten S. cytisi herbarium specimens occurring on Cytisus laburnum (= Laburnum anagyroides), were obtained from the U.S. National Fungus Collections (BPI) in Beltsville, Maryland, USA (Table 2). After microscopic inspection, the five specimens with the least amount of surface contamination (yeast and saprobes) where selected for DNA extraction (Table 2). Using a stereo microscope, ± 25 pycnidia, including their dried conidial cirri, where excised from each respective herbarium specimen, and suspended in tubes with 20 μL STL buffer from an E.Z.N.A. ® Forensic DNA Kit (Omegabiotek, Norcross). Special care was taken to keep the amount of contaminant leaf material, excised together with the fungal tissue, as low as possible. The fungal material was kept in STL buffer to rehydrate for 24 h at 4 °C, after which the fungal cell walls were degraded by two cycles of freezing with liquid nitrogen and immediate re-heating to 99 °C.

Table 2 Herbarium specimens of Laburnum anagyroides infected with Septoria cytisi, obtained from the U.S. National Fungus Collections (BPI), Maryland, USA. Specimens marked with an asterisk were selected for DNA extraction.

| BPI accession number | Host                | Year collected | Location          |
|----------------------|---------------------|----------------|-------------------|
| 0378986              | Laburnum anagyroides| 1913           | France            |
| 0378987              | Laburnum anagyroides| 1933           | Romania           |
| 0378988              | Laburnum anagyroides| 1893           | Italy             |
| 0378989              | Laburnum anagyroides| 1929           | ‘Czechoslovakia’  |
| 0378990              | Laburnum anagyroides| 1874           | Italy             |
| 0378991*             | Laburnum anagyroides| 1885           | ‘Czechoslovakia’  |
| 0378992              | Laburnum anagyroides| 1903           | Italy             |
| 0378993*             | Laburnum anagyroides| 1929           | Austria           |
| 0378994*             | Laburnum anagyroides| 1884           | ‘Czechoslovakia’  |
| 0378995              | Laburnum anagyroides| 1876           | Italy             |

Fig. 2 A diagrammatic representation of part of the nrDNA operon indicating the positions of the Septoria-specific primers used to generate ITS and LSU sequences of S. cytisi.
The genomic DNA extraction was performed using the ‘Isolation of DNA from dried blood’ protocol available in the E.Z.N.A. ® Forensic DNA Kit with one modification: in order to increase the final DNA concentration, only 50 μL of preheated (70 °C) elution buffer was used to elute the DNA from the column.

Genus-specific primers had to be designed because the use of generic fungal ITS and LSU primers only generated sequences of contaminants (mostly yeasts). For the amplification reactions concerning the herbarium specimens, the Verbatim High Fidelity DNA Polymerase Kit (Thermo Scientific) was used in combination with the Septoria-specific S18S-2 forward primer (annealing to the nuclear rDNA operon at the 3'-end of the 18S nrDNA gene (SSU); Table 2), together with the Septoria-specific SITS2_Fd reverse primer (annealing to the nuclear rDNA operon at the 5'-end of the 28S nrDNA gene (LSU); Table 2), in order to amplify a region spanning the 5.8S nrDNA gene and the first and second internal transcribed spacer regions (Fig. 2). This amplification reaction was set up in a volume of 12.5 μL using 5× High Fidelity buffer (with MgCl2), 0.8 μM of each primer, 2 μL of gDNA, 150 μM dNTP mix and 0.1 unit of Verbatim polymerase using a MyCycler thermal cycler (Bio-Rad). PCR amplification conditions were set as follows: an initial denaturation temperature at 98 °C for 2 min, followed by 50 cycles of denaturation temperature at 98 °C for 30 s, primer annealing at 52 °C for 30 s, primer extension at 72 °C for 30 s and final extension at 72 °C for 2 min. The resulting PCR products were then size-fractionated on a 3 % (w/v) agarose gel stained with ethidium bromide, excised from the gel and subsequently sequenced as described by Cheewangkoon et al. (2008).

Degradation and shearing of the S. cytisi herbarium gDNA made it impossible to directly amplify and sequence the approximate 1 300 bp needed to cover both the ITS and D1–D3 domains of the 28S nrDNA in a single reaction. Therefore, specific primers were developed from the obtained S. cytisi ITS1 sequence, spaced about 300 bp apart (Table 2, Fig. 2), which made it possible to sequentially amplify and sequence the entire regions of both the ITS, and the D1–D3 domains of the LSU of S. cytisi sequentially, and later to sequence it as described by Cheewangkoon et al. (2008).

**Fungal cultures**

Genomic DNA was extracted from mycelium growing on MEA (Table 1), using the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). These strains were screened for five loci, namely ITS, Actin (ACT), calmodulin (CAL), RNA polymerase II second largest subunit (RPB2) and β-tubulin (TUB) (Table 3). DNA amplification and sequencing reactions were performed as described by Cheewangkoon et al. (2008).

**Phylogenetic analysis**

To determine whether the multi-locus DNA sequence datasets were congruent, a partition homogeneity test (Farris et al. 1994) of all possible combinations was performed in PAUP v4.0b10 (Swofford 2003) with 1 000 replications. Parallel to this, a 70 % Neighbor-Joining (NJ) reciprocal bootstrap method with Maximum Likelihood distance (Mason-Gamer & Kellogg 1996, Lombard et al. 2010) was also employed to check congruency. The models of evolution for the NJ tree were estimated with Modeltest v3.7 (Posada & Crandall 1998) and bootstrap analyses (10 000 replicates) were performed in PAUP. Resulting NJ tree topologies were visually compared for conflicts between the individual gene regions. Maximum-parsimony genealogies for individual datasets and the combined dataset were estimated in PAUP using heuristic searches based on 1 000 random taxon addition sequences and the best trees were saved. All characters were weighted equally and alignment gaps were treated as missing data. Branches of zero length were collapsed and all multiple, equally most parsimonious trees were saved. Tree length (TL), consistency index (CI), retention index (RI) and the rescaled consistency index (RC) were calculated in PAUP for the equally most parsimonious trees and the resulting trees were printed with TreeView (Page 1996) and the alignments and phylogenetic trees were lodged in TreeBASE (www.treebase.org). All novel sequences derived from this study were deposited in GenBank (Table 1). Trees were either rooted to *Cladosporium brunnei* for the LSU tree, or to *Mycosphaerella punctiformis* for the multigene tree.

**Morphology**

Descriptions were based on fungal cultures sporulating in vitro on WAB, incubated under continuous near-ultraviolet light for 2–4 wk. Wherever possible, 30 measurements (×1 000 magnification) were made of structures mounted in lactic acid, with the extremes of spore measurements given in parentheses. Colony colours (surface and reverse) were assessed after 1 mo on MEA, PDA and OA at 25 °C in the dark, using the colour charts of Rayner (1970).

**RESULTS**

**ITS and LSU amplification and sequencing of S. cytisi**

The gDNA extractions from the S. cytisi herbarium samples were performed on the herbarium specimens indicated in Table 2, and both the ITS and a partial LSU regions where targeted for these isolates using *Septoria*-specific primers (Table 4). An ITS amplicon length of 486 bp was achieved from herbarium sample US0378993 while the other samples yielded only partial ITS amplicons varying in length from 440 bp in sample US0378994 to ± 200 bp in sample US0378990; amplicons of sample US0378991 only yielded contaminated sequences with general primers and did not amplify with either *Septoria*- or S. cytisi–specific primers.

A comparison between the full-length S. cytisi ITS sequence and 287 other *Septoria* ITS sequences that were generated as part of a larger unpublished study, broadly linked *S. cytisi* to a distinct ITS clade containing *S. astralagi* and *S. hippocastani*, basal to a clade consisting of the majority of sequenced *Septoria*

**Table 3** Primer combinations used during this study for generic amplification and sequencing.

| Locus | Primer | Primer sequence 5’ to 3’ | Orientation | Reference |
|-------|--------|--------------------------|-------------|----------|
| Actin | ACT-512F | ATGTGCCAGGGCGGTTCCGC | Forward | Carbone & Kohn (1999) |
| Actin | ACT2Ru | ARTRCRCGDCGCRGCAGCTTC | Reverse | Groenewald, unpubl. data |
| Calmodulin | CAL-22F | GAGTCAGGAGGGCCCTTCCGC | Forward | Carbone & Kohn (1999) |
| Calmodulin | CAL2Ru | TGRCTNGGCTCDCCGGATCTTC | Reverse | Groenewald, unpubl. data |
| β-tubulin | TUB2Fd | GTBCCACTCYCARACCGGCGTARTG | Forward | Aveskamp et al. (2009) |
| β-tubulin | TUB4Ru | CCRGAYTRGCRRAARACRAAGTTC | Reverse | Aveskamp et al. (2009) |
| RPB2 | RPB2-5F | GAGYAMGNGATCTTTYG | Forward | Liu et al. (1999) |
| RPB2 | RPB2-5F+14R | ACNMNNCCCARTGNGWRTTRTG | Reverse | Present study |
| LSU | LSU1Fd | GRATCAGGATTGRATCCCG | Forward | Crous et al. (2009a) |
| LSU | LR5 | TCCGGAGGAAAATCTCG | Reverse | Vilgalys & Hester (1990) |
Table 4  Septoria cytisi-specific ITS and LSU primers used for amplification and sequencing. Nucleotide positions were determined relative to the ITS/LSU sequence of Zymoseptoria tritici (GenBank accession FN428877).

| Primer name | Primer sequence 5' to 3' | Orientation | Relative position |
|-------------|--------------------------|-------------|------------------|
| S18S-2      | CGTAGGTTGAACYTGGCGGAGGATAGCTACGTGGA | Forward | 7 |
| 5.8S1Ff     | CTTCTGGTGGCTCAGAGGTC | Forward | 240 |
| SIT2_2_Fwd  | CGCCCGCACTGCGAGGGATTAGAACAC | Forward | 459 |
| SIT2_2_Rev  | GATTCTAAATGCGGAGGATGCGC | Reverse | 459 |
| LSU_Sep_230_Fwd | TATGTTGACGGCGCGACGGTCTTAC | Forward | 710 |
| LSU_Sep_230_Rev  | GTAAGGTTGTGGGGCCTGACATAA | Reverse | 710 |
| LSU_Sep_530_Fwd  | AGAGTTGACATGGAACTGAAGCTCAGTCATT | Forward | 999 |
| LSU_Sep_530_Rev  | AGAGGACTACATTGCTTAAGGTCATTT | Reverse | 999 |
| LSU_Sep_575_Fwd  | CTTGGGCGAGGTCGCGGT | Forward | 1059 |
| LSU_Sep_575_Rev  | AGCCGGACCTGCCAGAAG | Reverse | 1059 |
| LSU_Sep_785_Rev  | AGGACATCAGGAGGCGCGGAT | Reverse | 1225 |

Table 5  Polymorphisms found in the ITS and LSU sequence between the S. cytisi herbarium specimens. Data marked with – are not available.

| BPI specimen | Collection year | ITS position (bp) | LSU position (bp) |
|--------------|-----------------|------------------|------------------|
|              |                 | 93 219 411       | 176 377 446 536 561 563 |
| USO 378989 | 1929 | A – – | T T G T T C |
| USO 378993 | 1929 | A C C | C C C A A G – |
| USO 378994 | 1884 | C G T | C C G A G G |
| USO 378990 | 1874 | A G C | – – – – – – |

species (data not shown). Interspecific variation in the S. cytisi ITS sequences were present; however, it was limited to a few nucleotides per isolate sequenced (Table 5).

Amplification of the D1–D3 domains of the LSU region was attempted on the same species (data not shown). Interspecific variation in the LSU sequences was only obtained from a single sample (USO378994). The four remaining herbarium specimens only yielded LSU sequences varying in length from 500–800 bp. Interspecific variation in the LSU nucleotide sequences was limited to a few nucleotides per sequenced isolate (Table 5).

Phylogenetic analyses

LSU dataset

During phylogenetic analyses, the S. cytisi LSU sequence was aligned with LSU sequence data of 64 Capnodiales taxa, including 19 representative Septoria taxa, in order to determine which of these Septoria isolates belonged to Septoria s.str. (i.e. high association with S. cytisi) and to establish how this clade is related to other well-established genera within the Capnodiales. For the LSU tree, ± 759 characters were determined for 64 Capnodiales taxa, including 19 Septoria taxa as well as the two Cladosporium bruennii isolates that were used as outgroups (CPC 5101 and CBS 188.54). The phylogenetic analysis showed that 164 characters were parsimony-informative, 38 were variable and parsimony-uninformative and 557 were constant. Thirty-two equally most parsimonious trees were obtained from the heuristic search, the first of which is shown in Fig. 3 (TL = 574, CI = 0.495, RI = 0.848, RC = 0.419). The phylogenetic analysis of the Capnodiales LSU dataset, including S. cytisi, showed this species clustering in a well-defined clade incorporating the majority of the Septoria spp. used in this analysis, clearly delineating this clade as Septoria s.str. These results also show a distinct monophyletic clade that are referred to as Zymoseptoria gen. nov. below, which contains S. triticic and S. passerinii together with two other species in this genus.

Multi-locus dataset

For the multi-locus phylogenetic analyses of the graminicolous isolates, ± 220 nucleotides where determined for ACT, 345 for CAL, 513 for ITS, 350 for TUB, and 305 for RPB2 (see Table 3 for detailed primer description). The adjusted sequence alignment for each locus consisted of 69 ingroup taxa with Ramularia endophylla (Mycosphaerella punctiformis; strain CBS 113265) as outgroup.

The strict consensus tree (Fig. 4) based on the multi-locus maximum-parsimony analysis had an identical topology to those of the strict consensus trees obtained for the individual loci. The partition homogeneity tests for all of the possible combinations of the five gene regions consistently yielded a P-value of 0.001, and were therefore incongruent. However, the 70 % reciprocal bootstrap trees of the individual gene regions showed no conflicting tree topologies between the separate datasets. Based on the result of the 70 % reciprocal bootstrap trees (Mason-Gamer & Kellogg 1996, Cunningham 1997), the DNA sequences of the five gene regions (ACT, CAL, RPB2, TUB and ITS) were concatenated for the phylogenetic analyses.

The concatenated and manually aligned multi-locus alignment contained 70 taxa (including the outgroup sequence) and, out of the 1 723 characters used in the phylogenetic analysis, 233 were parsimony-informative, 291 were variable and parsimony-uninformative and 1 199 were constant. 810 equally parsimonious trees were obtained from the heuristic search, the first of which is shown in Fig. 4 (TL = 768, CI = 0.815, RI = 0.922, RC = 0.751). Phylogenetic results showed two well-supported new species emerging besides the conserved S. triticic and S. passerinii clades, with a significant amount of genetic variation within the S. triticic clade as previously found by Goodwin et al. (2007). This intraspecific variation is most likely the cause of the partition homogeneity test failure.

The overall genetic diversity of S. triticic, examined over five loci, was found to be quite significant within the 54 global isolates of S. triticic used for this study. Most of the existing phylogenetic variation observed between the S. triticic isolates used in the combined tree (Fig. 4) was caused by single insertion and deletion events of triplets within tandem repeats inside the ACT and RPB2 intron sequences of these isolates. The most significant
impact of these indel events can be seen in the phylogenetic cluster containing CPC 18099–18101 (on Aegilops tauschii, Iran), that arises in the *S. tritici* clade of the combined tree (Fig. 4). This small clade has a bootstrap support value of 94 %, suggesting that it could represent a cryptic or ancestral lineage of what is currently considered to be *S. tritici*. Further study using more isolates would be required to address this issue.

**Taxonomy**

Based on the LSU dataset (Fig. 3), *S. cytisi* was shown to cluster within the major *Septoria* clade, while the taxa occurring on graminicolous hosts clustered in a separate clade, distinct from *Septoria* (*S. cytisi*) and *Mycosphaerella* (*M. punctiformis*, represented by *R. endophytica*), suggesting that they represented a distinct genus in the *Mycosphaerellaceae*. Morphologically these phylogenetic differences were supported by the distinct yeast-like growth exhibited in culture by the graminicolous species, as well as their mode of conidiogenesis, e.g. phialidic, with periclinal thickening and occasional inconspicuous percurrent proliferation(s), but lacking blastic sympodial proliferation which occurs in many species of *Septoria* s.str. Based on these differences in culture, morphology and phylogeny, a new genus is hereby introduced for the taxa occurring on graminicolous hosts.
**Zymoseptoria** Quaedvlieg & Crous, gen. nov. — MycoBank MB517922

*Septoriae similis, sed aducto fermentoide, sine formatione blastica-sympodial conidiorum, in cultura typis conidiorum usque ad 3.*

*Type species.* **Zymoseptoria tritici** (Desm.) Quaedvlieg & Crous.

**Etymology.** *Zymo* = yeast-like growth; *Septoria* = *Septoria*-like in morphology.

**Conidiomata** pycnidial, semi-immersed to erumpent, dark brown to black, subglobose, with central ostiole; wall of 3–4 layers of brown *textura angularis*. **Conidiophores** hyaline, smooth, 1–2-septate, or reduced to conidiogenous cells, lining the inner cavity. **Conidigenous cells** tightly aggregated, ampulliform to doliiform or subcylindrical, phialidic with periclinal thickening, or with 2–3 inconspicuous, percurrent proliferations at apex. **Type I conidia** solitary, hyaline, smooth, guttulate, narrowly.
Zymoseptoria brevis M. Razavi, Quaedvlieg & Crous, sp. nov.
— MycoBank MB517923; Fig. 5

**Etymology.** Named after its conidia, which are shorter (**brevis**) than those of the other species.

**On sterile barley leaves on WA:** Conidiomata pycnidial, sub-stomatal, immersed to erumpent, globose, dark brown, up to 200 μm diam, with central ostiole, 5–10 μm diam; wall of 3–4 layers of brown *textura angularis*. Conidiophores reduced to conidiogenous cells, or with one supporting cell, lining the inner cavity. Conidiogenous cells hyaline, smooth, tightly aggregated, subcylindrical to ampulliform, straight to curved, 7–15 × 2–4 μm, with 1–2 inconspicuous, percurrent proliferations at apex, 1–1.5 μm diam. **Type I conidia** solitary, hyaline, smooth, guttulate, narrowly cylindrical to subulate, tapering towards acutely rounded apex, with truncate base, 0–1-septate, (12–)13–16(–17) × 2(–2.5) μm; on PDA, 9–21 × 2–3.5 μm; hila not thickened nor darkened, 1–2 μm. On OA and PDA yeast-like growth and microcyclic conidiation (**Type III conidia**) common on agar media.

Zymoseptoria halophila (Speg.) M. Razavi, Quaedvlieg & Crous, **comb. nov.**
— MycoBank MB517924; Fig. 6

**Basionym:** Septoria halophila Speg., Anales Mus. Nac. Hist. Nat. Buenos Aires, Ser. 3, 13: 382. 1910.

**Initial symptoms** of the disease were dark-brown lesions which soon became pale buff in the centre. The leaves were heavily mottled later, and the solitary, sometimes aggregated pycnidia formed on the lesions. The disease was more severe on the lower leaves. *Pycnidia* were observed on adaxial surface of the infected leaves, and were dark-brown, globose, measuring 90–150 μm, with an ostiole ± 10 μm diam. On sterile barley leaves on WA: **Conidiomata** pycnidial, semi-immersed to erumpent, dark brown to black, subglobose, up to 300 μm diam, with central ostiole, up to 30 μm diam; wall of 3–4 layers of brown *textura angularis*. Conidiophores reduced to conidiogenous cells, lining the inner cavity. Conidiogenous cells hyaline, smooth, tightly aggregated, ampulliform to doliiform, 10–15 × 4–7 μm, with 2–3 inconspicuous, percurrent proliferations at apex, 1–2 μm. On OA and PDA yeast-like growth and microcyclic conidiation (**Type III conidia**) common, also forming on aerial hyphae via solitary conidiogenous loci.

**Culture characteristics** — Colonies on PDA flat, spreading, with moderate aerial mycelium and feathery, lobate margins; surface olivaceous-grey, outer region dirty white, reverse iron-grey; on MEA more erumpent, with less aerial mycelium; surface iron-grey with patches of white, reverse greenish black; on OA somewhat fluffly with dirty white to pale olivaceous aerial mycelium, and submerged, olivaceous-grey margin; reaching 15 mm diam after 1 mo at 25 °C; fertile.

**Specimen examined.** Iran, Ilam province, Dehloran, on living leaves of *Phalaris minor*, M. Razavi, holotype CBS H-20542, cultures ex-type No 8S = CPC 18106 = CBS 128853.

**Notes** — *Zymoseptoria brevis* can easily be distinguished from the other taxa presently known within the genus based on its shorter conidia.

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**Fig. 5** Zymoseptoria brevis (CPC 18106) a. Pycnidium forming on barley leaves in vitro; b. colony sporulation on potato-dextrose agar; c. conidiogenous cells; d. colony on synthetic nutrient-poor agar, showing yeast-like growth; e. conidium undergoing microcyclic conidiation (arrows; **Type III**); f–h. pycnidiospores (**Type I**). — Scale bars = 10 μm.
phragmospores (Type II conidia), that again give rise to type I conidia via microcyclic conidiation; yeast-like growth and microcyclic conidiation (Type III conidia) common on agar media.

Culture characteristics — Colonies on PDA flat, spreading, with sparse aerial mycelium and feathery, lobate margins; centre olivaceous-grey, outer region iron-grey; reverse iron-grey; on MEA surface and reverse greenish black; on OA iron-grey, reaching 20 mm diam after 1 mo at 25 °C; fertile.

Specimen examined. Iran, Ilam province, Dehloran, on living leaves of Hordeum glaucum, 25 Apr. 2007, M. Razavi, specimens IRAN12892F, CBS H-20543, cultures ex-type GLS1 = IRAN1483C = CPC 18105 = CBS 128854.

Notes — The present collection of Z. halophila was initially reported from Iran as S. halophila by Seifbarghi et al. (2009) (GenBank HM100267, HM100266), based on the description of S. halophila provided by Priest (2006). Zymoseptoria halophila was originally described from Hordeum halophilum collected in Argentina, with conidia being (0–)1(–2)-septate, 36–58 × 1.5(–2) μm, and conidiogenous cells being 8–10 × 2.5–3.5 μm. It is likely that the various collections on Hordeum and Poa spp. from Australia listed by Priest (2006) could represent different species, but this can only be resolved once additional collections and cultures have been obtained to facilitate further molecular comparisons.

Zymoseptoria halophila is closely related to Z. passerinii, which is also reflected in its conidial size, which overlaps in length, but can only be distinguished based on their difference in width. It is possible that some published records of Z. passerinii could in fact represent Z. halophila, but more collections would be required to resolve its host range and geographic distribution.

Zymoseptoria passerinii (Sacc.) Quaedvlieg & Crous, comb. nov. — MycoBank MB517925; Fig. 7

Basionym: Septoria passerinii Sacc., Syll. Fung. (Abellini) 3: 560. 1884.

Specimens examined. Italy, Vigheffio, near Parma, on Hordeum murinum, June 1879 (F. von Thümen, Mycotheca Univ. No. 1997, isotype in MEL, see Priest 2006, f. 107). – USA, North Dakota, Foster county, on Hordeum vulgare, coll. S. Goodwin, isol. D. Long, epitype designated here CBS H-20544, culture ex-epitype P83 = CBS 120382.

Notes — Priest (2006) reported Z. passerinii from several Hordeum species collected in Western Australia and deposited them at IMI (now in Kew), and found them to be identical to type material examined, suggesting that this pathogen is widely distributed along with its host. Ware et al. (2007) reported a Mycosphaerella-like teleomorph from a heterothallic mating of isolates of Z. passerinii. Single ascospore isolates have been deposited as CBS 120384 (P71 × P83A) and CBS 120385 (P71 × P83B). Isolate P63, which is genetically similar to P83 on the loci sequenced in this study, has been used for whole genome analysis of Z. passerinii (E.H. Stukenbrock, pers. comm.).

Fig. 6 Zymoseptoria halophila (CPC 18105). a. Pycnidia forming on barley leaves in vitro, with oozing conidia cirrus; b–e. conidiogenous cells formed in pycnidia; f. conidia (Type I); g. colony with yeast-like growth on synthetic nutrient-poor agar; h, j–l. conidia formed as phragmospores in aerial hyphae (Type II); i, m. conidia formed via microcyclic conidiation (Type III). — Scale bars = 10 μm.
**Zymoseptoria tritici** (Desm.) Quaedvlieg & Crous, *comb.* nov.
— MycoBank MB517926; Fig. 8

Basionym: *Septoria tritici* Desm., *Ann. Sci. Nat., Bot.,* sér. 2, 17: 107 (1842).

Teleomorph: ‘*Mycosphaerella* graminicola’ (Fuckel) J. Schröt., in Cohn, *Krypt.-Fl. Schlesien* 3, 2: 340. 1894 (‘1893’).

Basionym: *Sphaeria graminicola* Fuckel, *Fungi Rhenani Exsiccati* no. 1578. 1865.
≡ *Sphaerella graminicola* (Fuckel) Fuckel, *Jahrb. Nassauischen Vereins Naturk.* 23–24: 101. 1870.

Specimens examined. FRANCE, on *Triticum* sp. (holotype of *Septoria tritici*; PC). – GERMANY, Oestrich, on *Triticum repens*, Fuckel, *Fungi Rhenani Exsiccati* no. 1578 (L, isotype of *Mycosphaerella graminicola*). – NETHERLANDS, Brabant West, on *Triticum aestivum*, coll. R. Daamen, 6 May 1981, isol. as single conidium, W. Veenbaas, 810507/1, 7 May 1981, epitype designated here CBS H-20545, including teleomorph material on *Triticum* leaf of heterothallic mating IPO 323 (MAT 1-1) × IPO 94269 (MAT 1-2), culture ex-epitype IPO 323 = CBS 115943.

Notes — The isolate designated here as ex-epitype (IPO 323 = CBS 115943) is also the strain used in the whole genome amplification and sequencing of this species ([http://genome.jgi-psf.org/Mycgr3/Mycgr3.download.html](http://genome.jgi-psf.org/Mycgr3/Mycgr3.download.html)).

**DISCUSSION**

For many years the genus *Mycosphaerella* has been treated as a wide general concept to accommodate a range of related and unrelated species and genera that have small ascomata, and hyaline, 1-septate ascospores, without pseudoparaphyses (Aptroot 2006). The observation that *Mycosphaerella*-like teleomorphs were linked to more than 40 different anamorphs (Crous 2009) was thus seen as rather odd, though acceptable within this wider concept used to accommodate these thousands of mostly phytopathogenic fungi. It was only in recent years when the higher order phylogenetic relationships of *Mycosphaerella* was addressed as part of the Assembling the Fungal Tree of Life initiative (Schoch et al. 2006), that *Mycosphaerella* was shown to be polyphyletic (Crous et al. 2007), even containing different families within the *Dothideomycetes* (Crous et al. 2009a, b, Schoch et al. 2009a, b).

The fact that *Septoria* also contains significant morphological variation was commented on by Sutton (1980), who stated that the genus is heterogeneous, and should be revised, containing conidiomata that ranged from acervuli to pycnidia, and conidiogenesis that ranged from blastic sympodial to annellidic (percurrent proliferation) or phialidic (with periclinal thickening). As can be seen with the taxa treated to date, however, these characters alone are also insufficient to delineate all natural...
genera, as several modes of conidio genesis or conidial types occur within the same genus in the Septoria-like complex. Part of the reason for the confusion surrounding the genus Septoria is based on the fact that until now no DNA sequence data were available for the type species, *S. cytisi*. Due to the lack of cultures of this species, DNA was subsequently extracted from several herbarium specimens. Using this technique, however, some intraspecific variation was observed in both the LSU and ITS sequences of *S. cytisi*. This could possibly be explained by geographical and temporal spread in the sampling sites, spanning 54 years from a region encompassing South and Central Europe, making some sequence variation within these specimens probable. Even if one or two nucleotides might actually be scored wrong in the US0378994-derived LSU sequence for *S. cytisi*, this would not have any impact on the phylogenetic position of *S. cytisi* within the Septoria s.str clade, its nearest sister genus being *Cercospora* in the Mycosphaerellaceae (Groenewald et al. 2006).

As shown in the present study (Fig. 2), the genus *Mycosphaerella* is unavailable to accommodate the taxa occurring on graminicolous hosts, as *Mycosphaerella* is restricted to species with *Ramularia* anamorphs (Verkley et al. 2004a, Crous et al. 2009b). Furthermore, *Septoria* s.str. also clusters apart from the species on cereals (Fig. 3), making the name *Septoria* unavailable for these pathogens.

In the present study we introduce a novel genus *Zymoseptoria* to accommodate the Septoria-like species occurring on graminicolous hosts. Although species of *Zymoseptoria* tend to have phialides with apical periclinal thickening, this mode of conidio genesis has also evolved in *Septoria* s.str. (e.g. *S. apicina*), and is not restricted to *Zymoseptoria*. More importantly, species of *Zymoseptoria* exhibit a yeast-like growth in culture, and have up to three different conidial types that can be observed, namely Type I (pynidial conidia), Type II (phragmospores on aerial hyphae), and Type III (yeast-like growth proliferating via microcyclic conidiation). Introducing a novel genus for this group of important plant pathogens was not taken lightly, as *Z. passerinii* causes septoria speckled leaf blotch (SSLB) on barley (*Hordeum vulgare*), and has been reported around the globe on this crop (Matthe 1997, Cunfer & Ueng 1999, Goodwin & Zismann 2001, Ware et al. 2007). *Septoria tritici* blotch (STB) is caused by *Z. tritici* (teleomorph *Mycosphaerella graminicola*), and is currently present in all major wheat growing areas. This disease is consistently ranked amongst the most damaging wheat diseases in Australia, Europe, North and South America, and in Europe more than 70 % of all the fungicides applied to wheat are to control STB (Eyal et al. 1987). Wheat, together with maize and rice directly contribute 47 % to global human consumption (Tweeten & Thompson 2009). Since 1961, wheat production has increased globally with almost 300 % on a virualy stable cultivation area of c. 200 M ha. This progress was largely achieved by increased average yields (FAO 2010). However, the annual growth rate of global wheat production cannot meet the global market requirements in the coming four decades (Fischer et al. 2009, Fischer & Edmeades 2010).

Although *Z. passerinii* and *Z. tritici* share many similarities (Goodwin et al. 2001) (Fig. 3, 4), both pathogens having a dimorphic lifestyle (Mehrabi et al. 2006); a major difference between them is that *Z. tritici* has a year-round and very active sexual cycle (Shaw & Royke 1993, Kema et al. 1996, Zhan et al. 2003), whereas there have been no reports of a sexual cycle for *S. passerinii* observed in nature, despite isolates of *S. passerinii* having opposite mating types being commonly found in natural populations, even on the same leaf (Goodwin et al. 2003), suggesting cryptic sex does exist for *Z. passerinii* (Ware et al. 2007). With respect to the two additional species treated in the present study, *Z. brevis* and *Z. halophila*, almost nothing is known about their relative importance, geographical distribution, host range or sexual behaviour. Given the importance of their known host crops, however, this complex is in dire need of further study.

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