Combined morphological and molecular approach for identification of *Stemphylium vesicarium* inoculum in pear orchards

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Abstract

Stemphylium vesicarium is the causal agent of brown spot of pear (BSP), an important disease reported in pear-growing areas of Europe. The pathogen is able to colonize pear leaf debris and dead tissues of herbaceous plants on the ground and produce abundant ascospores and conidia that are capable of infecting pear trees. Inoculum monitoring in pear orchards is mainly achieved through spore traps and species identification is based on conidial morphology, but the similarities on conidial traits among species of *Stemphylium* make correct identification difficult. In this work a total of thirty-seven *Stemphylium* isolates recovered from different sources in pear orchards were characterized at the morphological, pathogenic and molecular level. Correspondence among ITS and *gpd* sequences and morphological traits were evaluated in order to determine their applicability in identification of *S. vesicarium*. Species identification

based exclusively on morphological data was not feasible. Three different morphological groups were resolved according to colony and conidial morphology, but conidial dimensions of these groups were in the range described for *S. vesicarium*. Molecular analyses of the ITS and *gpd* sequences clustered field isolates in the *S. vesicarium - S. herbarum-S. alfalfae- S. tomatonis* and *S. sedicola* (84%), *S. eturmiunum* (13.5%), and *S. gracilariae* (2.5%) species groups. Combined morphological and molecular data were necessary for unambiguous identification of isolates in the *S. vesicarium* species group. Only isolates identified as *S. vesicarium* were pathogenic on pear. In this taxonomic group, differences in pathogenicity were observed, mainly related to their origin. Most isolates recovered from lesions (87%) were pathogenic, whereas 60% of isolates recovered from air samples or from nonhost plant species were nonpathogenic on pear. The study revealed that several species of *Stemphylium* coexist in pear orchards with *S. vesicarium*, the causal agent of BSP, and that combined morphological and molecular data are needed to differentiate them. Consequently, direct measurements of the airborne inoculum using volumetric spore traps and conidia may overestimate the actual pathogen population and its pathogenic potential.

KEY WORDS

Brown spot of pear; *gpd* partial sequence; internal transcriber spacer; pathogenicity; sequencing; phylogeny

ABBREVIATIONS

BSP: brown spot of pear

ITS: internal transcriber spacer

Gpd: glyceraldehyde 3-phosphate dehydrogenase

1. Introduction

The genus *Stemphylium* Wallr. was established in 1833 (Wallroth 1833) and comprises up to 150 species (Wang and Zhang 2006), with *S. botryosum* Wallr. as the type species. The sexual state in species with known teleomorphs is *Pleospora sp*. *Stemphylium sp*. has been described as saprotrophic and pathogenic on a wide range of plants (Farr *et al.* 1989). Pathogenic forms cause yield reduction and economic losses in horticultural and fruit tree crops. *S. vesicarium* is the causal agent of brown spot of pear (Ponti *et al.* 1982), as well as purple spot in asparagus, and leaf spot in alfalfa, onion and garlic (Falloon *et al.* 1984, Chaisrisook *et al.* 1995, Shishkoff and Lorbeer 1989).

Brown spot of pear (*Pyrus communis* L.) (BSP) is an important disease reported in pear-growing areas of Europe, including Spain, Italy, France, The Netherlands, Belgium and Portugal (Llorente and Montesinos 2006). Disease symptoms consist of necrotic lesions on leaves, twigs and fruit. Infected fruit show small necrotic spots that progressively enlarge, deepen and can rot, making fruit unmarketable (Llorente and Montesinos 2006, Rossi *et al.* 2007). Chemical control of BSP is based on preventive fungicide sprays applied during the vegetative period based on infection risk potential predicted by the BSPcast model (Montesinos *et al.* 1995, Llorente *et al.* 2000). *S. vesicarium* is able to colonize pear leaf debris and dead tissues of herbaceous plants on the ground and produce abundant ascospores and conidia that are capable of infecting pear trees (Rossi *et al.* 2005, Llorente *et al.* 2006). Disease control has been improved with sanitation methods that reduce inoculum in the orchard (Rossi and Pattori 2009, Llorente *et al.* 2010). Assessment of inoculum potential in the orchard environment

increases the efficacy of disease control methods. Generally, inoculum monitoring is achieved through spore traps and identification of *Stemphylium* species relies on morphological and developmental characters such as variation in conidia, conidiophore and ascospore morphology (Simmons 1969). However, many of these characters overlap among species in the genus, making it difficult to distinguish *S. vesicarium* from other *Stemphylium* species.

Phylogenetic studies based on analysis of the nuclear internal transcribed spacer (ITS), mitochondrial small subunit (mtSSU) and gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*) have inferred phylogenetic relationship among species in the genus *Stemphylium* and morphological and phylogenetic concordance in species delimitation (Câmara *et al.* 2002, Pryor and Bigelow 2003, Kodsueb *et al.* 2006, Inderbitzin *et al.* 2009). These studies concluded that *S. vesicarium*, *S. alfalfae* and *S. herbarum* are nearly identical in ITS and *gpd* loci and their separation into separate species is accomplished on the basis of morphological and developmental characters (Câmara *et al.* 2002, Inderbitzin *et al.* 2009).

The purpose of our work was to identify *Stemphylium* isolates recovered from different sources in pear orchards, using traditional morphological methods and molecular tools. Correspondence among ITS and *gpd* sequences, morphological traits and pathogenicity on pear was evaluated, in order to determine their applicability in identification of *S. vesicarium* and closely related species, and to increase our knowledge of *S. vesicarium* inoculum in pear orchards.

2. Materials and methods

Fungal isolates

Stemphylium spp. isolates were recovered from different sources in thirteen North-eastern Spanish pear orchards, in which the disease had been observed in previous years (Table 1). Isolates were obtained from air samples, lesions in pear leaves or fruit, necrotic pear leaf debris and necrotic or healthy tissues of nonhost plant species. All isolates were deposited in the Culture Collection of the Institute of Food and Agricultural Technology (INTEA, EPS Girona, Spain). For isolation of *Stemphylium* spp., 1×1 mm segments of selected healthy or necrotic tissues were placed on PDA plates and incubated for 10 days at 22.5 °C. Air samples from 1 m above ground were collected on *Alternaria radicina* selective agar plates (ARSA; Pryor et al. 1994) mounted on a microbial air sampler (Sampl'air LITE, AES Chemunex, bioMérieux Inc., France) at a flow rate of 100 l/min, and incubated for 5 days at 22.5 °C. Plates were observed at 100 X magnification and spores of *Stemphylium* spp. were transferred with a sterile needle to tomato agar plates (800 ml of distilled water, 10 g tomato concentrate, 2.3 g $CaCO_3$ and 12.8 g agar) and incubated for 7-10 days at 22.5°C with 16 hour-light photoperiod. The shape and size of conidia were used as the main criteria to distinguish and select *Stemphylium* spp. isolates (Simmons, 1969). Single-spore cultures of isolates were stored on PDA slants at 4°C.

A total of thirty-two isolates representing different sources and orchards were selected for this study. Additionally, five isolates recovered from pear orchards by European research laboratories were evaluated. Culture collection ex-type specimens of *S. vesicarium*, *S. botryosum* and *S. eturmiunum* were also included (Table 1).

Morphological analysis

Morphological observations were made from 10 day-old cultures grown on tomato agar at 22.5 ºC and 16 hour-light photoperiod in a growth cabinet (I-30BLL Percival Plant

126 Biology Chamber, Percival Scientific Inc., USA). Five agar plates were incubated per
127 isolate. Conidial suspensions in sterile distilled water were obtained for each plate at the
128 end of the incubation time and three 20 µl-aliquots were analyzed per plate.
129 Morphometric measures of conidia were done according to Simmons keys for
130 identification of *S. vesicarium* and other species (Simmons 1969, 1985). Maximum
131 length and width of 20 randomly selected mature conidia from each aliquot were
132 measured and the ratio length/width (l/w) was calculated. Observations were performed
133 at 200X and 400X with an optical microscope (Axio Scope A1, Carl Zeiss, Göttingen,
134 Germany). Photomicrographs were taken with a digital camera (AxioCam MR Carl
135 Zeiss Light Microscopy, Göttingen, Germany) mounted on the microscope, and conidial
136 dimensions were measured using the Axio Vision LE 4.7.1 (Carl Zeiss Microscopy
137 GmbH, Munich, Germany) software. Differences in conidial dimensions were
138 investigated with one way ANOVA by using the GLM procedure of the SAS system
139 (SAS Institute, Cary, NC, USA).
140
141 *Pathogenicity test*
142 Pathogenicity tests with isolates and ex-type strains were conducted on young detached
143 leaves of cv. Conference pear plants grown in the greenhouse. The leaves were
144 disinfected by immersion for 5 min in a sodium hypochlorite solution (1% active
145 hypochlorite), rinsed three times in sterile distilled water and inoculated with four 30 µl-
146 aliquots of the conidial suspensions (1-5 × 10^5 conidia ml^{-1}). Two inoculations were
147 made on each side of the midvein. The leaves were incubated into humid plastic boxes
148 for 7 days at 25°C/16-h light photoperiod in a controlled environmental chamber (MLR-
149 350 Growth Cabinet, SANYO, Japan). Three replicates of three leaves per replicate
150 were inoculated with each isolate. The experiment was conducted twice. Disease

151 incidence and severity (diameter of necrotic area in mm) were recorded at the end of the
152 incubation time. An isolate was considered pathogenic when progressive necrosis was
153 observed surrounding the inoculation point and mean severity values were higher than
154 2.5 mm diameter.
155
156 *DNA extraction, PCR amplification and sequencing*
157 Monosporic stock cultures of each isolate were grown in 50 ml of potato dextrose broth
158 in 250 ml Erlenmeyer flasks on an orbital shaker (150 rpm) at room temperature for 10
159 days. Mycelial mats from cultures were collected by filtration, transferred to sterile
160 plastic Petri dishes and frozen at -80 ºC (Ultra-Low Temperature Freezer, MDF-
161 U5186S, Sanyo, Japan). After drying, overnight samples were lyophilized
162 (Freezemobile 12SE, the Virtis Company, NY) and ground (25-50 mg) in liquid
163 nitrogen using a mortar and pestle. DNA was extracted from the powered tissue using a
164 DNeasy™ Plant Mini Kit (QIAGEN) according to the manufacturer's instructions.
165 DNA extracts were quantified with a spectrophotometer (Nanodrop ND-1000, UV-Vis
166 Spectrophotometer, Thermo Fisher Scientific, USA) and samples were stored at -20 ºC.
167 Sequences of the internal transcribed spacer ITS1, 5.8S and ITS2 regions of the nuclear
168 ribosomal DNA (rDNA) were amplified using primers ITS1F (Gardes and Bruns 1993)
169 and ITS4 (White *et al.* 1990). Sequences of *gpd* gene were amplified using primers
170 GPD1 and GPD2 (Berbee *et al.* 1999). 50 µl PCR mixture for each PCR amplification
171 contained PCR Buffer 1X, $MgCl_2$ 2 mM, dNTP's 0.4 mM, 0.4 µM of each primers
172 ITS1F and ITS4 (ITS) or GPD1 and GPD2 (*gpd*), 2 U Invitrogen Taq Polymerase and 5
173 µl of DNA (10-50 ng). Amplifications were performed for 35 cycles with an initial 3
174 min at 94 ºC for denaturation and a final 10 min at 72 ºC for the extension with a
175 GeneAmp® PCR System 9700 (Applied Biosystems, USA). Each cycle consisted of 30

s at 94 ºC, 1 min at 57 ºC and 1.5 min at 72 ºC. Successful amplification was checked by electrophoresis of a 8-µl aliquot of the reaction mixture in a 1.2% w/v agarose gel that was stained with etidium bromide and viewed by UV-illumination. PCR products were purified using a QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer. Sequencing of the purified PCR amplification products was done in both directions using the same primers as for the amplification, with the BigDye™ Terminator Cycle Sequencing system (v3.1 PeE kit, Applied Biosystems), using an ABI PRISM™ 310 DNA Sequencer (Applied Biosystems).

Phylogenetic analyses

Sequences were edited using Chromas 2.33 (Technelysium Pty. Ltd.). Alignments were manually inspected for ambiguities and adjustments were made when necessary by using BioEdit v7.0.9 (Hall 1999) and ClustalW (Thompson *et al.* 1994). In order to compare *Stemphylium* isolates used in this study with closely related species, sequences obtained from each isolate were combined with sequences obtained from GenBank, derived from ex-type cultures of 22 named species analyzed in previous phylogenetic studies (Câmara *et al.* 2002; Inderbitzin *et al.* 2009) (Table 2). *Alternaria alternata* (DQ323699 and AY278808, for ITS and gpd sequences, respectively) was used as the outgroup. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al.* 2011). The neighbor-joining (NJ) and the maximum parsimony (MP) methods of phylogenetic inference were used for construction of the phylogenetic trees. In the NJ analyses, all characters were weighted equally, and the Kimura 2-parameter distance calculation method was used. In the MP analysis trees were inferred by using the heuristic search option with tree bisection reconnection (TBR) algorithm and search level 2 in which the

initial trees were obtained by the random addition of sequences (100 replicates). Branch length was calculated using the average pathway method. In both analyses the topology was tested with 1000 bootstrap trials. Concordance between datasets was evaluated with the partition-homogeneity test implemented in PAUP phylogenetic software (version 4, Sinauer Associates Inc., Sunderland, Massachusetts). The combined analysis was run using the parameters described above. Alignments were submitted to TreeBASE as S14571.

3. Results

Morphological and pathogenic analysis

Growth on tomato agar of field isolates and reference strains of *Stemphylium* species was slow, reaching 90 mm diameter after 10 days incubation at 22.5 ºC. A teleomorph was observed at the end of incubation period in cultures of all field isolates. Colony and conidial morphology and dimensions are listed in Table 1. Mean conidial length (28.7±5.8 µm), width (15.3 ±4.2 µm) and ratio (1.9±0.4) of field isolates, except for EPS45, were included into the range described for *S. vesicarium*. Conidium size in EPS45 was intermediate between *S. botryosum* and *S. vesicarium* (Simmons 1967, 1969), and the l/w ratio (1.40 µm) was smaller than that of *S. vesicarium* ex-type strains ATCC18521 and CBS311.92, and similar to that of *S. botryosum* type strain CBS 714.68 (Table 1). Despite this, analysis of variance indicated that conidial dimensions of all field isolates were not significantly different ($P>0.1$), and they could be assigned to *S. vesicarium*. Regarding colony traits and conidial morphology, field isolates were distributed into three different morphological groups (Table 1). The largest group (I) included thirty-four isolates and the two *S. vesicarium* ex-type strains, which developed

cottony pale brown colonies with radial growth and white to brown mycelium. Mature conidia were oblong, brown with a densely verrucose external wall, with 3-5 transverse septa, 2-3 longitudinal septa and 3 transverse constrictions, consistent with type descriptions for *S. vesicarium* (Simmons 1969). Morphological group II included isolate EPS45, which produced velvety dark brown colonies with mycelium growing within the agar and the conidia were broadly ovoid or semispherical, golden-brown and verrucose, similar to characteristics reported for *S. botryosum* (Simmons, 1969) and to those observed in the type strain *S. botryosum* CBS 714.68. Group III comprised isolates EPS118 and EPS146 that produced white to gray non radial colonies that turned brown with age and the mature conidia were ellipsoid-oblong brown or dark brown. These isolates could not be clearly attributed to one of the described *Stemphylium* species.

In total 51% of field isolates were pathogenic in pear leaf assays. The pathogenic group included 80 % of the isolates recovered from lesions, 50 % of isolates from nonhost plants (epiphytic or saprophytic), and 25% of aerial isolates (Table 1). Pathogenic isolates developed necrotic lesions that expanded through the leaf. *S. vesicarium* ex-type strains ATCC 18521 and CBS 311.92 isolated from pea and onion, respectively, were nonpathogenic on pear leaves, nor were the ex-type strains of the other *Stemphylium* species included in the study.

PCR amplification, sequencing and alignment

PCR of 37 field isolates and 4 ex-type strains resulted in the amplification of 600-650 bp fragments using primers ITS1F and ITS4, and 580-600 bp fragments using primers GPD1 and GPD2. ITS and *gpd* sequences of the thirty-two Spanish *Stemphylium* isolates determined in this study were submitted to GenBank (GU065719, JQ988073 to JQ988103 for ITS, and GU065720, JX075152 to JX075182 for *gpd*). Five different

genotypes were obtained among the field isolates. Twenty-four isolates had identical sequences in the ITS and *gpd* regions, which were also identical to sequences of *S. vesicarium* ex-type strains CBS311.92 and ATCC18521, and five additional field isolates differed in only one or two substitution/deletions. These two groups included all isolates recovered from pear lesions and 40 % of isolates from air or nonhost plant samples, as well as isolate EPS45, assigned to morphological group II (Table 1). The third group differed from the former group by 5 (ITS) or 8 (*gpd*) substitution/deletions and was composed of isolates EPS39, EPS40, EPS51, EPS55 and EPS70. These isolates were recovered from air samples and assigned to morphological group I in this study. Saprophytic isolate EPS56, assigned morphological group I, differed from the former group by 2 deletions (ITS) and 6 substitutions (*gpd*). Finally, sequences of isolates EPS118 and EPS146, recovered from air samples and placed in morphological group III, differed by 4 insertions and 5 substitutions (ITS) and 37 substitutions, 6 insertions and one deletion (*gpd*) from the major genotypic group.

Phylogenetic analysis

Each locus was first analyzed separately, reducing each alignment to one isolate per genotype to speed up analyses. Alignment of ITS sequences of field isolates and ex-type strains with those of 22 named species of *Stemphylium* resulted in a 512 character dataset, of which 56 characters (10.9 %) were polymorphic and 12 (2.3%) were parsimony informative. Alignment of the *gpd* region resulted in a 517 character dataset, of which 134 (25.9 %) were polymorphic and 96 (18.6 %) were parsimony informative. The partial *gpd* sequence showed a higher number of variable sites (substitution or deletion) than the ITS region within the *Stemphylium* species. The two methods of phylogenetic inference used, MP and NJ, recovered the same topology for both loci.

Parsimony analysis of the ITS found 29 most-parsimonious trees (85 steps, CI=0.9058, RI=0.8840, and RC=0.8008). A total of 15 most parsimonious trees (230 steps, CI=0.6453, RI=0.8013, and RC=0. 5171) were obtained for the *gpd* gene. Species of *Stemphylium* formed a well-supported clade in parsimony analyses of the ITS region and *gpd* gene with bootstrap values of 100%. ITS and *gpd* trees were similar to those reported in previous studies using these loci (Câmara *et al.* 2002, Inderbitzin *et al.* 2009); more subgroups were supported in the *gpd* tree than in the tree constructed from the ITS data (data not shown). The partition homogeneity test of the combined ITS and *gpd* alignment revealed that the data were not significantly disconcordant (P=0.12), therefore NJ and MP analyses were conducted on the combined dataset. Maximum-parsimony analysis of combined ITS and *gpd* dataset yielded 20 most-parsimonious trees (296 steps, CI=0.770, RI= 0.792, RCI=0.610), one of which is shown (Fig. 1). The two loci resolved a monophyletic *Stemphylium* with seven well-supported groups (> 73% bootstrap), five of which corresponded to previously established A-C and E-F groups (Câmara *et al.* 2002, Inderbitzin *et al.* 2009). Group D, defined by *S. callistephi* and *S. solani* according to Câmara *et al.* (2002), formed a grade. These two species did not cluster together and were resolved separately from the other *Stemphylium* species (100% and 68% bootstrap, respectively), as reported previously (Wang *et al.* 2010) (Fig. 1). The phylogeny for the combined sequences resembled that of the individual *gpd* dataset, in which *S. vesicarium, S. herbarum, S. alfalfae, S. tomatonis* and *S. sedicola* clustered together in clade C and *S. gracilariae, S. majusculum* and *S.gigaspora* clustered in separated subgroups in this clade.

The analysis has provided an overview of relationships among the 37 isolates sequenced in this study. Field isolates were nested in two of the clades designated by Câmara *et al.* (2002) (Fig. 1). Most isolates, (N=35) clustered in group C, represented by the largest

number of *Stemphylium* species. Twenty-nine isolates (including isolate EPS45 identified as *S. botryosum* according to morphology) clustered together in a subgroup composed of *S. vesicarium, S. herbarum, S. alfalfae, S. tomatonis* and *S. sedicola* (91% bootstrap). Isolate EPS56 formed a monophyletic subgroup with *S. gracilariae* (99% bootstrap) and isolates EPS39, EPS40, EPS51, EPS55 and EPS70 clustered in the subgroup represented by *S. eturmiunum*. Isolates EPS118 and EPS146 clustered in a well-supported subgroup (98% bootstrap) close to *S. paludiscirpi* and *S. trigloghinicola*, in the monophyletic group E. Species assignment of these two isolates based on molecular data was not feasible. Finally, the ex-type strains sequenced in this study clustered in the corresponding species-group (Fig. 1).

4. Discussion

Previously reported descriptions and molecular analysis of *Stemphylium* species were carried out with isolates recovered from lesions in host plant species (Metha 2001, Inderbitzin *et al.* 2009, Câmara, *et al.* 2002, Köhl *et al.* 2009, Wang *et al.* 2010). In our work, epiphytic, saprophytic and aerial inoculum was also analyzed, thus revealing the difficulty in assigning isolates of *Stemphylium* to a species based exclusively on morphological or molecular data. However, combined molecular and morphological analyses permitted unambiguous identification of most isolates at the species level.

Although morphological analysis indicated that three groups of isolates could be recognized based on culture growth and colony morphology, all isolates were attributed to *S. vesicarium* according to conidial dimensions and septation pattern described by Simmons (1969). These results reveal that identification of *Stemphylium* species based exclusively on conidial size can lead to misidentification of species, mainly for those where dimensions overlap, as occurred in nine out of thirty-seven isolates. In fact,

additional morphological characters based on conidiophore or culture traits have been included in the taxonomy of *Stemphylium* species to provide complementary information for proper identification (Simmons, 1967, 1969, 2001, 2004).

Phylogenetic analysis of ITS and *gpd* sequences performed in the present study confirmed previous reports on the taxonomy of *Stemphylium* (Câmara *et al.* 2002, Inderbitzin *et al.* 2009), with the same groups delineated by these authors, except for group D. In agreement with Wang *et al.* (2010), *S. callistephi* and *S. solani* were resolved as phylogenetically distinct groups from the other species analyzed. *S. vesicarium*, *S. herbarum*, *S. alfalfae*, *S. tomatonis* and *S. sedicola* grouped in the same clade, and could not be differentiated on the basis of molecular data. As reported previously, according to multilocus studies, these species should be synonymized (Inderbitzin *et al.* 2009; Wang *et al.* 2010). Morphological and developmental characters were used to define species in this group (Simmons 2001). All isolates recovered from pear lesions and most epiphytic and saprophytic isolates belonged to genotypic group C, and morphological data were useful for their identification as *S. vesicarium*. Consequently, molecular and morphological data should be combined for unambiguous identification of *S. vesicarium* isolates.

By contrast, several isolates with morphological traits similar to *S. vesicarium* were unambiguously attributed to *S. gracilariae* and *S. eturmiunum* species based on phylogenetic analyses of *gdp* or the ITS-*gdp* combined data. *S. gracilariae* was first reported as a marine fungus, but it was detected later on cabbage plants (Inderbitzin *et al.* 2009). Our findings support the suggestion that this species is not confined to the marine habitat. *S. eturmiunum* has been described as the causal agent of leaf blight of onion (Fernández and Rivera-Vargas 2008), and postharvest mold in tomato (Andersen and Frisvad 2004). The presence of *S. eturmiunum* in air samples of pear orchards

agrees with its ubiquitous distribution in soil, dead or decaying plants, as well as being considered as an airborne mold. In addition, two isolates recovered from air samples formed a sister clade of *S. triglochinicola* and *S. paludiscirpi* in group E. Conidial morphology and culture growth of these isolates did not agree with descriptions of *S. triglochinicola*, reported as saprophytic on a dead leaf of *Triglochin maritimum* on the coasts of the United Kingdom and Norway (Webster 1969, Anonymous 2013), nor of other species in group E, including *S. paludiscirpi* and the newly described species *S. phaseolina* (Wang *et al.* 2010), *S. cucumis* and *S. lycii* (Pei *et al.* 2011). From five to more than thirty changes were observed in *gpd* sequences of Spanish field isolates compared to named species of group E. The clade that contained these two unnamed isolates might be a new species employing GCPSR criteria (Inderbitzin *et al.* 2009).

The ex-type strains isolated from lesions in pea and onion were nonpathogenic on pear, nor were the eight isolates identified as *S. gracilariae, S. eturmiunum* or unnamed species. Our results agree with previous studies in which *S. vesicarium* isolated from onion and asparagus, *S. herbarum* from different hosts and *S. botryosum* from *Medicago* have been reported as nonpathogenic on pear (Köhl el al 2008, 2009). These findings suggest some degree of host specificity in plant pathogenic species of *Stemphylium*. Additionally, not all field isolates identified as *S. vesicarium* were pathogenic on pear, which indicates some are saprophytic (Llorente *et al.* 2010). The nonpathogenic group included isolates recovered from pear lesions, nonhost plants and air samples.

Our findings point out that several species of *Stemphylium* coexist with *S. vesicarium* in pear orchards and that not all inoculum is pathogenic on pear. The presence of *Stemphylium* species in air inoculum or growing as saprobes in pear orchards, which are nonpathogenic on pear, have a direct impact in the inoculum potential assessment,

because direct measurements of airborne inoculum using volumetric spore traps may overestimate the *S. vesicarium* population. Moreover, differentiation of pathogenic and nonpathogenic isolates of *S. vesicarium* is important in order to estimate the actual pathogenic potential of inocula in an orchard. ITS and *gdp* sequences of *S. vesicarium* isolates have shown to be highly similar despite having different pathogenic profiles, so they are not useful for distinguishing pathogenic from nonpathogenic isolates. Preliminary characterization of *S. vesicarium* isolates for pathogenicity and for DNA polymorphism using different PCR-based techniques (RAPD and AFLPs) found a correlation between DNA polymorphism and pathogenicity (Ruz *et al.* 2012). Recently, a specific TaqMan-PCR technique has been developed to detect and quantify pear-pathogenic inoculum of *S. vesicarium* in pear orchards (Köhl *et al.* 2013).

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539 **FIGURE LEGENDS**

540 Fig. 1. One of 20 most parsimonious trees based on the combined regions of the ITS
541 and *gpd* of *Stemphylium* isolates from pear orchards and 22 species of *Stemphylium*.
542 Sequence data for related species were obtained from GenBank. The tree was rooted on
543 sequences of *Alternaria alternata*. The tree was generated by MEGA 5 by using the
544 heuristic search option with tree bisection reconnection algorithm. MP bootstrap values
545 (>50%) from 1000 replicates are indicated at the nodes. Phylogenetic groups from
546 Câmara *et al.* (2002) and Inderbitzin *et al.* (2009) are given by the narrow vertical lines
547 *ex-type strain.
548

1 Table 1. Morphological traits on tomato agar and pathogenicity on pear of *Stemphylium* isolates and *ex-type* strains used in this study.

Code [a]	Origin [b]		Colony [c]	Conidia [c]								Pat [e]	MG [f]
				Morphology		Septa [d]			Dimensions [d]				
	Country (orchard year)	Host plant/ habitat	Texture and color	Color	Shape	TS	LS	TC	Length (µm)	Width (µm)	Ratio (l/w)		
EPS14	ES (G01 1991)	Pear / lesion	Cottony, pale brown	Pale brown	Oblong	3-5	1-2	3	24.4	15.0	1.7	+	I
EPS26	ES (G12 1991)	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-3	3	29.2	14.7	2.0	+	I
EPS39	ES (G02 2007)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	3	23.2	13.2	1.8	-	I
EPS40	ES (G02 2007)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	1	22.5	12.9	1.8	-	I
EPS45	ES (G02 2007)	Pear orchard / air	Velvety, dark brown	Golden brown	Broadly ovoid	2-3	1-2	1-2	30.4	22.1	1.4	-	II
EPS47	ES (T01 2006)	NH/saprophytic	Cottony, pale brown	Brown	Oblong	3-5	1-2	1-2	28.3	16.2	1.8	-	I
EPS48	ES (T01 2007)	NH/saprophytic	Cottony, pale brown	Brown	Oblong	3-5	1-2	3	25.8	12.9	2.0	+	I
EPS49	ES (T02 2007)	NH/saprophytic	Cottony, pale brown	Golden brown	Oblong	3-5	2-3	1 / 3	35.1	21.4	1.7	-	I
EPS51	ES (G03 2007)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	25.7	15.9	1.6	-	I
EPS52	ES (G04 2007)	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	27.4	14.7	1.9	+	I
EPS54	ES (G03 2007)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	30.0	14.0	2.1	+	I
EPS55	ES (G05 2007)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	27.3	15.5	1.8	-	I
EPS56	ES (T02 2006)	NH/saprophytic	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	25.9	13.5	1.9	-	I
EPS61	ES (G06 2007)	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	2-3	25.9	11.7	2.2	+	I
EPS65	ES (T01 2007)	NH/saprophytic	Cottony, pale brown	Dark brown	Oblong	3-5	1-2	1 / 3	31.3	15.3	2.1	+	I

EPS66	ES (G03 2007)	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	3	27.4	14.8	1.9	-	I
EPS70	ES (G05 2007)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	26.2	12.0	2.2	-	I
EPS76	ES (G06 2006)	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	26.6	14.8	1.9	+	I
EPS77	ES (G05 2007)	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	28.2	15.8	1.8	+	I
EPS80	ES (G08 2007)	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	27.0	13.1	2.1	-	I
EPS81	ES (G09 2007)	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	25.8	14.5	1.8	+	I
EPS96	ES (G10 2006)	Pear / lesion	Cottony, pale brown	Dark brown	Oblong	3-5	1-2	1 / 3	28.7	16.2	1.8	-	I
EPS107	ES (G03 2007)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	32.2	16.4	2.0	-	I
EPS110	ES (G03 2008)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	30.8	15.1	2.0	+	I
EPS111	ES (G03 2008)	Pear orchard / air	Cottony, pale brown	Dark brown	Oblong	3-5	1-3	1 / 3	31.6	15.6	2.0	-	I
EPS118	ES (G03 2008)	Pear orchard / air	Cottony, grayish brown	Brown	Ellipsoid-oblong	3-5	1-3	1 / 3	32.2	15.9	2.0	-	III
EPS119	ES (G11 2008)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	2-3	29.6	15.2	2.0	+	I
EPS126	ES (G02 2008)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	28.9	16.4	1.8	+	I
EPS127	ES (G05 2008)	Pear orchard / air	Cottony, pale brown	Dark brown	Oblong	3-5	1-2	3	26.9	13.1	2.1	-	I
EPS141	ES (G05 2008)	Pear orchard / air	Cottony, pale brown	Brown	Oblong	3-5	1-3	1 / 3	31.5	16.8	1.9	-	I
EPS146	ES (G03 2008)	Pear orchard / air	Cottony grayish brown	Brown	Ellipsoid-oblong	3-5	1-3	3	30.0	15.0	2.0	-	III
EPS157	ES (G03 2008)	NH/epiphytic	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	30.3	17.2	1.8	+	I
Fr 3906	FR	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	2-3	3	25.4	14.3	1.8	+	I
PRI 850	NL	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	30.3	16.1	1.9	+	I
PRI 852	NL	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	3-4	33.8	14.6	2.3	+	I
PRI 869	NL	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	30.2	16.4	1.9	+	I
PRI 890	NL	Pear / lesion	Cottony, pale brown	Brown	Oblong	3-5	1-2	1 / 3	31.3	13.7	2.3	+	I

Ex-type strains													
*S. vesicarium** ATCC 18521	CA	Pea	Cottony, pale brown	Brown	Oblong	3-5	1-2	3	31.0	14.9	2.1	-	I
*S. vesicarium** CBS 311.92	NE	Onion	Cottony, pale brown	Brown	Oblong	3-5	1-2	3	32.0	15.8	2.0	-	I
*S. botryosum*** CBS 714.68	CA	Alfalfa	Velvety, dark brown	Golden brown	Broadly ovoid	2-3	1-3	1 / 3	29.1	18.9	1.5	-	II
*S. eturmiunum*** CBS109845	NZ	Tomato	Cottony, dark brown	Brown	Oblong	3	1-3	1 / 3	28.9	15.1	1.9	-	-

* Ex-type strain. ** Type strain deposited as *Pleospora herbarum var. herbarum* and *Pleospora eturmiuna*, respectively.

[a] ATCC: American Type Culture Collection, Manassas (USA). CBS: Centraal Bureau voor Schimmelcultures, Baarns (The Netherlands). EPS: Institute of Food and Agricultural Technology, Girona (Spain). Fr: provided by M. Giraud (Centre Ctifl de Lanxade, France). PRI: Plant Research International, provided by J. Köhl, Plant Research International (The Netherlands).

[b] CA: Canada, ES: Spain, FR: France, NL: The Netherlands, NZ: New Zealand. NH: nonhost plant

[c] Descriptions based on Simmons (1967, 1969, 1985, 2001, 2004)

[d] Septa values (TS: tranverse, LS: logitudinal and TC: transverse constrictions) are the range of 95% confidence interval (180 conidia). Dimension values are the mean of measurements of three replicates of sixty conidia per replicate.

[e] Pat: Pathogenicity on cv Conference detached pear leaves. +: progressive necrosis >2.5 mm diameter; -: necrosis located to inoculated point.

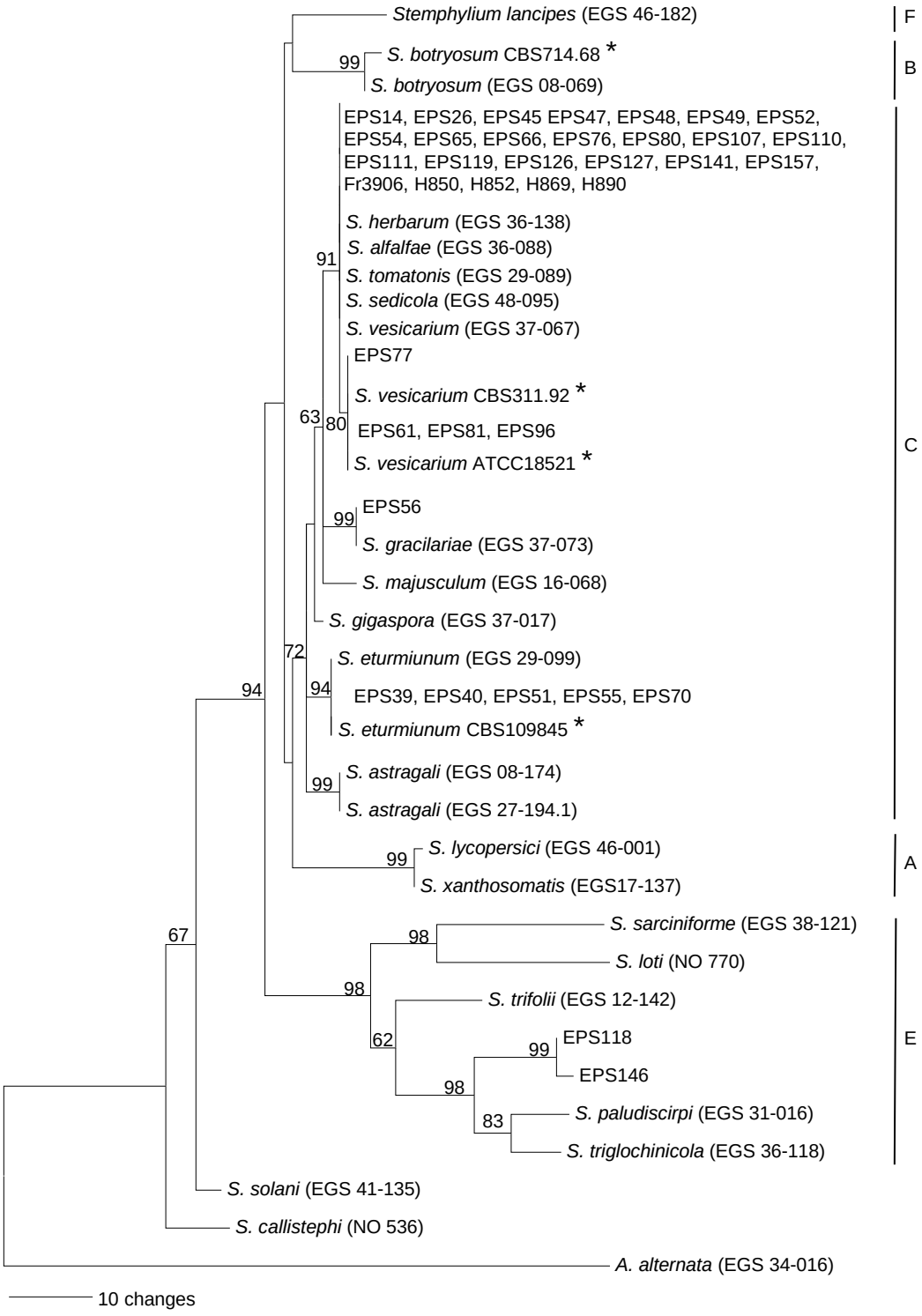
[f] MG: Morphological group assigned in this study according to colony and conidial morphology (conidial dimensions are not considered).

Table(s)

Table 2. Selected *Stemphylium* sequences included in the phylogenetic analysis of ITS and *gpd* regions with GenBank accession numbers.

Species	ID Number	GenBank Accession No.	
		ITS	*gpd*
Stemphylium alfalfae	EGS 36-088	AY329171	AY316971
S. astragali	EGS 08-174	AY329178	AY316980
S. astragali	EGS 27-194.1	AF442777	AF443876
S. botryosum	EGS 08-069	AY329168	AY316968
S. callistephi	NO 536	AF442783	AF443882
S. eturmiunum	EGS 29-099	AY329230	AY317034
S. gigaspora	EGS 37-017	AY329177	AY316978
S. gracilariae	EGS 37-073	AY329217	AY317021
S. herbarum	EGS 36-138	AY329169	AY316969
S. lancipes	EGS 46-182	AY329203	AY317007
S. loti	NO 770	AF442789	AF443888
S. lycopersici	EGS 46-001	AY329216	AY317020
S. paludiscirpi	EGS 31-016	AY329231	AY317035
S. majusculum	EGS 16-068	AY329228	AY317032
S. sarciniforme	EGS 38-121	AY329213	AY317017
S. sedicola	EGS 48-095	AY329232	AY317036
S. solani	EGS 41-135	AY329214	AY317018
S. tomatonis	EGS 29-089	AY329229	AY317033
S. trifolii	EGS 12-142	AY329218	AY317022
S. triglochinicola	EGS 36-118	AY329175	AY316976
S. vesicarium	EGS 37-067	AY329212	AY317016
S. xanthosomatis	EGS 17-137	AY329206	AY317010

Figure(s)


Stemphylium lancipes (EGS 46-182)
F
99
S. botryosum CBS714.68 *
S. botryosum (EGS 08-069)
B
EPS14, EPS26, EPS45 EPS47, EPS48, EPS49, EPS52, EPS54, EPS65, EPS66, EPS76, EPS80, EPS107, EPS110, EPS111, EPS119, EPS126, EPS127, EPS141, EPS157, Fr3906, H850, H852, H869, H890
S. herbarum (EGS 36-138)
S. alfalfae (EGS 36-088)
91
S. tomatonis (EGS 29-089)
S. sedicola (EGS 48-095)
S. vesicarium (EGS 37-067)
EPS77
S. vesicarium CBS311.92 *
63
80
EPS61, EPS81, EPS96
S. vesicarium ATCC18521 *
C
99
EPS56
S. gracilariae (EGS 37-073)
S. majusculum (EGS 16-068)
S. gigaspora (EGS 37-017)
72
S. eturmiunum (EGS 29-099)
94
94
EPS39, EPS40, EPS51, EPS55, EPS70
S. eturmiunum CBS109845 *
99
S. astragali (EGS 08-174)
S. astragali (EGS 27-194.1)
99
S. lycopersici (EGS 46-001)
S. xanthosomatis (EGS17-137)
A
67
98
S. sarciniforme (EGS 38-121)
S. loti (NO 770)
98
S. trifolii (EGS 12-142)
62
99
EPS118
EPS146
98
83
S. paludiscirpi (EGS 31-016)
S. triglochinicola (EGS 36-118)
E
S. solani (EGS 41-135)
S. callistephi (NO 536)
A. alternata (EGS 34-016)
10 changes