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
conidal 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. alfalfa*- *S. tomatonis* and *S. sedicola* (84%), *S.
eturnium* (13.5%), and *S. gracilariarue* (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
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, Kodsubeb *et al.* 2006, Inderbitzin *et al.* 2009). These studies concluded that *S. vesicarium*, *S. alfalfa* 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 (Samp'l'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 CaCO₃ 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 °C and 16 hour-light photoperiod in a growth cabinet (I-30BLL Percival Plant
Biology Chamber, Percival Scientific Inc., USA). Five agar plates were incubated per isolate. Conidial suspensions in sterile distilled water were obtained for each plate at the end of the incubation time and three 20 µl- aliquots were analyzed per plate.

Morphometric measures of conidia were done according to Simmons keys for identification of *S. vesicarium* and other species (Simmons 1969, 1985). Maximum length and width of 20 randomly selected mature conidia from each aliquot were measured and the ratio length/width (l/w) was calculated. Observations were performed at 200X and 400X with an optical microscope (Axio Scope A1, Carl Zeiss, Göttingen, Germany). Photomicrographs were taken with a digital camera (AxioCam MR Carl Zeiss Light Microscopy, Göttingen, Germany) mounted on the microscope, and conidial dimensions were measured using the Axio Vision LE 4.7.1 (Carl Zeiss Microscopy GmbH, Munich, Germany) software. Differences in conidial dimensions were investigated with one way ANOVA by using the GLM procedure of the SAS system (SAS Institute, Cary, NC, USA).

**Pathogenicity test**

Pathogenicity tests with isolates and ex-type strains were conducted on young detached leaves of cv. Conference pear plants grown in the greenhouse. The leaves were disinfected by immersion for 5 min in a sodium hypochlorite solution (1% active hypochlorite), rinsed three times in sterile distilled water and inoculated with four 30 µl- aliquots of the conidial suspensions (1-5 × 10⁵ conidia ml⁻¹). Two inoculations were made on each side of the midvein. The leaves were incubated into humid plastic boxes for 7 days at 25°C/16-h light photoperiod in a controlled environmental chamber (MLR-350 Growth Cabinet, SANYO, Japan). Three replicates of three leaves per replicate were inoculated with each isolate. The experiment was conducted twice. Disease
incidence and severity (diameter of necrotic area in mm) were recorded at the end of the incubation time. An isolate was considered pathogenic when progressive necrosis was observed surrounding the inoculation point and mean severity values were higher than 2.5 mm diameter.

**DNA extraction, PCR amplification and sequencing**

Monosporic stock cultures of each isolate were grown in 50 ml of potato dextrose broth in 250 ml Erlenmeyer flasks on an orbital shaker (150 rpm) at room temperature for 10 days. Mycelial mats from cultures were collected by filtration, transferred to sterile plastic Petri dishes and frozen at -80 °C (Ultra-Low Temperature Freezer, MDF-U5186S, Sanyo, Japan). After drying, overnight samples were lyophilized (Freezemobile 12SE, the Virtis Company, NY) and ground (25-50 mg) in liquid nitrogen using a mortar and pestle. DNA was extracted from the powered tissue using a DNeasy™ Plant Mini Kit (QIAGEN) according to the manufacturer’s instructions.

DNA extracts were quantified with a spectrophotometer (Nanodrop ND-1000, UV-Vis Spectrophotometer, Thermo Fisher Scientific, USA) and samples were stored at -20 °C.

Sequences of the internal transcribed spacer ITS1, 5.8S and ITS2 regions of the nuclear ribosomal DNA (rDNA) were amplified using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Sequences of *gpd* gene were amplified using primers GPD1 and GPD2 (Berbee et al. 1999). 50 µl PCR mixture for each PCR amplification contained PCR Buffer 1X, MgCl₂ 2 mM, dNTP’s 0.4 mM, 0.4 µM of each primers ITS1F and ITS4 (ITS) or GPD1 and GPD2 (*gpd*), 2 U Invitrogen Taq Polymerase and 5 µl of DNA (10-50 ng). Amplifications were performed for 35 cycles with an initial 3 min at 94 °C for denaturation and a final 10 min at 72 °C for the extension with a GeneAmp® PCR System 9700 (Applied Biosystems, USA). Each cycle consisted of 30
s at 94 °C, 1 min at 57 °C and 1.5 min at 72 °C. 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 °C. 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
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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 discordant (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. alfalfa, 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. alfalfa*, *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. eturmiumum*. 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,
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. alfalfa*, *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).

**ACKNOWLEDGEMENTS**

This research was supported in part by grants from Ministerio de Educación y Ciencia (AGL2006-04987/AGR and AGL2009-09829/AGR) of Spain; Comissió Interdepartamental de Recerca i Tecnologia from the Generalitat de Catalunya (2009SGR00812) and BR 10/17 from the University of Girona.

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FIGURE LEGENDS

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

<table>
<thead>
<tr>
<th>Code</th>
<th>Origin</th>
<th>Colony</th>
<th>Conidia</th>
<th>Pat</th>
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<td></td>
<td></td>
<td>Host plant/</td>
<td>Morphology</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>habitat</td>
<td>Color</td>
<td>Shape</td>
<td>TS</td>
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<tr>
<td>EPS14</td>
<td>ES (G01 1991)</td>
<td>Pear / lesion</td>
<td>Cottony, pale brown</td>
<td>Pale brown</td>
<td>Oblong</td>
</tr>
<tr>
<td>EPS26</td>
<td>ES (G12 1991)</td>
<td>Pear / lesion</td>
<td>Cottony, pale brown</td>
<td>Brown</td>
<td>Oblong</td>
</tr>
<tr>
<td>EPS39</td>
<td>ES (G02 2007)</td>
<td>Pear orchard / air</td>
<td>Cottony, pale brown</td>
<td>Brown</td>
<td>Oblong</td>
</tr>
<tr>
<td>EPS40</td>
<td>ES (G02 2007)</td>
<td>Pear orchard / air</td>
<td>Cottony, pale brown</td>
<td>Brown</td>
<td>Oblong</td>
</tr>
<tr>
<td>EPS45</td>
<td>ES (G02 2007)</td>
<td>Pear orchard / air</td>
<td>Velvety, dark brown</td>
<td>Golden brown</td>
<td>Broadly ovoid</td>
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<tr>
<td>EPS47</td>
<td>ES (T01 2006)</td>
<td>NH/saprophytic</td>
<td>Cottony, pale brown</td>
<td>Brown</td>
<td>Oblong</td>
</tr>
<tr>
<td>EPS48</td>
<td>ES (T01 2007)</td>
<td>NH/saprophytic</td>
<td>Cottony, pale brown</td>
<td>Brown</td>
<td>Oblong</td>
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**Ex-type strains**

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<th>Strain</th>
<th>Country</th>
<th>Host</th>
<th>Description</th>
<th>Colour</th>
<th>Shape</th>
<th>Septa</th>
<th>Longevity</th>
<th>LD50</th>
<th>Pathogenicity</th>
<th>MG</th>
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<td>1-3</td>
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* Ex-type strain. ** Type strain deposited as Pleospora herbarum var. herbarum and Pleospora eturmiuna, respectively.

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7 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.

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

10 MG: Morphological group assigned in this study according to colony and conidial morphology (conidial dimensions are not considered).
Table 2. Selected *Stemphylium* sequences included in the phylogenetic analysis of ITS and gpd regions with GenBank accession numbers.

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