The ‘forma specialis’ issue in Fusarium: A case study in Fusarium solani f. sp. pisi

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The Fusarium solani species complex (FSSC) has been studied intensively but its association with legumes, particularly under European agro-climatic conditions, is still poorly understood. In the present study, we investigated phylogenetic relationships and aggressiveness of 79 isolates of the FSSC collected from pea, subterranean clover, white clover and winter vetch grown under diverse agro-climatic and soil conditions within Temperate and Mediterranean Europe. The isolates were characterized by sequencing tef1 and rpb2 loci and by greenhouse aggressiveness assays. The majority of the isolates belonged to two lineages: the F. pisi comb. nov. lineage (formerly F. solani f. sp. pisi) mainly accommodating German and Swiss isolates, and the Fusisorium (Fusarium) solani lineage accommodating mainly Italian isolates. Based on the results of aggressiveness tests on pea, most of the isolates were classified as weakly to moderately aggressive. In addition, using one model strain, 62 accessions of 10 legume genera were evaluated for their potential to host F. pisi, the species known mainly as a pathogen of pea. A total of 58 accessions were colonized, with 25 of these being asymptomatic hosts. These results suggest a broad host range for F. pisi and challenge the forma specialis naming system in Fusarium.

Fusarium solani (sexual morph Nectria haematococca; syn. Haematonectria haematococca) is a filamentous fungus of significant agricultural importance that has been accommodated as a single species in the section Martiella within the genus Fusarium1. Re-evaluation of species taxonomy based on molecular phylogenetic analyses has revealed that F. solani is a species complex (FSSC) which includes at least 60 distinct phylogenetic species2,3. Members of the complex are globally distributed and of considerable ecological plasticity, causing infections in both plants and humans3,5.

Phytopathogenic species within the FSSC include some of the most economically important plant pathogens associated with vascular wilts and root rots in over 100 crops6. Despite the broad host range of the complex as a whole, individual species are often associated with only one or a few plant hosts. Consequently, populations of F. solani pathogenic on plants have been divided into 12 formae speciales and two races7–9.

Early studies on sexual compatibility of special forms and races has already shown that F. solani represents at least seven biological species classified as mating populations (MPs) I-VII, with Nectria haematococca as the most commonly referred sexual morph. Biological species correlated with a host range, as successful sexual crosses were found only among heterothallic isolates within each special form or race11. However, the designation forma specialis may lead to incorrect assumptions concerning the aggressiveness and host specificity of individual isolates. For example, studies on the host range of F. solani f. sp. pisi (MP VI), named by its specific pathogenicity on pea (Pisum sativum), revealed that the species was also pathogenic on chickpea (Cicer arietinum) as well as several other non-legume hosts, such as ginseng (Panax ginseng) and mulberry tree (Morus alba)12,13. Similar results have
been reported for the host range and aggressiveness of *F. virguliforme* (formerly *F. solani f. sp. glycines*) and *F. solani f. sp. eumartii*15. Thus, the term *forma specialis* is often misleading and will most likely need to be reconsidered in the future.

Traditional taxonomic methods for identifying special forms and races rely on morphological criteria, aggressiveness tests and sexual compatibility. They are time consuming, labor intensive and often inconclusive. The use of morphological criteria for diagnostic purposes requires extensive knowledge of classical taxonomy and still remains difficult due to overlapping morphological characters among many closely related species. In the case of aggressiveness tests on a specific host plant, the environmental factors and genetic makeup of the host may have significant influence on the bioassay outcome. Similarly, the biological species concept on the basis of sexual crosses in *F. solani* has several problems including unequal frequencies of mating type alleles in different populations, failure of compatible isolates to reproduce due to male or female dominance, or simply environmental conditions suppressing sexual reproduction1

In the past 10 to 15 years, molecular phylogenetic approaches have been extensively employed to facilitate accurate species identification in the genus *Fusarium*16,17. Polymorphisms in DNA sequences of the translation elongation factor 1 alpha (*tef1*) and the second largest subunit of RNA polymerase II (*rpb2*) have provided a robust and reliable means for phylogenetic species recognition within the FSSC and the genus *Fusarium*18,19. These protein coding gene regions show a high level of sequence polymorphism among closely related species, do not have non-orthologous copies and can be amplified from all species of the genus using single pairs of universal primers20.

The main objectives of this study were to investigate diversity, geographical patterns of host preference and phylogenetic relationships among the FSSC isolates collected from pea, subterranean clover, white clover and winter vetch grown under diverse agro-climatic and soil conditions within Temperate and Mediterranean Europe. To that end, we conducted gene sequence analysis, aggressiveness bioassays and sought to clarify the host range of *F. solani* *f. pisi* by determining the potential of various legumes to symptomatically or asymptptomatically host the fungus. Based on our results, we propose a taxonomic recombination by assigning isolates of *Fusarium solani f. sp. pisi* to *Fusarium pisi* comb. nov. Therefore, in this article the fungus is being referred to as *Fusarium pisi*.

**Results**

**Phylogeny.** Phylogenetic analyses inferred from the *tef1* and the *rpb2* sequences resolved the phylogenetic positions of the 83 isolates studied (including *F. redolens*) in relation to currently recognized monophyletic species in the FSSC complex. The *tef1* data set included 122 strains and consisted of 720 characters including alignment gaps, of which 220 characters were parsimony informative; the *rpb2* data set included 59 strains and consisted of 910 characters with alignment gaps, of which 168 were parsimony informative, and the concatenated *tef1* and *rpb2* gene sequence included 56 strains and comprised 1600 characters including alignment gaps, of which 262 were parsimony informative.

The isolates studied formed four different lineages, all nested within the FSSC clade 3 (Fig. 1). According to the single locus phylogenetic analysis, based on the *tef1* tree, 51 isolates were placed in two closely related subclades in the *F. pisi* lineage: the first group of 27 isolates matched with *F. pisi* comb. nov. (NRRL 22820) with a 75% bootstrap value, and the second group of 24 isolates matched with *Fusarium solani* (FRC 5485) with a 68% bootstrap value. Based on the tree generated from *rpb2* sequencing with representative isolates from both sub-clades, the topological differences did not receive any additional significant support on either *rpb2* or concatenated *tef1* - *rpb2* trees. The same strains that previously formed two sub-clades (*tef1* tree, Fig. 1) were nested in one clade that matched *F. pisi* (NRRL 22820) with some strains showing low intraspecific variation (Figs 2 and 3). Thus, based on the phylogenetic network obtained from concatenated gene trees, all 51 strains were assigned to a single species i.e. *F. pisi*. With the exception of Fs66 recovered from the roots of subterranean clover grown in Italy and Fs76 recovered from white clover roots grown in Sweden, the strains nested in the *F. pisi* lineage originated from different localities in Germany and Switzerland. These strains were recovered from all four legume hosts and included 25 strains from subterranean clover, 7 strains from winter vetch, 16 strains from pea, one strain from faba bean roots and one strain recovered from yard waste compost.

A group of 25 isolates were placed in the *Fusarium solani sensu stricto* (FSSC 5) lineage, recently assigned an epitype specimen by Schroers et al.2 to define the species *Fusarium solani s. str.*, which was recombined by the same authors in *Fusisporium as Fusisporium solani*. Similar to *F. pisi*, the relative distances using the single and the combined gene analyses (two locus tree) revealed intraspecific variation in *Fusisporium (Fusarium) solani s. str.* (Figs 1–3). This lineage primarily accommodated isolates recovered from subterranean clover and winter vetch roots collected in Italy. The few exceptions included six strains collected in Germany and Switzerland, namely two pea, one cherry and two white clover strains from Germany and one subterranean clover strain from Switzerland (Figs 1–3).

The results of the *tef1* tree topology also revealed one strain (FK70) matching with *F. keratoplasticum* (FRC S2477, Fig. 1). Strains Fs29 and Fs30, recovered from compost and hibiscus, respectively, did not cluster with any of the currently recognized FSSC species (Figs 1–3). Based on distances from the nearest neighbor species and high bootstrap support values (>96%) between sub-clusters using both the single and the two gene phylogenetic analyses (*tef1* and the *rpb2* sequences), our results suggest that these two isolates represent at least one new lineage within the FSSC clade 3.

All of the tested isolates were scattered within clade 3 without evidence of phylogenetic structure with respect to host. Four isolates of *F. redolens*, recovered from white clover grown in Sweden were placed in the *F. fujikuroi* species complex and used as an outgroup for species level resolution within the *tef1* locus.

**Taxonomy.** *Fusarium pisi* (Jones) A. Šišić, J. Bačanović-Šišić, S. A. Ahmed & A. M. S. Al-Hatmi, comb. nov. =*Fusarium martii* Appel & Wollenw var. *pisi* Jones, J. Agric. Res., Washington 26: 459 (1923) (basionym).
Following descriptions were based on CBS 142372 strain (Fs21 current study) growing in the darkness at 27 °C after 7 days on PDA, MEA and CLA. Colonies grew rapidly to a final diameter of 45 mm. Observed aerial mycelium cottony, white on MEA and white to milky on PDA (Fig. 4a, b). Reverse pigmentation yellowish to orange-brown. Sporodochia emerged after 7 days of incubation as white flowers on pieces of carnation leaf placed on CLA (Fig. 4c). Abundant production of erect, mononematous conidiophores from the agar surface and the aerial mycelium was also observed. Mononematous conidiophores were acremonium like and unbranched or occasionally branched once (Fig. 4d–f). Long conidiophores ranged from 2.1 to 162.2 μm with a mean length of 45.1 μm and 4.8 μm width. Short conidiophores ranged from 3.0 to 35.2 μm with mean length of 11.2 μm and 4.0 μm width at the base.

Figure 1. Phylogenetic tree resulting from RAxML analysis for the tef1 gene sequences. The data set comprised 720 characters with alignment gaps, and included 122 sequences with reference strains. Maximum Likelihood analysis was performed by RAxML with non-parametric bootstrapping using 1000 replications. The tree was rooted with four strains of *F. redolens* collected for the purpose of this study together with reference strains *F. redolens* NRRL 25123 and *F. thapsinum* H105-557S-1DCPA. Isolate ID number is followed by host plant and country of origin, where IT = Italy, CH = Switzerland, DE = Germany, SE = Sweden; Based on the results of the greenhouse experiment 1, the symbols −, +, ++, and +++ indicate non-aggressive, weakly aggressive, moderately aggressive and highly aggressive isolates on pea, respectively; n/a = not included in aggressiveness test.
terminating into a single sub-cylindrical phialide. Tip of the phialide with inconspicuous preclinical thickening, collarette not flared (4d–4f). Macroconidia was abundant, 4.3–46.1 × 5.4–6.2 μm, 2–4 septate, slightly curved or arcuate with a rounded apical cell and wedge-shaped, weakly pedicellate basal cell (Fig. 4g–j). Microconidia ovoidal or with a rounded apex and truncate base, 2.5–12.7 × 1.5–2.1 μm (Fig. 4d–f). Chlamydospores absent.

Cardinal growth temperature test showed that the strain evaluated in this study had optimal development at 25–30 °C attaining diameter of 17 and 21 mm on PDA and SNA, respectively. Colonies showed no visible growth at 5 °C and at 35 and 40 °C on PDA. On SNA strain was still able to grow at 5 and 35 °C but not at 40 °C.

**Aggressiveness of selected FSSC isolates to pea in greenhouse assay.** The severity of pea root rot averaged over isolates varied significantly among different species within the FSSC. *Fusarium pisi* (mean Disease Index, DI = 60.91) together with *F. redolens* (DI = 63.41) caused the highest overall disease severity, followed by *Fusisporium solani* (DI = 50.23) and the *F. solani* group (DI = 39.65) (Tukey HSD, P < 0.05). There were no significant differences in mean fresh or dry plant biomass of inoculated treatments and un-inoculated controls (data not shown).

**Figure 2.** Phylogenetic tree resulting from RAxML analysis for the rpb2 gene sequences. The data set comprised 910 characters with alignment gaps, and included 56 sequences with reference strains. Maximum Likelihood analysis was performed by RAxML with non-parametric bootstrapping using 1000 replications. The tree was rooted with one strain of *F. thapsinum* CBS 130176. Isolate abbreviations are provided in the caption of Fig. 1.
Significant variation in aggressiveness was also observed among individual isolates of *F. pisi*, *Fusisporium solani*, and those included in the *F. solani* group (Fig. 5). The majority of the isolates tested were pathogenic to pea. In general, weakly to moderately aggressive strains dominated the populations of the tested *Fusarium* species. Among the 48 *F. pisi* isolates, three isolates recovered from subterranean clover roots (Fs24, Fs36, Fs43) and one isolate collected from compost (Fs28) did not differ significantly in root rot severity from the un-inoculated control and were classified as non-aggressive (4/48, 8%). Among the pathogenic isolates, three (6%) were highly aggressive, 24 (50%) moderately aggressive, and 17 (35%) were weakly aggressive. Similar results were also observed for *Fusisporium (Fusarium) solani* (Fig. 5a). Among the 24 isolates tested, three (Fs26, Fs60 and Fs71)
recovered from the roots of white clover, subterranean clover and winter vetch, respectively, were classified as non-aggressive. The remaining isolates induced mild to modest symptoms on pea roots and were differentiated into weakly (12/24, 50%) and moderately aggressive (9/24, 38%). The two *F. solani* isolates collected from compost (Fs29) and hibiscus (Fs30), placed into one separate clade (lineage 1, Fig. 1) were rated as non- and weakly aggressive, respectively. The *F. keratoplasticum* isolate recovered from winter vetch in Italy (FK70), sorted in the *F. solani* group in Fig. 5, was weakly aggressive. Among the *F. redolens* isolates, two were weakly and two were moderately aggressive. Aggressiveness was not related to isolate phylogenetic position or the host plant from which it was recovered (Figs 1–5a).

In contrast to root rot severity, the effect of the tested isolates on pea biomass was much less pronounced. Significant reductions in fresh biomass compared to the corresponding control were observed only in plants inoculated with *F. pisi* isolates Fs1, Fs2, Fs17 and Fs18 recovered from pea roots, as well as for isolate Fs22 recovered

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**Figure 4.** Morphological description of *Fusarium pisi* comb. nov. CBS 142372. (a) Growth on MEA (b) growth on PDA; (c) Sporodochia after 7 days appearing as white flowers on pieces of carnation leaf placed on CLA; (d–f) Long monophialides with false head and microconidia; (f) Minute conidia formed on short aerial conidiophores arising from hyphae; (g–j) Macroconidia abundant. Scale bar = 10 µm.
Significant variation in response to *F. pisi* varied greatly (Table 1). For *Lathyrus*, with the exception of *L. aphaca* (L045), all accessions developed disease severity rating > 2. Mean root rot severity ranged between 2.2 for *L. gorgoni* acc. L1663 and 5.2 for *L. sativus* acc. L1668. Similar responses were observed for *Medicago* accessions. Inoculated *M. arabica* acc. 1735 and 211 and *M. polymorpha* acc. 365 did not develop significant disease symptoms (DSR < 2), whereas *M. arabica* acc. 624 and *M. orbicularis* acc. 44 and 46 had DSR of 3.4, 2.8 and 2.3, respectively.

Significant variation in response to *F. pisi* was also observed for *Trifolium* accessions. Out of the 25 accessions tested, only seven were found to be susceptible (DSR > 2), with five belonging to *T. subterraneum* (DSR between 3.2 and 4.3), one to *T. diffusum* (acc. 906, DSR = 3.2) and one to *T. palestinum* (acc. 910, DSR = 4.0). The remaining 18 accessions had no symptoms or developed lower levels of disease (DSR < 2). These included all *T. repens* accessions (n = 12), two *T. subterraneum* and one of each *T. angustifolium*, *T. arvense* and *T. campestris*.

Among the 14 *Vicia* accessions, six were considered susceptible based on the disease severity symptoms whereas five did not develop disease symptoms higher than two. For susceptible accessions, the mean DSR ranged from subterranean clover roots (Fig. 5b). In comparison to the un-inoculated control, dry plant biomass was significantly reduced only by isolate Fs1 (data not shown).

**Host range of *F. pisi* in greenhouse assays.** *Fusarium pisi* was re-isolated from the surface sterilized roots in 58 out of the 62 accessions tested. Among the 58 successfully colonized accessions, 33 (including the three *Pisum* cultivars) were asymptomatic and 25 accessions were asymptomatic hosts of *F. pisi* (Table 1). The fungus was not re-isolated from one *T. subterraneum* (acc. 1001) and three of the *T. repens* accessions (acc. 1965, 1968 and 2010). As assessed by plating surface sterilized root segments, these accessions were considered as non-hosts for the tested *F. pisi* isolate. In addition, several potentially pathogenic fungal species were isolated from the un-inoculated roots in 13 of the tested accessions: one *Pisum* cultivar, seven *Lathyrus*, four *T. subterraneum* and one *T. repens* accessions. Contaminants were usually *F.avenaceum* and *F. oxysporum*, several other *Fusarium* spp. and in one case *Didymella pinodella* (Table 1).

Inoculation with *F. pisi* resulted in significantly different levels of root rot disease severity among the tested legumes (Table 1). The highest overall disease severity rating (DSR) was observed on *Trigonella foenum-graecum* (mean DSR = 7.8), followed by *Scorpiurus muricatus* (DSR = 5.4), *Pisum* cultivars (DSR = 5.1), *Melilotus albus* (DSR = 4.8) *Crotalaria ochroleuca* (DSR = 4.0) and *Lathyrus* accessions (DSR = 3.4). Inoculated *Vicia*, *Medicago*, *Trifolium*, and *Galega* accessions showed lower overall disease symptoms, with mean severity ratings of 2.1, 2.0, 1.6 and 1.4, respectively. Symptoms of fungal infection were not observed in *Lotus pedunculatus* (acc. 1965, 1968 and 2010). As assessed by plating surface sterilized root segments, these accessions were considered as non-hosts for the tested *F. pisi* isolate. In addition, several potentially pathogenic fungal species were isolated from the un-inoculated roots in 13 of the tested accessions: one *Pisum* cultivar, seven *Lathyrus*, four *T. subterraneum* and one *T. repens* accessions. Contaminants were usually *F.avenaceum* and *F. oxysporum*, several other *Fusarium* spp. and in one case *Didymella pinodella* (Table 1).

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| Legume host | Common name | Code | DSR<sup>1</sup> control | DSR inoculated | FW<sup>1</sup> control (g) | FW<sup>2</sup> change g and (%) | DW<sup>1</sup> control (g) | DW<sup>2</sup> change g and (%) | Host plant<sup>a</sup> |
|-------------|-------------|------|--------------------------|----------------|--------------------------|-------------------------------|--------------------------|-------------------------------|-----------------|
| P. sativum L. | Spot medick | 1735 | 0.0 FA 0.4 0.1 1.2 2.3 | 0.07(−3.2) | 0.40 | +0.02(4.7) | AS |
| N. sylvestris | Flat vetchling | 1695 | 0.0 0.4 0.2 2.3 | 0.07(−6.8) | 0.14 | −0.01(−7.9) | AS |
| M. arctica | Spotted medick | 211 | 0.1 1.3 1.78 | 0.07(−4.2) | 0.27 | +0.03(9.8) | AS |
| M. arctica | Spotted medick | 624 | 0.0 3.4 2.34 | 0.11(−4.6) | 0.37 | +0.04(11.8) | S |
| M. orbicularis | Button medick | 44 | 0.0 2.8 0.74 | +0.32(43.4) | 0.11 | +0.08**(65.7) | S |
| M. orbicularis | Button medick | 46 | 0.1 2.3 0.99 | +0.02(2.2) | 0.15 | +0.04(28.3) | S |
| M. polymorpha | Burr medic | 365 | 0.0 1.6 | 0.01(−5.2) | 0.28 | −0.04(−13.6) | S |
| T. repens | White clover | 1935 | 0.0 1.0 0.68 | −0.01(−4.3) | 0.06 | +0.01(0.2) | AS |
| T. repens | White clover | 1936 | 0.0 0.5 1.04 | −0.18(−17.1) | 0.13 | −0.04(−34.6) | AS |
| T. repens | White clover | 1937 | 0.5 1.3 0.85 | −0.07(−8.2) | 0.11 | 0.00(0.0) | AS |
| T. repens | White clover | 1954 | 0.0 0.4 0.77 | −0.01(−1.1) | 0.09 | −0.01(−7.5) | NH |
| T. repens | White clover | 1959 | 0.0 FO 0.6 0.86 | +0.14(16.3) | 0.12 | +0.02(15.0) | AS |
| T. repens | White clover | 1960 | 0.2 1.0 0.55 | −0.01(−1.6) | 0.07 | −0.01(−0.6) | AS |
| T. repens | White clover | 1965 | 0.0 0.2 0.52 | −0.12(−23.5) | 0.08 | −0.02(−23.5) | NH |
| T. repens | White clover | 1968 | 0.0 0.4 0.54 | +0.01(2.0) | 0.08 | −0.01(−1.1) | NH |
| T. repens | White clover | 1976 | 0.0 1.0 1.06 | −0.24(−22.3) | 0.11 | −0.02(−16.2) | AS |
| T. repens | White clover | 1977 | 0.0 0.5 0.76 | −0.13(−17.6) | 0.09 | −0.02(−28.6) | AS |
| T. repens | White clover | 2001 | 0.0 0.8 0.66 | −0.20(−29.6) | 0.08 | −0.01(−8.6) | AS |
| T. repens | White clover | 2010 | 0.0 1.0 0.72 | +0.64(88.5) | 0.08 | +0.05(67.1) | AS |
| T. subterraneum | Subterranean clover | 1001 | 1.4FO 1.8 2.20 | −0.21(−9.4) | 0.36 | −0.03(−7.6) | NH |
| T. subterraneum | Subterranean clover | 1021 | 2.0FO+FA 3.0 1.32 | −0.15(−11.0) | 0.17 | −0.03(−18.3) | AS |
| T. subterraneum | Subterranean clover | 1040 | 0.0 3.3 1.01 | −0.38(−37.3) | 0.12 | −0.05(−39.4) | S |
| T. subterraneum | Subterranean clover | 1042 | 1.6FO 3.2 1.25 | −0.15(−11.6) | 0.18 | −0.03(−16.4) | S |
| T. subterraneum | Subterranean clover | 1065 | 3.6FO 3.4 1.91 | −0.14(−7.3) | 0.26 | −0.02(−6.6) | AS |
| T. subterraneum | Subterranean clover | 1067 | 0.0 3.4 1.09 | −0.35(−32.1) | 0.13 | −0.03(−26.9) | S |
| T. subterraneum | Subterranean clover | 1068 | 0.0 4.3 0.93 | −0.53(−56.8) | 0.10 | −0.06(−56.7) | S |
| T. subterraneum | Subterranean clover | Campedea | 0.5 3.6 0.54 | +0.02(3.2) | 0.08 | −0.01(−8.2) | S |
Table 1. Plant species and accessions tested for susceptibility to *F. pisi*, and the symptomatic (S), asymptomatic (AS) or non-host (NH) classification based on their response to infections measured 35 days after inoculation under controlled conditions. 1DSR = disease severity rating of un—inoculated control plants, and additionally re-isolated fungi from surface sterilized roots, where *FA = Fusarium avenaceum*, *FO = F. oxysporum*, *FC = F. culmorum*, *FG = F. graminearum*, *FT = F. tricinctum* and *PP = Peyronellaea pinodella*; 2FW/DW = fresh/dry plant biomass of un—inoculated plants; 3FW/DW change = expressed as gram change in the biomass of the inoculated plants compared to corresponding un—inoculated control, and in parenthesis the biomass of the inoculated plants was expressed as a percentage change of the biomass of corresponding un—inoculated control plants. 4Symptomatic (S), asymptomatic (AS) and non-host (NH) accessions; With exception of *Pisum sativum* accession IPR83 provided by Instituto Agronomico do Parana (IAPAR), Brasil, all of the accessions were provided by Technical University of Munich, Germany. Data were pairwise (2 by 2) analyzed by comparing inoculated treatments and corresponding un—inoculated controls using Dunn’s test. The symbols ***, **, and * indicate significance levels of *P < 0.001, < 0.01, and < 0.05, respectively.

between 2.2 for *V. articulata* acc. 924 and 3.6 for *V. sativa* accessions 1576 and 1577. *Vicia sativa* acc. 1576 and *V. villosa* subsp. *varia* acc. 1644 were also classified as symptomatic hosts in the absence of visible disease symptoms due to a significant loss of biomass (Table 1).

Low variability in response to *F. pisi* was found among the tested *Pisum* cultivars. Winter pea cv. EFB 33 (DSR = 4.1) was generally less susceptible to root rot compared to spring pea cv. Santana and cv. IPR83 (both DSR = 5.6) (Table 1).

Compared to the corresponding controls, fresh or dry plant biomass of inoculated treatments was significantly reduced only in *Pisum sativum* cv. IPR83, *Lathyrus inopiscus* acc. L1672, *Trifolium diffusum* acc. 906, *T. palestini- num* acc. 910, *Vicia articulata* acc. 924, *V. sativa* acc. 1576 and 1590, *V. villosa* subsp. *varia* acc. 1644, *Scorpiurus muricatus* acc. 69 and *Trigonella foenum-graecum* acc. 409. Additionally, in some accessions, inoculation with *F. pisi* even resulted in a significant biomass increase (Table 1).

Discussion

In the current study, the single gene phylogeny inferred from *tef1* gene sequences that included 79 isolates, as well as the single *rpb2* and the concatenated *tef1 – rpb2* phylogenetic tree topologies inferred for a selected subset of 28 isolates placed the examined strains in four lineages within the FSSC clade 3. Most isolates were associated
with two major lineages, the *F. pisi* (*F. solani f. sp. pisi*) lineage accommodating mainly German and Swiss isolates, and the *Fusisporium (Fusarium) solani* s. str. lineage accommodating mainly Italian isolates. The aggressiveness tests on pea that included a subset of 75 isolates confirmed the pathogenicity of most of the FSSC isolates tested. Aggressiveness was not related to isolate phylogenetic position or the host plant from which it was recovered.

The predominant share of the identified strains examined here (*n* = 51) belonged to *F. pisi*, suggesting a significant pathogenic potential of this species and/or a common prevalence in different host plants. Previous studies have established *F. pisi* as a primary causal agent of pea root rot and one of the main reasons for the decline of pea production worldwide. While the result of the aggressiveness tests on pea in our study showed that the most aggressive isolates belonged to *F. pisi*, it is important to note that the tested population was dominated by weakly and moderately aggressive strains. In addition, non-pathogenic strains were also present in the population of the species. More importantly, our study showed that both, pathogenic and non-pathogenic isolates of *F. pisi* can be found in a variety of habitats under diverse agro-ecological conditions, and that the fungus is, in addition to pea, able to colonize roots of various hosts such as subterranean clover, white clover, winter vetch and faba bean under field conditions.

The ability of *F. pisi* to occupy diverse ecological niches and the significant variation in the aggressiveness of individual isolates observed in this study is consistent with the previous work of VanEtten, who found large variation in pea plant symptom severity following inoculation with 152 *F. pisi* isolates collected from diverse habitats and geographical locations. While some isolates were highly aggressive to pea, the authors also reported the presence of non-pathogenic strains. Additional studies on the aggressiveness factors of *F. pisi* revealed that a number of enzymes released from the fungi have a major influence on their ability to cause disease. The capacity of the pathogen to degrade the phytoalexin pisatin by the activity of pisatin demethylase is one of the main determinants of its aggressiveness in pea. All naturally occurring isolates without this ability were essentially non-pathogenic.

Out of the 26 additionally identified isolates in our study, 25 belonged to *Fusarium solani* (Fusarium solani sensu stricto), the species mainly considered as a causal agent of dry rot in potatoes and known as an opportunistic human pathogen. However, the fungus has not been reported as part of the root rot complex of pea. As for *F. pisi*, our results suggest a lack of host specificity and the ability of *Fusisporium (Fusarium) solani* s. str. isolates to colonize various hosts under field conditions, as well as their potential to cause significant damage to pea. In addition, with few notable exceptions, the population of *F. pisi* isolates in our study mainly originated from German and Swiss environments, whereas the Italian isolates mainly comprised the *Fusisporium (Fusarium) solani* s. str. lineage. These results indicate a biogeographic distribution pattern of the FSSC species distribution with respect to the host plants.

One additionally identified isolate belonged to *F. keratoaplasticum*, the species mainly associated with human eye infections. Our data suggest considerable ecological plasticity of the fungus which possesses pathogenic potential on pea, and points to soil and plant debris as potential environmental sources of human infections. Included in *F. solani* group, the isolates Fs29 and Fs30 which were recovered from compost and hibiscus, respectively, represent at least one new phylogenetic lineage in the complex. Additional studies are needed to fully understand their ecological importance.

The greenhouse data on the host range of *F. pisi* further supports our observations that the species is not adapted to a particular host. Data from the single isolate inoculation indicate that the host range of *F. pisi* should be expanded to include 33 symptomatic and 25 asymptomatic hosts. Only one accession of subterranean clover and three accessions of white clover tested could be classified as non-hosts for *F. pisi* isolate Fs21 pointing to potential sources of resistance. A broader host range has been demonstrated for other special forms of *F. solani* previously. Studies on *F. solani f. sp. cuminerti*, named by its specific pathogenicity to potato, revealed that the species was also pathogenic to pepper, eggplant and tomato. Besides the Solanaceae family, the pathogen also infected maple (*Acer* sp.) and citrus (*Citrus* sp.) trees. Similarly, *F. phaseoli* comb. nov. (formerly *F. solani f. sp. phaseoli*) generally considered as a root rot pathogen of bean (*Phaseolus vulgaris*), has been associated with at least four other legume host plants. In an extensive survey of *F. solani f. sp. glycines* associated with sudden death syndrome of soybean, Aoaki et al. found that the disease is in fact caused by two phylogenetically and morphologically distinct species, *F. virguliforme* (formerly *F. solani f. sp. glycines*) and *F. tucumaniae* in North and South America, respectively. More recent studies conducted by Kolander et al. showed that *F. virguliforme* can cause disease in a range of legumes and non-legumes including alfalfa, red clover, white clover, pea, bean, sugar beet and canola. In addition, the authors demonstrated the ability of *F. virguliforme* to asymptptomatically infect wheat, maize, ryegrass and lambsquarters, which are commonly grown in rotations with legumes. More recently, two new special forms have been assigned to the FSSC, namely *F. solani f. sp. passiflorae* and *phalaenopsis*. However, the authors did not provide sufficient molecular data that would allow comparison of their strains with already existing species within the FSSC, and based their results solely on the pathogenicity to the specific host evaluated on a narrow range of closely related plant species. Thus, whether such problems exist in these and other special forms within the complex remains to be investigated.

In addition to causing economically important crop diseases, *Fusarium* spp. in general and the members of the FSSC in particular that were previously considered solely as plant pathogens are being increasingly reported as causal agents of superficial and systemic infections in humans and animals. For example, these so-called trans-kingdom fungal pathogens have been demonstrated for *F. oxysporum f. sp. lycopersici, Fusisporium solani* s. str., *F. keratoaplasticum* and *F. falciforme*. Similarly, *F. pisi* has recently come into focus as a species of clinical relevance associated with human eye infection in the Netherlands (Al-Hatmi, unpublished data). This confirms that the species is adapted to many different habitats and supports the idea that *Fusarium* spp. can serve as a model for studying trans-kingdom pathogenicity in fungi.

Our results provide new insights into the diversity of the FSSC associated with the legume host plants in Europe and provide another example for a wide host range of a single lineage within the complex. Currently the concept of forma specialis represents an informal rank in taxonomic classification, and may deserve revision and formal taxonomic treatment.
Material and Methods

Isolates. A total of 79 FSSC isolates were collected for this study (Table 2). Among these, 18 isolates were recovered from pea (*Pisum sativum*), 39 from subterranean clover (*Trifolium subterraneum*), 3 from white clover (*T. repens*) and 14 from winter vetch (*Vicia villosa*). The isolates originated from Germany (n = 28), Switzerland (n = 24), Italy (n = 21) and Sweden (n = 1). Additionally, we included one isolate from faba bean (*V. faba*), and expanded the isolates associated with legumes with two isolates collected from compost and two (one of each) recovered from an infected hibiscus (*Hibiscus* sp.) and cherry tree (*Prunus* sp.), all from Germany (Table 2). All isolates were collected in the period between 2009 and 2016, morphologically identified as *F. solani* and maintained as single-spore cultures at the Internal Culture Collection of the Ecological Plant Protection Department at University of Kassel. Four isolates of *F. redolens*, recovered from white clover grown in Sweden, were additionally included in this study.

DNA extraction, PCR amplification and sequencing. Genomic DNA was extracted from cultures actively growing on half strength PDA agar plates (½ strength PDA; 19 g/l Difco PDA and 10 g/l agar) using the CTAB (cetyltrimethylammonium bromide) protocol described by Doyle and Doyle. All DNA were diluted 20 times in milli-Q water and stored at −20 °C before use.

A portion of the translation-elongation factor 1 alpha (*tef1*) gene was amplified using primer pairs EF1 and EF2. Based on the *tef1* tree topology, 28 strains were selected and the second-largest subunit of RNA polymerase II (*rpb2*) was amplified using primers RP2B-5F2 and RP2B-7cR. Each polymerase chain reaction (PCR) had a total volume of 50 μl and contained 1 μl of diluted genomic DNA, 10× TrueStart Hot Start Taq Buffer [200 mM Tris-HCl (pH 8.3 at 25 °C), 200 mM KCl, 50 mM (NH₄)₂SO₄], 2.5 mM MgCl₂, 0.2 mM of each of the dNTP, 0.4 μl of each primer, and 1 unit TrueStart Hot Start Taq DNA Polymerase (ThermoFisher Scientific, Darmstadt, Germany). The PCR reactions were performed in a Biometra TAdvanced Thermal Cycler (Applied Biosystems, Foster City, California, USA). Conditions for amplification for the *tef1* gene region were an initial denaturation step of 3 min at 95 °C, followed by 30 cycles of denaturation (95 °C for 30 s), annealing (53 °C for 30 s) and elongation (72 °C for 45 s). The final elongation step was conducted at 72 °C for 7 min. For the *rpb2* loci amplification consisted of 5 cycles of 45 s at 94 °C, 45 s at 60 °C and 2 min at 72 °C, then 5 cycles with a 58 °C annealing temperature and 30 cycles with a 54 °C annealing temperature.

Amplons were purified using the DNA Clean & Concentrator kit (Zymo Research, Freiburg, Germany) according to the manufacturer’s instructions and sequenced in both directions either by Eurofins Genomics (Ebersberg, Germany) or by Macrogen (Amsterdam, Netherlands) with the above-mentioned primers.

Phylogenetic analyses. Obtained row sequences were assembled and errors identified and corrected in MEGA v6. Partial sequences of *tef1* and *rpb2* were used as queries for the Fusarium-ID v. 1.0 database and the Fusarium MLST databases to confirm the taxonomic assignments of the isolates. The sequences were aligned with sequences of reference strains retrieved from the GenBank using MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/index.html) and the alignments were adjusted manually with MEGA v6. The phylogenetic analyses, including the majority of known *Fusarium* species within the FSSC, was performed on *tef1* and *rpb2* sequences separately as well as on a combined data set (28 selected strains). The strains used in these analyses and GenBank accession numbers of the sequences are listed in Table 2.

A bootstrapped Maximum-Likelihood (ML) analysis was performed using the RAxML-VI-HPC v. 7.0.3 (Ebersberg, Germany) or by MacroGen (Amsterdam, Netherlands) with the above-mentioned primers. Based on the *rpb2* ML tree topology, 23 FSSC strains from non-pea hosts are capable of causing disease on pea, a total of 75 isolates were tested in a greenhouse assay. The aggressiveness test included 48 isolates of *F. pisi*, 24 isolates of *Pusisorium solani*, 2 isolates of *F. solani*, and 1 isolate of *F. keratoplasticum*. In this study, the isolates that formed distinct groups based on the phylogenetic analysis and showed no strong phylogenetic relationship to any of the previously defined species within the FSSC.
| Isolate ID<sup>a</sup> | Species<sup>b</sup> | Host/Substrate<sup>c</sup> | Geographical origin | Year<sup>d</sup> | GenBank accession numbers<sup>e</sup> |
|----------------------|-----------------|-----------------|-------------------|------------|-----------------|
| Fs1                  | *Fusarium pisi* | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556491 — |
| Fs2                  | *F. pisi*       | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556463 — |
| Fs3                  | *F. pisi*       | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556448 — |
| Fs4                  | *Fusisporium solani* | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556500 — KY556544 |
| Fs5                  | *Fusisporium solani* | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556511 — |
| Fs6                  | *F. pisi*       | *Pisum sativum* | Germany, Neu Eichenberg, Hessen | 2013 | KY556459 — |
| Fs7                  | *F. pisi*       | *Pisum sativum* | Germany, Neu Eichenberg, Hessen | 2013 | KY556466 — |
| Fs8                  | *F. pisi*       | *Pisum sativum* | Germany, Neu Eichenberg, Hessen | 2013 | KY556450 — |
| Fs9                  | *F. pisi*       | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556452 — |
| Fs10                 | *F. pisi*       |                | Germany, n/a | 2009 | KY556452 — |
| Fs11                 | *F. pisi*       | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556497 — |
| Fs12                 | *F. pisi*       | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556447 — |
| Fs13                 | *F. pisi*       | *Pisum sativum* | Germany, Neu Eichenberg, Hessen | 2013 | KY556433 — |
| Fs14                 | *F. pisi*       | *Pisum sativum* | Germany, Frankenhausen, Hessen | 2013 | KY556449 — |
| Fs15                 | *F. pisi*       | *Pisum sativum* | Germany, Neu Eichenberg, Hessen | 2012 | KY556492 — |
| Fs16                 | *F. pisi*       | *Pisum sativum* | Germany, Neu Eichenberg, Hessen | 2011 | KY556493 — |
| Fs17                 | *F. pisi*       | *Pisum sativum* | Germany, Neu Eichenberg, Hessen | 2011 | KY556471 — |
| Fs18                 | *F. pisi*       | *Pisum sativum* | Germany, Neu Eichenberg, Hessen | 2011 | KY556458 — KY556526 |
| Fs19                 | *F. pisi*       | Subterranean clover (*Trifolium subterraneum*) | Germany, Neu Eichenberg, Hessen | 2013 | KY556472 — KY556535 |
| Fs20                 | *F. pisi*       | Subterranean clover | Germany, Freising-Weißenstephan, Bavaria | 2015 | KY556488 — KY556536 |
| Fs21                 | *F. pisi*       | Subterranean clover | Germany, Neu Eichenberg, Hessen | 2013 | KY556454 — KY556537 |
| Fs22                 | *F. pisi*       | Subterranean clover | Germany, Neu Eichenberg, Hessen | 2014 | KY556473 — KY556527 |
| Fs23                 | *F. pisi*       | Subterranean clover | Germany, Neu Eichenberg, Hessen | 2013 | KY556455 — KY556538 |
| Fs24                 | *F. pisi*       | Subterranean clover | Germany, Neu Eichenberg, Hessen | 2013 | KY556474 — |
| Fs25                 | *F. pisi*       | Faba bean (*Vicia faba*) | Germany, Freising, Bavaria | 2015 | KY556460 — |
| Fs26                 | *Fusisporium solani* | White clover (*Trifolium repens*) | Germany, Neu Eichenberg, Hessen | 2014 | KY556517 — KY556542 |
| Fs27                 | *Fusisporium solani* | White clover (*Trifolium repens*) | Germany, Neu Eichenberg, Hessen | 2014 | KY556501 — |
| Fs28                 | *F. pisi*       | Compost | Germany, Hannover, Lower Saxony | 2014 | KY556475 — KY556528 |
| Fs29                 | *F. solani*     | Compost | Germany, Hannover, Lower Saxony | 2014 | KY556524 — KY556552 |
| Fs30                 | *F. solani*     | Hibiscus dying branch (*Hibiscus sp.*) | Germany, Wittenhausen, Hessen | 2015 | KY556525 — KY556553 |
| Fs31                 | *Fusisporium solani* | Cherry dying branch (*Prunus sp.*) | Germany, Wittenhausen, Hessen | 2016 | KY556520 — KY556549 |
| Fs32                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556476 — |
| Fs33                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556486 — KY556529 |
| Fs34                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556484 — KY556530 |
| Fs35                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556482 — KY556539 |
| Fs36                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556487 — |
| Fs37                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556495 — |
| Fs38                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2015 | KY556456 — |
| Fs39                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2011 | KY556477 — |
| Fs40                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556478 — |
| Fs41                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556464 — |
| Fs42                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556485 — KY556531 |
| Fs43                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556479 — KY556532 |
| Fs44                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556470 — KY556533 |
| Fs45                 | *Fusisporium solani* | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2013 | KY556521 — KY556543 |
| Fs46                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2015 | KY556467 — KY556534 |
| Fs47                 | *F. pisi*       | Subterranean clover | Switzerland, Reckenholz, Canton Zurich | 2015 | KY556461 — |
| Fs48                 | *F. pisi*       | Winter vetch | Switzerland, Reckenholz, Canton Zurich | 2015 | KY556489 — |
| Fs49                 | *F. pisi*       | Winter vetch | Switzerland, Reckenholz, Canton Zurich | 2015 | KY556490 — KY556540 |
| Fs50                 | *F. pisi*       | Winter vetch | Switzerland, Reckenholz, Canton Zurich | 2015 | KY556480 — |
| Fs51                 | *F. pisi*       | Winter vetch | Switzerland, Reckenholz, Canton Zurich | 2015 | KY556468 — |

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16 °C night temperature. Natural day light was additionally supplemented with high-pressure sodium lamps arranged in a completely randomized design. Experimental plants were kept in the greenhouse at 19 °C day and controls were mock inoculated with sterile distilled water. Four replicate pots were sown per treatment and

were included in the *F. solani* group. Four isolates of *F. redolens* were also included in this experiment. The geographical origin and the host plants from which the isolates are collected are given in Table 2.

| Isolate ID | Species | Host/Substrate | Geographical origin | Year | GenBank accession numbers |
|------------|---------|----------------|---------------------|------|--------------------------|
| Fs52       | *F. pisi* | Winter vetch | Switzerland, Canton Zurich | 2015 | KY556496 — |
| Fs53       | *F. pisi* | Winter vetch | Switzerland, Canton Zurich | 2015 | KY556465 — |
| Fs54       | *F. pisi* | Winter vetch | Switzerland, Canton Zurich | 2015 | KY556483 — |
| Fs55       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556515 — |
| Fs56       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556502 — |
| Fs57       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556503 KY556545 |
| Fs58       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556498 KY556546 |
| Fs59       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556504 — |
| Fs60       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556518 KY556547 |
| Fs61       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556505 — |
| Fs62       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556509 — |
| Fs63       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556519 — |
| Fs64       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556510 — |
| Fs66       | *F. pisi* | Subterranean clover | Italy, Viterbo | 2015 | KY556469 — |
| Fs67       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556499 — |
| Fs68       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556506 — |
| Fs69       | *Fusarium solani* | Winter vetch | Italy, Viterbo | 2015 | KY556522 KY556550 |
| FK70       | *F. keratoplasticum* | Winter vetch | Italy, San Piero a Grado, Tuscany | 2014 | KY556523 — |
| Fs71       | *Fusarium solani* | Winter vetch | Italy, Viterbo | 2015 | KY556507 KY556548 |
| Fs72       | *Fusarium solani* | Winter vetch | Italy, San Piero a Grado, Tuscany | 2014 | KY556512 KY556551 |
| Fs73       | *Fusarium solani* | Winter vetch | Italy, San Piero a Grado, Tuscany | 2015 | KY556508 — |
| Fs74       | *Fusarium solani* | Winter vetch | Italy, San Piero a Grado, Tuscany | 2015 | KY556514 — |
| Fs75       | *Fusarium solani* | Winter vetch | Italy, San Piero a Grado, Tuscany | 2015 | KY556513 — |
| Fs76       | *F. pisi* | White clover | Sweden, Upsala | 2014 | KY556462 — |
| Fs77       | *F. pisi* | Subterranean clover | Germany, Neu Eichenberg, Hessen | 2014 | KY556457 KY556541 |
| Fs78       | *F. pisi* | Subterranean clover | Switzerland, Canton Zurich | 2013 | KY556494 — |
| Fs79       | *Fusarium solani* | Subterranean clover | Italy, Viterbo | 2015 | KY556516 — |
| Fs80       | *F. pisi* | Subterranean clover | Germany, Neu Eichenberg, Hessen | 2014 | KY556481 — |
| FR1        | *F. redolens* | White clover | Sweden, Upsala | 2014 | KY556443 — |
| FR2        | *F. redolens* | White clover | Sweden, Upsala | 2015 | KY556444 — |
| FR3        | *F. redolens* | White clover | Sweden, Upsala | 2014 | KY556445 — |
| FR4        | *F. redolens* | White clover | Sweden, Upsala | 2014 | KY556446 — |

Table 2. *Fusarium isolates* subjected to phylogenetic analysis and evaluated for aggressiveness to pea in greenhouse experiment 1. All isolates with exception of Fs77, Fs78, Fs79 and Fs80 were tested for aggressiveness on pea in greenhouse experiment 1; isolates that formed distinct groups based on the phylogenetic analysis and showed no strong phylogenetic relationship to any of the previously defined species within the FSSC were termed as *F. solani*; Unless indicated differently isolates were collected from infected root system; GenBank accession numbers for translation elongation factor 1-alpha (tef1) partial sequences and the second-largest subunit of RNA polymerase II (rpb2) gen region (selected isolates). n/a = not available.
| Species                  | Strain number | GenBank accession numbers |
|-------------------------|---------------|---------------------------|
| **Fusarium solani**     | CBS 119996    | HE647962.1 n/a            |
| **Fusarium petroliphilum** | CBS 135955 | n/a                       |
| **Fusarium falciforme** | CBS 138963    | KTT716213.1 n/a           |
| **Fusarium phaseoli**   | CBS 265.50    | HE647964.1 n/a            |
| **Fusarium panamaense** | CML 1988      | KF597819.1 n/a            |
| **Fusarium solani f. sp. piperis** | CML 2190 | JX657675.1 n/a |
| **Fusarium rectiphorum** | FRC S1831    | DQ247509.1 n/a            |
| **Fusarium haematococcum** | FRC S1832  | DQ247516.1 n/a            |
| **Fusarium keratoplasticum** | FRC S2477 | KR673939.1 KR673969 |
| **Fusarium ambrosium**  | NRRL 20438    | AF178332.1 n/a            |
| **Fusarium illudens**   | NRRL 22090    | AF178326.1 JX171601       |
| **Fusarium sp. cucurbitae** | MPI NRRL 22098 | n/a EU329489          |
| **Fusarium sp.**        | NRRL 22101    | n/a EU329490.1            |
| **Fusarium solani f. sp. pisi** | NRRL 22157 | AF178359.1 EU329493      |
| **Fusarium sp. robiniæ** | NRRL 22161  | n/a EU329494              |
| **Fusarium solani f. sp. xanthoxyli** | NRRL 22163 | AF178328.1 n/a          |
| **Neocosmospora vasinfecta** | NRRL 22166 | AF178350.1 EU329497      |
| **Fusarium sp.**        | NRRL 22178    | n/a EU329498              |
| **Fusarium sp.**        | NRRL 22230    | n/a EU329499              |
| **Fusarium phaseoli**   | NRRL 22276    | n/a JX171608              |
| **Fusarium sp.**        | NRRL 22278    | n/a EU329501              |
| **Fusarium ambrosium**  | NRRL 22354    | n/a EU329504              |
| **Fusarium kuranmegalense** | NRRL 22387 | n/a EU329505             |
| **Fusarium rectiphorum** | NRRL 22396   | n/a EU329508              |
| **Fusarium sp. batatus** | NRRL 22400  | n/a EU329509              |
| **Fusarium solani f. batatus** | NRRL 22402 | AF178344.1 n/a          |
| **Fusarium solani (FSSC6)** | NRRL 22404 | DQ247594.1 n/a           |
| **Fusarium sp.**        | NRRL 22436    | n/a EU329511              |
| **Fusarium sp. piperus** | NRRL 22570    | n/a EU329513              |
| **Fusarium sp.**        | NRRL 22579    | n/a EU329515              |
| **Fusarium sp. (FSSC 13)** | NRRL 22586  | AF178353.1 n/a            |
| **Fusarium plagianthi**  | NRRL 22632    | AF178354.1 n/a            |
| **Fusarium sp. plagianthi** | NRRL 22632 | n/a EU329519              |
| **Fusarium brasiliense** | NRRL 22678    | JQ670133.1 n/a            |
| **Fusarium tuscomamiae** | NRRL 22744   | DQ247651.1 n/a            |
| **Fusarium solani f. sp. pisi** | NRRL 22820 | AF178355.1 EU329532      |
| **Fusarium virguliforme** | NRRL 22825 | n/a GU170599             |
| **Fusarium solani**     | NRRL 25083    | JF740714.1 n/a            |
| **Fusarium redolens**   | NRRL 25123    | JF740748.1 n/a            |
| **Fusarium lichenicola** | NRRL 28030   | DQ246877.1 n/a            |
| **Fusarium brasiliense** | NRRL 31757    | n/a EU329565              |
| **Fusarium sp. (FSSC 12d)** | NRRL 32309 | DQ246937.1 n/a           |
| **Fusarium lichenicola** | NRRL 34123    | n/a EU329635              |
| **Fusarium virguliforme** | NRRL 36899  | FJ919494.1 n/a            |
| **Fusarium sp.**        | NRRL 45880    | n/a EU329640              |
| **Fusarium pseudensiforme** | NRRL 46517 | KC691555.1 KC691674      |
| **Fusarium solani (FSSS)** | NRRL 46643  | GU250544 GU250729        |
| **Fusarium eswallaceae** | NRRL 54722    | JQ038007.1 n/a            |
| **Fusarium petroliphilum** | NRRL 54988  | KC808210.1 n/a           |
| **Fusarium sp.**        | 44a GJS 09–1459 | KT313606.1 n/a          |

Continued
roots were then cut transversally across the lesions and internal disease severity was rated, where 0 = no symptoms, 1 = brown discoloration of cortical tissues, 2 = cortex tissue is completely rotten, 3 = shedding of the cortex tissue and endodermis, and 8 = dead plant. Consequently, a disease severity index (DI) between 0 and 100 was calculated for each pot using equation (1):

\[
\text{DI} = \frac{\sum (\text{SR} \times \text{NR})}{\text{Nt} \times \text{MR}} \times 100
\]

where, SR = Mean external and internal disease severity rating (DSR), NR = Number of infected plants having that DSR, Nt = Total number of plants assessed, MR = Maximum rating scale number.

Four distinct aggressiveness classes were then assigned relative to the un-inoculated control and based on the gradual increase of severity of symptoms following inoculation, where: DI = 0–36 = non-aggressive; DI = 37–55 = weakly aggressive; DI = 56–85 = moderately aggressive; and DI = 86–100 = highly aggressive. A threshold disease index of 36 for classifying an isolate as aggressive was chosen because factors other than inoculation caused low disease levels of root discoloration in un-inoculated control plants. Up to this level the DI of inoculated treatments was in the same range as the DI of the un-inoculated control; e.g. mean DI of un-inoculated controls was 24 (±12) while the mean DI of inoculated treatment was ≤36. In addition, up to this level there was no statistically significant difference in DI of inoculated treatments and un-inoculated control.

Twenty one different inoculation treatments were selected at random and the fungi were re-isolated from the surface sterilized roots (1% NaOCl, 3 roots per treatment) and identified morphologically to confirm recovery of the isolate.

Evaluation of host range of Fusarium pisi. To determine the host range and evaluate plant response to inoculation with F. pisi, 60 accessions of 10 legume genera were tested in a greenhouse assay. This study was conducted over a set of four consecutive experiments. In each experiment two field pea cultivars, cv. Santana and cv. EFB33, were included as additional controls (Table 1).

The F. pisi isolate (Fs21) classified as moderately aggressive to pea in experiment 1 was selected for the inoculation experiments. The inoculum was prepared by incubating the strain for 10 days in aerated malt extract broth (MEB, 17 g/l) at 20 °C under constant agitation/shaking at 100 rpm. After 10 days of incubation, conidia were collected by filtration and enumerated in suspension as described above.

Preliminary studies on seed germination using untreated seeds showed that the majority of accessions chosen for this experiment had a very low germination rate. Thus, to ensure adequate seedling emergence, seeds of all plant accessions, with the exception of pea, were treated with 97% sulfuric acid for 4 min, rinsed in distilled water and germinated for 48 h on wet filter paper in Petri dishes at room temperature. Pea seeds were treated with 70% ethanol prior to placing on wet filter paper. Single pre-germinated seeds were then transplanted into 200 ml pots filled with autoclaved sand. Each treatment consisted of five replicates with one germinated seed sown per pot. The experiment was arranged in a completely randomized design and the pots were inoculated 24 h after transplanting with 2 × 10⁵ spores g⁻¹ substrate. Plants were kept in the greenhouse for five weeks under the conditions described for experiment 1. After five weeks of growing, plants were harvested and the biomass and disease severity (external root tissue discoloration levels only) were assessed as described above.

Cultural methods in combination with disease severity data were used to determine the host range of F. pisi on tested plants. Three randomly selected roots from each treatment were surface-sterilized in 0.5% NaOCl for 10 s, thoroughly washed in distilled water and placed on filter paper under a laminar flow hood for 1 h to dry. Subsequently, the roots were cut into approximately 1 cm long fragments and placed in Petri dishes containing ½ strength PDA medium and incubated under alternating cycles of 12 h BLB fluorescent light and 12 h darkness.

Table 3. Reference strains sourced from the NCBI GenBank database used to examine phylogenetic relationships among collected isolates. CBS = Centraalbureau voor Schimmelcultures—Fungal Biodiversity Center, Utrecht, The Netherlands; CML = Coleção Micológica de Lavras, Departamento de Fitopatologia, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil; FRC = Specimen number in the Fusarium Research Center, Pennsylvania State University; NRRL Agricultural Research Service Culture Collection, Peoria, Illinois USA; ¹Reference strains GenBank accession numbers for translation elongation factor 1-alpha (tef1) partial sequences and the second-largest subunit of RNA polymerase II (rpb2) gen region. ²Unknown culture collections; n/a = the sequences were either not available or not applicable to the current study.

| Species                  | Strain number | GenBank accession numbers¹ |
|--------------------------|---------------|----------------------------|
| *Fusarium cf. solani*    | B665²         | HM862045.1 n/a             |
| *Fusarium solani* f. sp. cucurbitae | Fm73¹         | KC711041.1 n/a             |
| *Fusarium thapsinum*     | H05-5575-1 DCPA² | JX268965.1 n/a             |
| *Fusarium striatum*      | SQHH003¹      | KP715415.1 n/a             |

As follows: disease severity rating (DSR) 0 = no symptoms, 1 = streaks at the transition zone, epicotyl or hypocotyl, 2 = brown lesion cover up to 50% of root perimeter, 3 = brown-black lesion cover 51 to 99% of root perimeter, 4 = black lesion cover 100% of stem perimeter, 5 = black lesion spread up to 30–49% of the tap root, 6 = black lesions spread up to 50 to 70% of the tap root, 7 = black lesions spread >70% of the tap root, 8 = dead plant. The roots were then cut transversally across the lesions and internal disease severity was rated, where: DI = 0–36 = non-aggressive; DI = 37–55 = weakly aggressive; DI = 56–85 = moderately aggressive; and DI = 86–100 = highly aggressive. A threshold disease index of 36 for classifying an isolate as aggressive was chosen because factors other than inoculation caused low disease levels of root discoloration in un-inoculated control plants. Up to this level the DI of inoculated treatments was in the same range as the DI of the un-inoculated control; e.g. mean DI of un-inoculated controls was 24 (±12) while the mean DI of inoculated treatment was ≤36. In addition, up to this level there was no statistically significant difference in DI of inoculated treatments and un-inoculated control.
After 10 to 15 days of incubation, fungal colonies developing from the root pieces were sub-cultured separately in Petri dishes containing PDA and SNA agar, incubated as described previously and identified based on cultural characteristics and microscopic examination of conidigenous cells.

The response of the tested legume species to F. pisi was determined according to criteria adopted in a slightly modified form from Kolander et al. The accessions were considered symptomatic if the inoculated isolate was re-isolated from surface sterilized roots, the average disease severity rating was higher than 2 and significantly greater than in un-inoculated control plants. Accessions were also considered symptomatic if DSR > 2 but there was a significant reduction in mean plant biomass of inoculated treatment compared to the corresponding control. Some of the control plants showed moderate symptoms on the roots (mean DSR > 2) caused by factors other than inoculation with F. pisi, and in this case the host was considered susceptible if the final disease severity level of inoculated treatments was significantly higher than that of the corresponding control plants. The accessions were considered asymptomatic hosts if disease rating was less than or equal to 2, there was no significant reduction in biomass, and the fungus was re-isolated from the root parts following surface sterilization.

Data analysis. All statistical analyses were done using R statistical software (version 3.3.1). For aggressiveness assay of selected FSSC isolates to pea, the normality of data distribution and homogeneity of variances were tested by Shapiro-Wilk’s-Test and Levene’s test, respectively. Prior to statistical analysis, disease severity index (DSI) values were square root transformed. The data were first subjected to one way ANOVA to analyze differences in mean effects of different phylogenetic groups on root rot severity and plant biomass. Mean separations were made by Tukey HSD test. Differences among single isolates were tested separately by comparing means from inoculated treatments (each isolate) and un-inoculated control using Dunnett’s test. Treatments were considered significantly different if \( P \leq 0.05 \).

Due unequal variances of many groups, the data on the host range of Fusarium pisi were subjected to non-parametric analyses. Differences in root rot severity ratings and biomass of inoculated treatments and corresponding un-inoculated controls were compared with 2 by 2 comparisons using the non-parametric ranking procedure of the Dunn’s test.

Data availability. The partial translation elongation factor 1-alpha (tef1) and the second-largest subunit of RNA polymerase II (rpb2) sequences of all strains used in this study were submitted to GenBank database. All fungal and plant materials are maintained at University of Kassel and are available for researce purposes upon request (representative fungal strains were deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands).

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Author Contributions
A.S., and J.B.S. designed the project, conceived and conducted laboratory and greenhouse experiments. A.S., J.B.S., A.M.S.A., and S.A.A. performed taxonomy related expriments; A.S., and A.M.S.A. analysed the results, prepared data presentation and drafted the manuscript. M.F. supervised the research study and revised the manuscript. W.M., P.K., and G.S.H. contributed materials and data interpretation. All the authors discussed the results, revised and approved the manuscript.

Additional Information

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