Virulence of soil-borne pathogens and invasion by *Prunus serotina*

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Summary

- Globally, exotic invaders threaten biodiversity and ecosystem function. Studies often report that invading plants are less affected by enemies in their invaded vs home ranges, but few studies have investigated the underlying mechanisms.
- Here, we investigated the variation in prevalence, species composition and virulence of soil-borne *Pythium* pathogens associated with the tree *Prunus serotina* in its native US and non-native European ranges by culturing, DNA sequencing and controlled pathogenicity trials.
- Two controlled pathogenicity experiments showed that *Pythium* pathogens from the native range caused 38–462% more root rot and 80–583% more seedling mortality, and 19–45% less biomass production than *Pythium* from the non-native range. DNA sequencing indicated that the most virulent *Pythium* taxa were sampled only from the native range. The greater virulence of *Pythium* sampled from the native range therefore corresponded to shifts in species composition across ranges rather than variation within a common *Pythium* species.
- *Prunus serotina* still encounters *Pythium* in its non-native range but encounters less virulent taxa. Elucidating patterns of enemy virulence in native and nonnative ranges adds to our understanding of how invasive plants escape disease. Moreover, this strategy may identify resident enemies in the non-native range that could be used to manage invasive plants.

Introduction

Exotic invaders are a global threat to biodiversity and ecosystem function. Several nonmutually exclusive hypotheses have been proposed to explain the success of nonnative invasive species (Hierro *et al.*, 2005). A growing body of literature suggests that plants are less affected by natural enemies in nonnative than native ranges (Wolfe, 2002; Reinhart *et al.*, 2003; Callaway *et al.*, 2004; DeWalt *et al.*, 2004; Knevel *et al.*, 2004; Reinhart & Callaway, 2004; Vilá *et al.*, 2005). These findings contribute to the enemy release hypothesis (ERH), which predicts that invasiveness is enhanced when species are released from their native pathogens (Keane & Crawley, 2002). However, given that most natural systems have a diversity of resident pathogens that infect the local fauna or flora (Mitchell & Power, 2003), it is unlikely that successful invaders colonize areas devoid of enemies. Instead, invaders likely encounter nonadapted, and probably therefore less-damaging enemies that differ in density, species composition, and/or diversity relative to their native ranges. Many invasive plants are associated with fewer foliar pathogens (Mitchell & Power, 2003; but see van Kleunen & Fischer, 2009) and root-feeding nematodes (Van der Putten *et al.*, 2005) in their nonnative than native ranges. However, fewer enemy species does not necessarily translate to less damage to the host plant (Brinkman *et al.*, 2005).

Hierro *et al.* (2005) argue that determining whether plant performance varies between native and nonnative ranges and whether natural enemies are the cause of this variation (DeWalt *et al.*, 2004) are necessary for testing ERH. Hierro *et al.* (2005) also emphasize the need for

experiments that help reveal the mechanisms behind variation in enemy effects and plant performance. The ability of a pathogen to regulate its host population depends in part on its virulence but few studies have examined the virulence of specific pathogens and their effect on plant performance in native vs invaded ranges. Determining whether the virulence of natural enemies is significantly different in native vs non-native ranges requires comparison of their effects on hosts in controlled environments. To our knowledge, this approach has never been applied in studies of biological invasions or of soil pathogens. Reduced pathogen virulence in the invaded range represents a potential mechanism contributing to escape from natural enemies.

Here, we test whether soil-borne *Pythium* pathogens (kingdom Stramenopila, phylum Oomycota) are more virulent to *Prunus serotina* (black cherry) in *P. serotina*'s native vs nonnative ranges. We also evaluate whether differences in virulence between ranges result from variation in *Pythium* species composition or genotypic differences within species common to both ranges. *Pythium* spp. are well suited for geographical comparisons of pathogenicity because they are globally distributed and can be isolated from soil and used in controlled experiments. Worldwide, *Pythium* species are considered the most important plant pathogens infecting plant seeds or seedlings before emergence from the soil (Hendrix & Campbell, 1973). These are important because they often have a wide host range, can severely reduce plant fitness, and can survive as saprophytes in the soil (Burdon, 1987; Jarosz & Davelos, 1995).

The host species, *P. serotina,* is a temperate tree species native to forests throughout eastern North America, and has naturalized in many countries in central Europe (see the Supporting Information, Fig. S1). *Prunus serotina* is known to serve as host to a wide variety of plant pathogens in North America (*c.* 300 fungi listed in Farr *et al.*, 1989) and many of them may be less prevalent or absent in the introduced European range. Nevertheless, *Pythium* are present in the nonnative range (Smith *et al.*, 1988) and are the only pathogen species shown to significantly affect host population density and dynamics in its native range (Videos S1,S2) (Packer & Clay, 2000; Reinhart *et al.*, 2005; Reinhart & Clay, 2009). Comparing the virulence of *Pythium* from the native and invasive ranges of *P. serotina* provides an experimental test of virulence differences between the two ranges as a potential mechanism explaining enemy release.

Materials and Methods

Soil collection and isolation of *Pythium*

In order to obtain a random set of *Pythium* isolates over a wide geographical range for testing, we collected soil samples from 22 populations of *P. serotina* Ehrh. in six states in the USA (soil from 62 trees total) and 17 populations in four European countries (soil from 51 trees total; Fig. S1). Three trees were generally sampled per population. This sampling approach might not detect maximum levels of species diversity at the local scale but is unlikely to miss common and widespread *Pythium* at a larger scale. Differences in soil types, abiotic environments, forest structure, composition, and forest management between US and Europe may also contribute to differences in *Pythium* communities. Sampling in the USA occurred from May 24 to June 24, 2004 and in Europe from August 21 to September 4, 2004. Eight soil cores (2.5 cm diameter) were collected per tree from a depth of 0–10 cm. Two cores were taken 10 cm apart at four cardinal directions and at a distance of 1.5–2 m away from the trunk of each focal tree. The eight soil cores were broken up manually and homogenized into one sample per tree. The soil probe was sterilized before sampling around each tree to ensure independence of samples from different trees. Following collection, soils were transported in a cooler to either Indiana University (USA samples) or the Netherlands Institute of Ecology (European samples). The soil samples were air dried at room temperature for 7 d. This is standard practice for storing soil for later isolation of *Pythium*. The gradual drying helps induce dormancy and formation of durable spore structures (Martin, 1992). After drying, European soil samples were shipped by air to Indiana University, similar to procedures in related studies (Callaway *et al.*, 2004; Reinhart & Callaway, 2004). All dried soil samples were then stored at 10°C at Indiana University until isolations were conducted approx. 2 yr later. Previous research has demonstrated that *Pythium* can be baited from air-dried soil stored for 6 yr (Hoppe, 1959). Thus, gradual drying of the soil and long-term soil storage should not introduce any biases between samples from the USA and Europe. All procedures complied with KOR's USDA-APHIS Permit to Move Live Plant Pests (Permit type 526).

Pythium was isolated from small aliquots of the soil samples in spring 2006 for pathogenicity Expt 1 and again in January–February 2007 for Expt 2. Isolates were obtained from the soil samples with techniques standard for the culturing of *Pythium* (Martin, 1992; Abad *et al.*, 1994). The second set of isolations provided fresh isolates that might not have been identical to those obtained during the first set of isolations. This may have increased our isolate pool and avoided instability of isolates following prolonged culturing. All isolates used in the two pathogenicity experiments were selected with a stratified random sampling procedure where we randomly selected one isolate per host population if *Pythium* was isolated. This approach maximizes the geographic representation of our *Pythium* isolates. The species identities of the isolates

were unknown until after the experiments were completed, when isolates were identified by DNA sequencing. Some non*Pythium* isolates were identified by sequencing (i.e. *Mortierella* spp., capable of growing on the selective growth media P_5ARP; Martin, 1992), especially during initial isolations in 2006, and were therefore discarded from the data set. To avoid culturing *Mortierella* in Expt 2, we identified morphological characters of colonies on agar that distinguished the two genera.

Hyphal tips of *Pythium* isolates were replated on fresh selective media (P_5ARP) and allowed to grow 2–5 d and then transferred to nonselective growth media (cornmeal agar plates). *Pythium* cultures for use as inoculum in the two pathogenicity experiments consisted of grass blade cultures created by transferring approx. 15 sterile grass blades into a Petri plate containing sterile deionized water (Abad *et al.*, 1994). A fragment of agar with hyphal tips was then transferred into the grass blade culture and incubated at room temperature (*c.* 25°C) for ≥ 3 d and examined using a compound microscope.

Pythium identification and DNA sequencing

For molecular phylogeny determination, the isolates were grown on hempseed and water for several weeks. Mycelium was picked out, transferred to an Eppendorf tube with sterile water and freeze dried. The dry mycelium was ground, and DNA was prepared as previously described (Klassen *et al.*, 1996). The genomic region encoding the 3′-end of the 18S rRNA gene, internal transcribed spacer 1 (ITS1), 5.8S rRNA gene, ITS2, and the 5′-end of the 28S rRNA gene was amplified by PCR with the primers UN-UP18S42 (5′-CGTAACAAGGTTTCCGTAGGTGAAC-3′) and UN-LO28S576B (5′-CTCCTTGGTCCGTGTTTCAAGACG-3′). Amplifications were carried out in 50 μl volumes containing 0.1–10 ng genomic DNA, 0.2 mM dNTPs, 0.2 μM of each primer, 2 U *Taq* polymerase and 1 × PCR buffer. Amplifications were done using the PTC-200 DNA Engine cycler (MJ Research Inc., Watertown MA, USA) with the following cycling conditions: 95°C for 3 min, followed by 20–25 cycles of 95°C for 1 min, 68°C for 30 s, 72°C for 2.5 min, and a final extension for 10 min at 72°C. The PCR products were purified with the QIAquick PCR purification kit, and sent to BaseClear B.V. (Leiden, the Netherlands) for sequencing with the primers listed. A consensus sequence of the different reads was constructed with BIOEDIT and manually edited. The ITS1, 5.8S rRNA gene, ITS2 region, was identified by multiple alignment with sequences present in GenBank. Parsimonious tree construction, bootstrap analysis and consensus tree construction was done with PHYLIP (Felsenstein, 1989). Branch lengths were estimated with the DNAML program, a maximum likelihood program in the PHYLIP software package, taking the consensus tree as a user tree.

Pathogenicity experiment 1

We tested the effect of *Pythium* isolates from the native and nonnative ranges on seedlings of *P. serotina* from one seed source in the native range and one source in the nonnative range. The experiment was conducted in experimental vessels under controlled environmental conditions. Because the seeds broke dormancy at different times (*c.* 70 vs 100 d), the portion of the pathogenicity experiment using commercial seed from the native range (Louisiana, USA) was started May 5, 2006, and the portion using seed from the nonnative range (Belgium) was started June 23, 2006. This ensured that the experiments were started with seedlings of similar ontogeny for each seed source.

Before the start of the experiment, seeds were surface sterilized and cold-stratified for 70–100 d. Grass blade cultures were established and used as inoculum (Abad *et al.*, 1994). *Pythium* isolates were selected using a stratified random sampling design from a larger pool of isolates where we randomly selected at least one isolate from each population with *Pythium* isolates. We selected fewer isolates from more populations (vs more isolates from fewer populations) in order to obtain isolates representing a broad geographical range. Although this experiment used 42 randomly selected isolates (21 from each range), we only report results for those confirmed as *Pythium* (13 of 42; 10 from eight populations in the native range and three from three European populations). Seven seedlings were placed in each experimental vessel with three replicate vessels per isolate (13 isolates × 3 replicates per isolate).

Vessels were used to prevent cross-contamination and to maintain a relatively constant moisture environment (shown in Fig. S2). Petri plates were filled with water agar (Difco Bacto agar, Becton, Dickinson and Company, Sparks, Maryland, USA). The water agar functioned as a sterile, hydrated, and nutrient-poor medium for the seedlings to grow and interact with the inoculum, and permitted us to easily view the roots *in situ* and quantify root rot. The vessels were fabricated using Petri plate lids and bottoms, Parafilm and plastic strips. Pathogenicity tests using seed from Louisiana were conducted in smaller experimental vessels (65 vs 100 mm diameter vessels). At the start of the experiments, all seeds had germinated with emergent radicles. Some Louisiana seedlings also had developing shoots.

Seedlings were placed on top of the agar and then inoculated with one piece of grass from the grass blade cultures with visible mycelium growth (i.e. one isolate per vessel and one grass blade per vessel). The grass blade was placed in contact with one seedling. After covering the vessel with the Petri plate lid, the walls of the vessel were sealed with Parafilm. Vessels were placed on metal shelves with fluorescent lighting on a 12-h timer located in the laboratory. At least once per week, vessels were checked and randomly repositioned on the metal shelves. Aggressive isolates rapidly

infected the nearest host and spread to other seedlings (see Videos S1 and S2). For every vessel each seedling was scored as either alive or dead and with or without root rot *c.* 25 d after the experiment was initiated.

To compare disease symptoms (mortality and root rot) of seedlings inoculated with *Pythium* isolates from native vs non-native ranges, two generalized linear mixed models were tested using Proc GLIMMIX in SAS version 9.13 (SAS Institute Inc., Cary, NC, USA). Individual tests were performed for each response variable (mortality and root necrosis). Isolate Origin (native vs non-native ranges) and Seed Source (Louisiana and Belgium) were the independent variables. As the experiment was conducted in two parts, methodological variation between the parts could potentially affect interpretation of effects of seed source and the interaction between seed source and isolate origin (i.e. Type II errors). Thus, only isolate origin (USA vs Europe) was included as a fixed effect in the model for each response variable. Isolate was included as a random effect and nested within Origin. A binomial distribution and a logit link function were used as data represent the number of affected seedlings out of seven.

Pathogenicity experiment 2

Similar to Expt 1, the virulence of 17 random *Pythium* isolates from 13 populations in the native range and 10 random isolates from six populations in the European range was compared. Using a stratified random sampling design, we selected a representative *Pythium* isolate for the pathogenicity experiment from each of 19 populations. Because our sampling procedure was random, and blind to taxonomic identity, it is unlikely that we missed widespread and important species. The experiment was started June 10–11, 2007 using 100 mm diameter vessels with three seedlings per vessel. After conducting Expt 1, we reduced the number of seedlings per vessel to improve our ability to identify the portions of the root system that had rot. Because we found little difference in pathogen sensitivity between seed sources from the native and nonnative ranges in pathogenicity Expt 1, the second experiment was performed with recently germinated *P. serotina* seed from two distinct geographical sources in its native range (Louisiana (n = 3 replicate vessels per isolate; Louisiana Forest Seed Co., Lecompte, LA, USA) and Pennsylvania (n = 2 replicate vessels per isolate, because of fewer available germinated seed; Sheffield's Seed Co., Inc. Locke, NY, USA)). This allowed us to test whether pathogenic effects depended upon the origin of the plant material from the native range. Previous phylogenetic research concluded that populations in the non-native European range were probably founded by seeds from Pennsylvania, the center of *P. serotina* genetic diversity (Petitpierre, 2008).

Twenty-five days following the start of the experiment, root necrosis (Fig. S2), seedling mortality, and shoot biomass (stems and leaves) were quantified. At this point, each seedling was assigned a 100%, 50%, or 0% chance of survival. With fewer seedlings per vessel in Expt 2 (three vs seven), we were able to accurately assign more levels of disease severity. If a shoot had not emerged or appeared dead or wilted, it was scored as dead (0% chance of survival). Further, if a plant had a severely diseased root that interrupted the root system (i.e. extensive root rot or rot on the primary root) then it was also scored as having a 0% chance of survival (see 'severe disease' examples in Fig. S2). By contrast, a portion of root rots were limited to a subset of lateral roots and did not invariably cause mortality (50% chance of survival). Seedlings free of disease symptoms were scored as having a 100% chance of survival. Highly virulent isolates caused plants to become extensively necrotic after *c.* 1–2 wk (Videos S1 and S2). At the conclusion of the experiment, shoots were clipped, dried and weighed.

To compare the disease symptoms (i.e. mortality and necrosis) of seedlings treated with *Pythium* isolates from native vs non-native ranges, generalized linear mixed models were tested using Proc GLIMMIX in SAS version 9.13 (SAS Institute Inc.). Isolate Origin (native vs nonnative ranges) and Seed Source (Louisiana and Pennsylvania) were the two independent variables. Seed source and isolate origin were considered fixed effects and isolate (nested within Origin) as a random effect. A binomial distribution and a logit link function were used as data represent the number of affected seedlings out of three. When analysing the data, the denominator degrees of freedom in F-tests were calculated using Satterthwaite's approximation. Shoot biomass data were analysed using two-way ANOVA using Proc MIXED in SAS. Origin, Seed, and Isolate were explanatory variables where Origin and Seed were considered fixed effects and Isolate (nested within Origin) a random effect. Analyses were also repeated using only data from the most abundant species (*Pythium attrantheridium*) that was isolated from both ranges.

Results

Pythium composition

There were two trials isolating *Pythium* from soil samples for each population. Consistent with predictions for ERH, *Pythium* was isolated from 40–59% of soil samples of populations in the native range vs 18–35% of populations in the nonnative range (Table S1). Among the soil samples from the nonnative range, *Pythium* was isolated from seven (70%) populations from France, Germany and the Netherlands but no isolates were obtained from seven populations from Belgium. In the native range, *Pythium* was isolated from 14 (88%) *P. serotina* populations in Indiana, Kentucky, Pennsylvania, and Tennessee but not from three

populations in Florida and three in Mississippi. We suspect that more intensive sampling would have yielded isolates from all sample sites.

Across both pools of isolates used in Expts 1 and 2, *P. attrantheridium* (≥ 99% sequence homology with a representative *P. attrantheridium*) was the most commonly isolated taxon (Fig. 1). In Expt 1, *P. attrantheridium* represented five of ten isolates from the native range and two of three from nonnative range. In Expt 2, it represented 10 of 17 isolates from native range and seven of 10 from nonnative range (Fig. 1).

Some taxa were isolated only from one range. In Expt 1, isolates with sequence similarity to *Pythium macrosporum*, *Pythium intermedium*, and another from a distinct group similar to *P. intermedium/attrantheridium* were only found in samples from Kentucky, Tennessee, and Pennsylvania, respectively, in the native range. Isolates similar to *P. intermedium/attrantheridium* were also identified in Expt 2 from three populations in Tennessee and two in Kentucky) (Fig. 1). In pathogenicity Expt 2, *P. sylvaticum* was also identified only from one population in Kentucky. Two taxa identified from only the nonnative range included *P. parvum* (Expt 1, one population in France) and an unknown *Pythium* sp. (Expt 2, one population in Germany) most closely aligned with *P. violae* and *P. iwayamai*. In Expt 1, two *P. heterothalicum* isolates were identified from two samples from the native range (Kentucky and Indiana) and from two samples from the nonnative range (France and Germany) in Expt 2.

The *Pythium* phylogenetic tree (Fig. 1) provides evidence of relatedness among groups beyond species designations. For example, all isolates on the same branch (bootstrap support of 891 out of 1000 replications (89.1%)) as *P. attrantheridium* (e.g. II-IN6-1 as well as I-IN1-1 and II-NLD1-2) have > 99% homology with *P. attrantheridium*. Several of these isolates exhibit ITS sequence variation in only a few nucleotides. By contrast, *Pythium diclinum*, *Pythium marinum* and *Pythium lutarium* are considered as separate species but exhibit 99.8% homology.

Pathogenicity experiment 1

There was a marginally significant effect of isolate origin on seedling mortality (generalized linear mixed models, $F_{1,10.06} = 4.42$, $P_{origin} = 0.061$, Fig. 2a). Specifically, isolates from the native range caused 183% and 583% more mortality of seedlings from Louisiana and Belgium, respectively, than isolates from the nonnative range. There was also a marginally significant effect of isolate origin on root rot ($F_{1,9.40} = 4.04$, $P_{origin} = 0.074$, Fig. 2b). Isolates from the native range caused 305% and 462% more root rot of seedlings from Louisiana and Belgium, respectively, than isolates from the nonnative range. Root rot values did not perfectly mirror mortality.

The four isolates causing the greatest mortality (mean across seed types causing > 70% mortality) were identified using molecular phylogenetic analysis: 1 most virulent, *P. attrantheridium* (Indiana); 2–3, *P. macrosporum* (two isolates from separate soil samples from a population in Kentucky); and 4, an isolate similar to *P. intermedium/attrantheridium* (Pennsylvania). Other isolates in the native range in descending virulence were identified as *P. attrantheridium* (ranked 5, 6, 7, and 9), and *P. heterothallicum* (8 and 10). *Pythium attrantheridium* displayed variation in pathogenic activity with one isolate from the native range causing considerable mortality (Indiana, USA) while the others were less virulent. All European isolates of *P. attrantheridium* were relatively avirulent.

Pathogenicity experiment 2

On average, *Pythium* isolates from the native range caused 90% and 80% more seedling mortality, 46% and 38% more root rot and resulted in 19% and 45% less shoot biomass of seedlings from Louisiana and Pennsylvania, respectively, than isolates from the nonnative European range (Table 1, Fig. 2c–e). Seedling mortality and root rot were lower for seedlings from Louisiana than Pennsylvania, although Pennsylvania seedlings attained greater shoot mass (Table 1, Fig. 2c–e). There was also a significant interactive effect of seed source and isolate origin on seedling shoot biomass (Table 1, Fig. 2e, Fig. S3).

Additional analyses were performed to determine if this variation in virulence across ranges was primarily a result of variation in species composition of *Pythium* or variation among strains of a common *Pythium* species. The effects of individual *Pythium* isolates, especially those from the native range, exhibited wide variation in pathogenicity (Fig. 3, Fig. S3). The seven most virulent isolates from the native range, in terms of causing mortality (Fig. 3, Fig. S3), represent unique taxa (i.e. *P. sylvaticum*, *P. intermedium*, and *P. intermedium/attrantheridium*) not found among the isolates from the nonnative range, as determined by molecular phylogenetic analysis (Fig. 1).

Because isolates that exhibited > 99% sequence homology with *P. attrantheridium* were relatively common among both pools of isolates (i.e. 10 of 17 isolates from native range, and 7 of 10 from nonnative range), this large subset of data was analysed independently to test if *P. attrantheridium* isolates from the native range were more pathogenic than those from the nonnative range. This analysis revealed no effect of pathogen origin on seedling mortality ($F_{1,17.63} = 0.63$, $P = 0.44$) but a marginally significant effect on shoot biomass ($F_{1,16} = 3.14$, $P = 0.095$) (Fig. S4), where isolates of *P. attrantheridium* from the native range tended to reduce growth relative to isolates from the nonnative range.

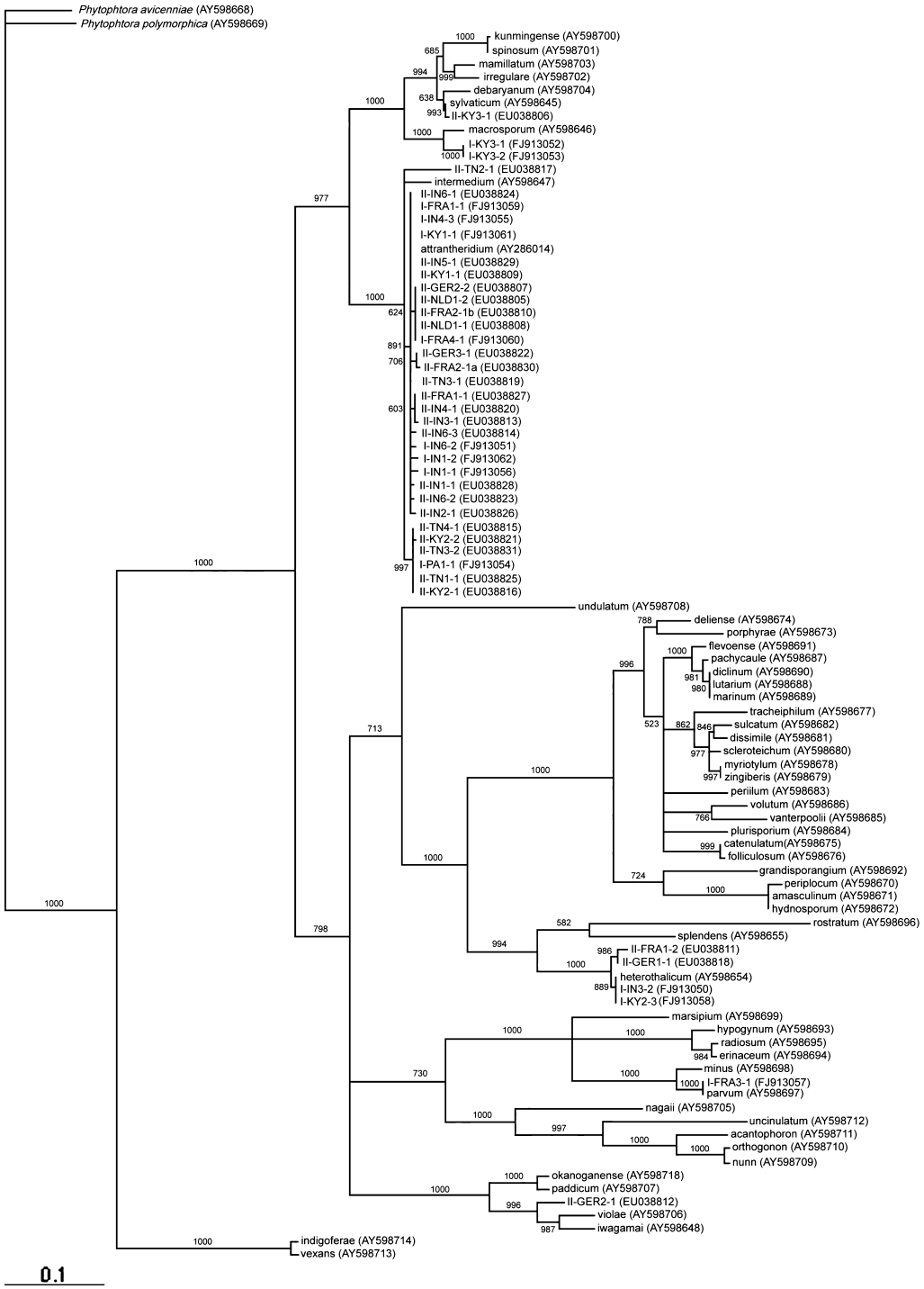

Fig. 1 One of four equally parsimonious trees based on the ITS1, 5.8S rRNA gene, ITS2 nuclear rDNA region showing relationships among the different isolates with identified *Pythium* species spanning the whole genus, as previously described (Lévesque & de Cock, 2004). *Phytophthora polymorphica* and *Phytophthora avicenniae* were used as outgroups. Numbers within the tree represent bootstrap values (1000 replications) and branches that had less than 50% (i.e. < 500) support were removed. Numbers between parentheses are the GenBank accession numbers. Isolates from the first and second pathogenicity experiment are labeled with a 'I' or 'II', respectively. Numerical abbreviations following origin codes (e.g. KY3-1) indicate population number and tree number. FRA2-1a and FRA2-1b are isolates from the same soil sample (i.e. location = France, population = 2, and tree = 1) all other isolates were associated with unique trees.

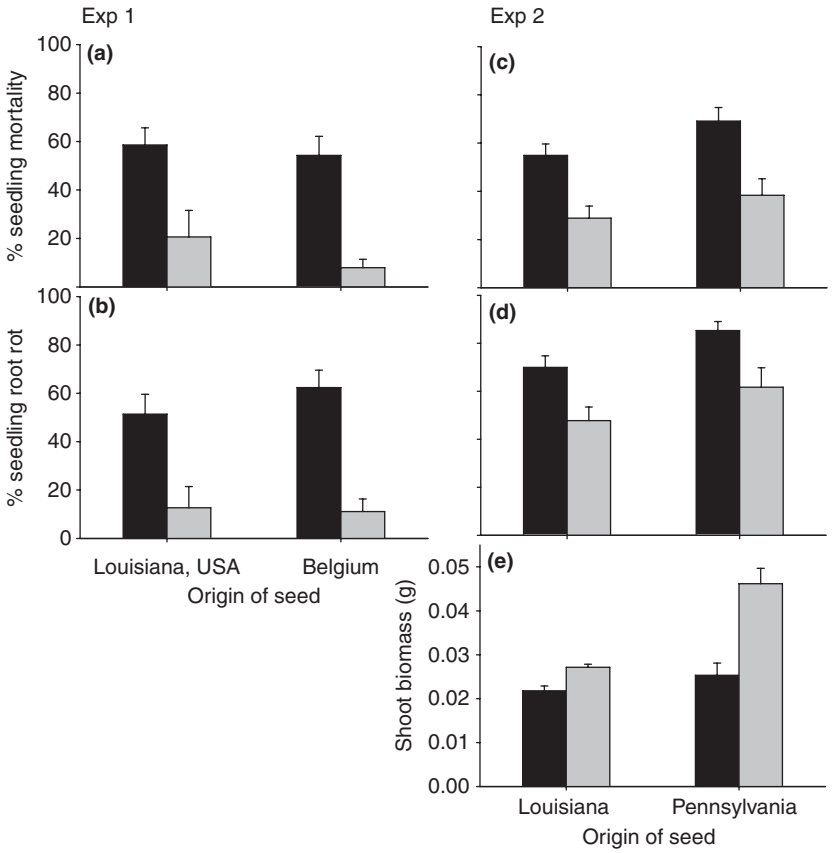

Fig. 2 Results from pathogenicity Expts 1 (a,b) and 2 (c–e). Percentage mortality (a,c), root rot (b,d) and shoot biomass (e) of *Prunus serotina* seedlings caused by *Pythium* isolates from soil collected near *P. serotina* trees in its native (USA, 10 (Expt 1) and 17 isolates (Expt 2)) and nonnative ranges (Europe, 3 (Expt 1) and 10 isolates (Expt 2)). Expt 1 used seedlings from the native (Louisiana, n = 3 replicate vessels per isolate) and nonnative ranges (Belgium, n = 3), and Expt 2 used seedlings from two different regions within the native range (Louisiana, n = 3 replicates per isolate, and Pennsylvania, n = 2). Expt 1 used seven seedlings per vessel and Expt 2 used three seedlings per vessel. Pathogenicity experiments lasted *c*. 25 d. Results are means ± SEM based on the total number of vessels per experiment. Origin of *Pythium* isolates: closed bars, native range (USA); tinted bars, nonnative range (Europe).

Table 1 Statistical results from factorial tests for the effects of seed origin and *Pythium* origin (native vs nonnative range) on the survival, root necrosis and shoot biomass of *Prunus serotina* seedlings (Expt 2)

Fixed effects	df	*F*	*P*
Seedling survival			
Seed	1,131	6.54	**0.012**
Pythium Origin	1,21.48	7.82	**0.011**
Seed × Origin	1,131	0.47	0.50
Root necrosis			
Seed	1,131	11.20	**0.0011**
Pythium Origin	1,18.19	7.64	**0.013**
Seed × Origin	1,131	0.99	0.32
Shoot biomass			
Seed	1,106	47.93	**<0.0001**
Pythium Origin	1,25	15.98	**0.0005**
Seed × Origin	1,106	22.76	**<0.0001**

Significant results (P < 0.05) are shown in bold type.

Discussion

Variation in virulence

Laboratory pathogenicity trials with *Pythium* isolates indicated that the probability of encountering a virulent *Pythium* near established *P. serotina* trees was significantly greater in the native than nonnative ranges. The most virulent *Pythium* taxa were sampled only from the native range of *P. serotina*, suggesting that they are rarer or absent in invaded European forests. Expt 1 revealed an exception: an isolate of the cosmopolitan *P. attrantheridium* was found to be highly virulent while most other isolates of this taxon appeared avirulent. Although some variation in seed sources was observed in Expt 2 (Figs 2,3), results from both experiments suggest that differences in *Pythium* virulence are robust even with variation among seed sources.

No previous study has conducted pathogenicity trials in controlled environments to test the virulence of specific enemies of an invasive plant from its native vs non-native ranges. While some studies (Reinhart *et al.*, 2003; Callaway *et al.*, 2004; Knevel *et al.*, 2004; Reinhart & Callaway, 2004) generally found that the net effect of soil biota in the native range is more negative than from the non-native range, they did not investigate the identity and pathogenicity of enemies from both ranges (reviewed in Reinhart & Callaway, 2006). Here we show that the prolific establishment of *P. serotina* in invaded European forests (Fig. S5) is correlated with both lower prevalence of *Pythium* in *P. serotina* populations (Table S1) and the rarity or absence of highly virulent *Pythium* commonly found in the native range.

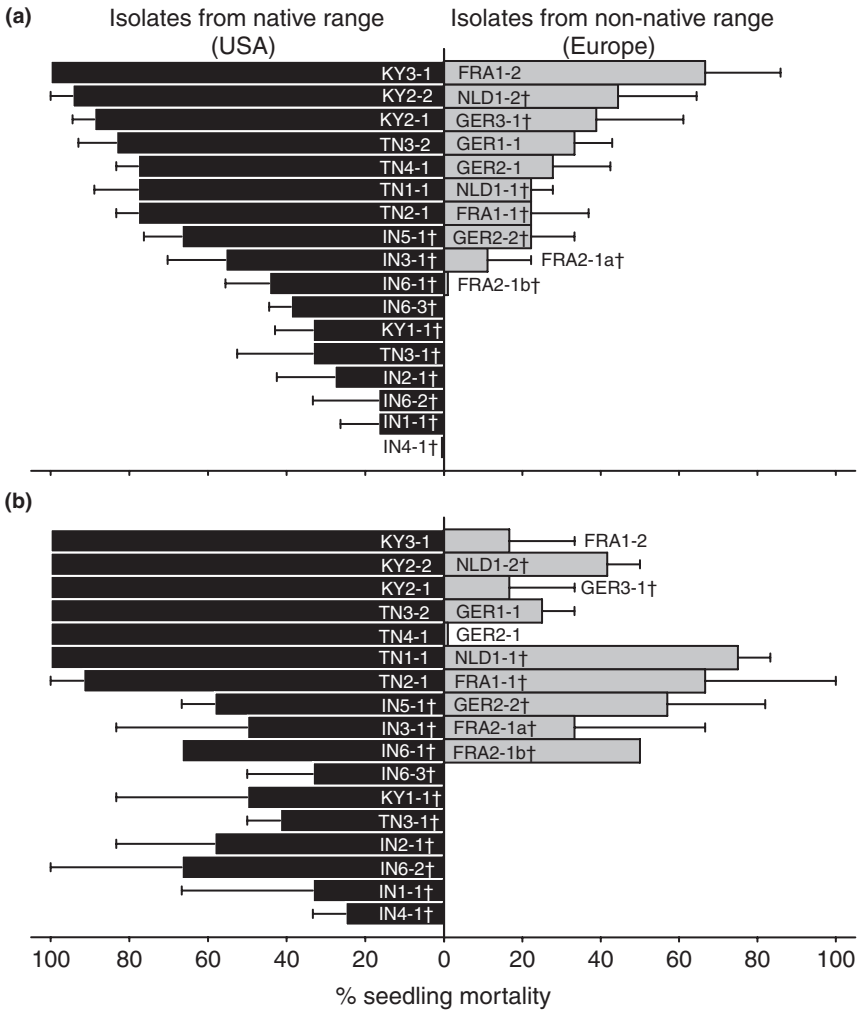

Fig. 3 Percentage mortality of *Prunus serotina* seedlings caused by *Pythium* isolates from soil collected near *P. serotina* in its native (USA, 17 isolates) and nonnative ranges (Europe, 10 isolates). The experiment used seed from either Louisiana (a, $n = 3$ replicates per isolate) or Pennsylvania (b, $n = 2$), USA. Responses to individual isolates shown in (a) and (b) are rank ordered based on (a). Isolates from USA originated from populations in Indiana (IN), Kentucky (KY), or Tennessee (TN). Isolates from Europe originated from populations in France (FRA), Germany (GER) or the Netherlands (NLD). Abbreviations of origin codes, populations, and trees (e.g. KY3-1) preceding accession numbers are described in Fig. 1 legend. Results are means ± SEM. († ≥ 99% sequence homology with *Pythium attrantheridium*).

Results from Expt 1 suggest that pathogenic activity is greater among *Pythium* from the native than nonnative ranges ($P \leq 0.07$), consistent with the highly significant results from Expt 2. Virulence patterns for the two seed sources (native and non-native ranges, Expt 1) were strongly correlated suggesting that overall variation in pathogenic activity between *Pythium* from the native vs non-native ranges was relatively consistent (see Fig. 2). However, effects of seed origin were not tested in Expt 1 because of the high probability of committing a Type II error (see the Materials and Methods section, 'Pathogenicity experiment 1'). Further, we found a significant difference between two native seed sources for seedling survival, root necrosis and biomass (Table 1, Fig. 2), where seedlings from Pennsylvania seed sources were more susceptible to disease. However, the Pennsylvania seedlings exhibited higher growth, suggesting a possible trade-off in susceptibility vs growth. There was also a significant interactive effect of seed source and isolate origin on seedling biomass (Fig. 2e, Fig. S3), indicating that the pathogenic effects of isolates from the native range were more consistent across seed sources than isolates from the non-native range. The evidence for variation in susceptibility among seed sources should be viewed in light of the small sample size (two seed sources) and other differences between seed sources (e.g. morphology and ontogenetic stage). *Pythium* pathogens have been described as having intermediate host-specificity (Augspurger & Wilkinson, 2007), making tight coevolutionary linkages between host and pathogen less likely.

Pythium community composition

In our samples the variation in pathogenic effects resulted primarily from variation in species composition of *Pythium* rather than variation in virulence of isolates common to both the native and invaded ranges of *P. serotina*. The most frequent isolate found in both ranges was *P. attrantheridium* and there was no effect of isolate origin on seedling survival ($P = 0.43$, Expt 2). Further, in Expt 2 isolates of this species

were less pathogenic than other *Pythium* taxa (e.g. Fig. 3). However, we observed a marginally significant ($P = 0.095$) effect of *P. attrantheridium* origin on shoot biomass, suggesting that isolates from the native range may reduce seedling growth rate, putting seedlings at a competitive disadvantage and decreasing their probability of survival compared with isolates from the European range. In Expt 1, the most virulent *Pythium* isolate overall was an isolate of *P. attrantheridium* originating from the native range. Despite these two pieces of evidence for virulence of *P. attrantheridium*, the greater overall pathogenic activity of isolates from the native vs non-native range reflects the preponderance of highly virulent taxa (e.g. *P. intermedium*, *P. intermedium/ attrantheridium*, *P. macrosporum* and *P. sylvaticum*) and the apparent absence and/or rarity of similar taxa in the nonnative range. Although we only isolated virulent taxa from soil samples associated with *P. serotina* in its native range, *P. intermedium* and *P. sylvaticum* have been reported in Europe (Smith *et al.*, 1988) but were not found among our isolates.

Enemy release hypothesis

The varied approaches used in numerous studies to assess ERH are complementary and provide critical information on: (1) biogeographical variation in enemy community composition (Mitchell & Power, 2003; Van der Putten *et al.*, 2005); (2) the effect of enemy communities as measured by either quantifying damage in the field in native vs nonnative ranges (Wolfe, 2002; Vilá *et al.*, 2005) or by quantifying damage of plants treated vs untreated with selective biocides in experimental plots in native vs nonnative ranges (DeWalt *et al.*, 2004); and (3) the virulence of specific enemies in the native vs invaded ranges. The study by DeWalt *et al.* is a strong test of ERH, as described by Keane & Crawley (2002) and Hierro *et al.* (2005) because it links plant performance to enemy impacts using a biogeographical comparison (i.e. native vs nonnative comparison). However, DeWalt *et al.* does not provide any information on why enemy impacts differ between ranges.

Comparisons of virulence of specific enemies in the native and invasive ranges, as reported here, provide another type of evidence for evaluating ERH and provide important mechanistic information about variation in enemy prevalence, community composition and effects on hosts. Our experimental measures of *Pythium* virulence, coupled with greater frequency of isolating *Pythium* from samples from the native range (Table S1), demographic patterns suggesting the absence of strong pathogenic effects in the nonnative range (Fig. S5) and the lack of *Pythium* origin × seed source interactions for seedling survival and root necrosis (Table 1, Fig. 2) all indicate that the success of *P. serotina* invading European forests is correlated with escape from highly virulent *Pythium* taxa.

Complicating factors

Measures of virulence may be complicated by a number of factors including: seedling ontogeny; post-invasion evolution, local adaptation (e.g. gene–gene interactions) and historic origin of *Pythium*. Ontogeny is important because seedlings are most susceptible at the earliest stages of development (Augspurger, 1990). However, it can be difficult to perform experiments where seedlings are in identical stages of development when seed from multiple populations have variable dormancy requirements. Post-invasion evolution has also been shown to affect some traits of invasive plants and their interactions with enemies, especially toxin–detoxifier systems between plants and herbivores (Siemann & Rogers, 2001; Maron *et al.*, 2004). We do not know if traits affecting *Prunus–Pythium* interactions might have undergone post-invasion evolution. Unlike the advances in understanding elicitor–receptor relationships of foliar disease systems (e.g. gene–gene interactions) (reviewed in Jones & Dangl, 2006), much less is known about plant resistance responses to belowground diseases (van West *et al.*, 2003). As *Pythium* from the non-native range appear to respond differently to individual seed sources, it is not clear how they would interact with local vs foreign host seedlings. Currently, little is known about the distribution of *Pythium*, especially in natural plant communities (Paulitz & Adams, 2003; Schurko *et al.*, 2003). It is possible that some *Pythium* spp. interacting with *P. serotina* in its native range could also be nonnative and vice versa.

The isolations are dependent on the techniques and samples used in this study. Culture-based methods were necessary to acquire isolates and determine the pathogenic activity of *Pythium.* They were coupled with DNA sequencing methods to identify the isolates. However, culture-dependent microbiological methods are less accurate than modern molecular methods for fully characterizing microbial communities in the soil and roots (Le Floch *et al.*, 2007). The collection, treatment and storage of soil samples and isolation methods may have constrained our ability to obtain all *Pythium* species present in our soil samples. Additional logistical constraints associated with the pathogenicity trials further limited the number of isolates that we could reasonably manage. Therefore, we used a random subset of *Pythium* isolates from our sampled populations.

The isolates used in our pathogenicity experiments probably do not represent all the species and genotypes of *Pythium* interacting with *P. serotina*, a broadly distributed tree species. It is possible that a common and aggressive *Pythium* exists in the nonnative range but was not sampled or included in the pathogenicity trials. However, the isolates that we did obtain for characterizing virulence were from a large number of *P. serotina* populations and should be an

unbiased representation of the variation of *Pythium* in both ranges. Furthermore, the demographic results from *P. serotina* throughout each range and results from plant responses to the total soil biota determined in a previous study (Reinhart *et al.*, 2003) are consistent with the results reported here.

Our results provide information about variation in the composition and virulence of *Pythium* communities in the native and European ranges, but they do not directly reveal the effects of these organisms in extant *P. serotina* populations in nature beyond inferring process from demographic data (Fig. S5). Other factors such as environmental differences, postinvasion adaptations and effects of other biological interactions with competitors, mutualists and other enemies may also play significant roles in the invasive success of *P. serotina* in Europe. Nevertheless, our results for *P. serotina* demonstrate: strong pathogenic effects of *Pythium* in the native range; the absence of strong pathogenic effects in the field in the nonnative range (Fig. S5); reduced prevalence of *Pythium* in the nonnative range (Table S1); and absence/rarity of virulent *Pythium* taxa associated with the invader in its nonnative range (Fig. 3). Although other, nonmutually exclusive processes may facilitate invasion in Europe (Deckers *et al.*, 2005; Godefroid *et al.*, 2005; Verheyen *et al.*, 2007), our results provide clear evidence that invasive *P. serotina* experiences reduced effects of belowground enemies compared with its native range. This reduction is correlated with a shift from exposure to virulent and avirulent pathogens in the native range to predominantly avirulent pathogens in the nonnative range.

Applications

Contrasting host–pathogen interactions between native and nonnative ranges has several important ecological implications. As non-native species such as *P. serotina* proliferate in foreign locations, resident enemies are predicted to eventually adapt to these exotic and abundant resources (Thompson, 2005; Nijjer *et al.*, 2007; Clay *et al.*, 2008). Because host-switching by pathogens should be greatest among more closely related species (Gilbert & Webb, 2007), *Prunus* species endemic to central Europe (e.g. *P. avium*, *P. padus* and *P. spinosa*) (Van der Meijden, 2005) increase the probability of host-switching to *P. serotina* by resident *Pythium* spp. At present, resident enemies in the nonnative range appear to have relatively minor affects on invasive *P. serotina* populations (Fig. S5). However, the observed variation in pathogenic activity of European *Pythium* isolates, the regional presence of *Pythium* species (*P. intermedium* and *P. sylvaticum*) that have strong negative effects on *P. serotina* in its native range, and native European *Prunus* spp., may all offer opportunities for the eventual control of this invasive species.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Video S1 Effect of an aggressive *Pythium* isolate on *Prunus serotina* seedlings.

Video S2 Root necrosis caused by *Pythium* infection.

Notes S1 Methods and Results.

Fig. S1 Native range of *Prunus serotina* (USA) and countries in Europe invaded by *Prunus serotina* and sampled.

Fig. S2 *Prunus serotina* growth and disease symptoms after 25 d of interacting with different *Pythium* isolates.

Fig. S3 Stem biomass of *Prunus serotina* seedlings when interacting with *Pythium* isolates from soil collected 1.5–2 m away from *P. serotina* in its native (USA) and nonnative ranges (Europe).

Fig. S4 Percentage of mortality and stem biomass of *Prunus serotina* seedlings caused by isolates of *Pythium attrantheridium*, the most commonly isolated *Pythium* species in both the native (USA) and nonnative ranges (Europe) of *P. serotina*.

Fig. S5 Density and relative dominance data collected around focal *Prunus serotina* trees from multiple populations of *P. serotina* in its native (USA) and non-native ranges (Europe).

Table S1 Number of *Prunus serotina* populations with *Pythium* isolated relative to the total number of populations sampled from each location.

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