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Abstract

Infection biology and invasion strategies of *Kretzschmaria deusta* were investigated through the analysis of patterns of colonization and population diversity in three case studies, comprising different host species. Molecular analysis and isolation assays performed on stem sections at different heights indicated a prevalent heart rot mode of expansion. Random Amplified Microsatellites - RAMs and somatic incompatibility assays on isolates allowed detection, in one case, of a genet occupying the entire decay column and, in the other cases, several different genets in each individual tree. Hypotheses on modes of arrival and entrance of *K. deusta* in trees are discussed on the basis of the distribution of genets and population genetics analyses. Significant correlation (Spearman $\rho = 1.0$; $p < 0.001$) between the number of genets and the number of areas delimited by pseudosclerotial plates (PSPs) on the decayed portions of stem sections suggests PSPs are interaction zone lines between different individuals of *K. deusta* occupying adjacent decay columns.

Keywords: Infection biology, *Kretzschmaria deusta*, Linkage Disequilibrium, Multiplex PCR, RAMs, Patterns of colonization, Pseudosclerotial plates, Somatic incompatibility, Wood decay.

Introduction

Although often regarded merely as a saprotroph, the xylariaceous ascomycete *Kretzschmaria deusta* is locally reported as a primary root rot, butt rot, trunk rot and canker rot of several tree species (Hawksworth 1972; Sinclair *et al*. 1987). In tropical and subtropical regions, *K. deusta* can pose a threat for commercial plantations, e.g. oil palms, red grapefruit, rubber and tea trees (Sinclair *et al*. 1987; Palacios *et al*. 2008), whereas in temperate regions it is reported as one of the main reasons for decreased safety and windthrows of *Acer* spp., *Aesculus* spp., *Fagus* spp., *Tilia* spp. and *Ulmus* spp. trees (Wilkins 1934, 1939; Greig 1989; Gibbs and Greig 1990; Terho & Hallaksela 2008; Michelotti *et al*. 2012). Sudden limb failures are also often associated with *K. deusta* (Lonsdale 1999). The detection of *K. deusta* during tree inspection is often problematic, even at an advanced stage of colonization. Indeed, signs of its presence, mainly consisting of inconspicuous conidial and perithecial stromata, can easily be missed during visual tree inspections. Even common instrumental analysis, including those based on penetrometers and acoustic diagnostic devices, can often fail to detect wood decay of *K. deusta*, which acts predominantly as a soft rot during tree colonization (Schwarze *et al*. 1995; Brandstetter 2007).

As a consequence, most studies on *K. deusta* have focused either on advanced techniques for the detection of decay (Pearce *et al*. 1994; Schwarze *et al*. 1995; Rabe *et al*. 2004; Deflorio *et al*. 2008a) and its early identification in trees (Nicolotti *et al*. 2009), or on the dynamics of fungal invasion and host response through artificial inoculation assays (Baum & Schwarze 2000; Schwarze & Baum 2000; Deflorio *et al*. 2008b, 2009). Based on the results of these studies, the micro-morphological features of the brittle decay caused by this ascomycete and aspects concerning its colonization strategy, i.e. mechanisms of reaction zone penetration and ability to colonize sapwood of certain tree species, have been exhaustively elucidated.

Conversely, although parasitism of *K. deusta* towards several tree species was demonstrated and deeply investigated in earlier case studies performed by Wilkins (1936, 1938, 1939, 1943), no clear evidences defining its infection biology, i.e. the modes of spread and entrance, has been reported so

far. Such evidence can be provided by studies on the genetic structure of pathogen populations within and among hosts. The abundance, size and distribution of different genets (genetic individuals) may be considered an indirect reflection of fungal mode of spread, entrance and establishment among and within the trees (Rayner & Boddy 1986). Briefly, vegetative mycelium through root contacts or soil provides the opportunity for one genet to spread from tree to tree, whereas arrival by propagules, i.e. sexual and asexual spores, may give rise to a more heterogeneous population. Similarly, within a tree, population diversity is expected to be higher where infection took place. In fact, during decay progress many genets can be filtered out due to spatial competition for domain and/or selectivity of the environment (Rayner & Boddy 1986). Analysis of population structure can further be useful to improve knowledge of the role of pseudosclerotial plates (PSPs) often characterizing the colonization of *K. deusta* in trees. Although these crust-like aggregations of melanised hyphae are commonly reported as barriers to maintain a dry moisture regime favourable for *K. deusta* growth (Rayner & Boddy 1988), no studies have been performed to test the hypothesis that PSPs may be interaction zone lines between decay columns occupied by different individuals.

The presence of different genets within a population can be detected by testing for somatic incompatibility (SI) between vegetative mycelia (Lane 1981). This method has allowed determination of intraspecific diversity in populations of xylariaceous fungi both on a local scale, i.e. within single ascomata or trees and among adjacent trees in a woodland site (Chapela & Boddy 1988; Griffin *et al*. 1992; Rodrigues *et al*. 1995; Hendry *et al*. 1998; Johannesson *et al*. 2001), and on a larger scale, i.e. thoroughout wide geographical areas (Pérez Jiménez *et al*. 2002). Molecular methods based on randomly amplified polymorphic DNA (RAPD) markers proved to be even more effective in discriminating genets within fungal populations (Jacobson *et al*. 1993). While the SI assays can reveal the genetic differences associated with the loci involved in the self/ non-self recognition, molecular genotyping can access genome-wide polymorphisms (Douhan *et al*. 2011). Although reproducibility of RAPD markers is often questionable, a variant of this technique, based

on random amplification of DNA regions flanked by microsatellites (Random Amplified Microsatellites-RAMs or Inter Simple Sequence Repeats-ISSR), allows more reproducible fingerprints (Gente *et al*. 2002) and has proved to be a valuable tool for assessing genetic diversity within populations of parasitic, saprotrophic and mutualistic symbiotic fungi (Vainio *et al*. 1998; Gherbi *et al*. 1999; Paavolainen *et al*. 2001).

The present study was aimed at investigating the infection biology and invasion strategies, i.e. modes of arrival, entrance and establishment, of *K. deusta* in three case studies, each involving a different tree species located either in urban environment or in a forest site. Each case study was thus focused on: (i) assessing the patterns of colonization of *K. deusta* in trees through both isolation assays and multiplex PCR (M-PCR)-based molecular analysis performed of wood samples collected from stem sections at different heights and on soil samples surrounding trees; (ii) determining the population structure of *K. deusta* through RAMs analysis and SI assays within each tree and stem section, and among trees in one site. Results of population structure analysis have further been used to test the hypothesis that PSPs are the result of mycelial interaction between different individuals occupying adjacent decay columns.

Material and methods

Tree material and sampling procedures

The three case studies comprised four trees in which *K. deusta* had been previously detected through a drill-based technique combined with M-PCR-based molecular analysis (Guglielmo *et al*. 2010). The first case included an *Acer platanoides* (ID: A1) and an *A. pseudoplatanus* (A2) tree located in the Turin city area 15 meters apart along the same street (45°03’40”N; 7°39’22”E); the second case consisted of a *Fagus sylvatica* tree (F) located in the forest of the nature reserve of Sacro Monte di Varallo, northern Italy (45°49’08”N; 8°15’21”E); the third case concerned a 200-year-old roadside *Platanus acerifolia* tree (P) on a central street in the city of Cirié, northern Italy (45°13’58”N; 7°36’14”E). All trees were first examined for external signs and symptoms of disease,

as well as for the presence of wounds. After felling, three to four transversel trunk sections, about 5 cm thick, were collected from the basal stem of each tree up to the upper edge of the visible decay column, transferred to the laboratory and maintained in a plastic sample bag at 4°C prior to sampling. Height above ground level and diameter were recorded for each trunk section (Table 1). Cross-sectional extents of wood visually classified as decayed, discoloured or sound-looking, as well as hollow, were calculated using the program ArcGIS 9 (Esri, Redlands, CA, USA) applied on photographs of each trunk section (Table 1). Number of areas delimited by PSPs in each stem section was assessed to calculate their density on the total surface area of decayed wood (density= № of areas delimited by PSPs / m^2 decayed area).

Samplings were performed using a sterilized scalpel on both sides sections. At least one sample was taken from discoloured, sound-looking and hollow-surrounding wood, whereas multiple samples, corresponding to different areas separated by PSPs, were excised from decayed wood. Each sample was subjected part to isolation assays, part to a 24 hr-lyophilisation prior to DNA extraction and M-PCR.

For the trees A1, A2 and P further samplings were performed from stumps and branches, as well as from soil (Table 1). Wood samples from the hollowed and decayed areas of stumps and branches were collected using a sterilized scalpel as previously described. Eight different soil samples were collected, using a soil auger, at a depth of 10-15 cm in the four cardinal directions at 5 and 10 cm from each tree. Soil samples were maintained in plastic bags at 4°C prior to a 24 hr-lyophilisation, DNA extraction and M-PCR.

Fungal culture isolation assays

Isolation assays were performed by plating, for each wood sample excised from trunk sections, branches and stumps, five fragments of 3-4 mm x 1-2 mm on 9 cm-Petri dishes of 2% (w/v) Malt Extract Agar (MEA) amended with 225 mgL^{-1} of Streptomycin sulphate (AppliChem GmbH, Darmstadt, Germany). Prior to plating, wood fragments were surface-sterilized by dipping them into

0.5% (w/v) sodium hypochloride (NaClO) solution for 5 s and rinsing in sterile distilled water. The Petri dishes were incubated at room temperature for 4 weeks and observed daily for fungal growth from wood fragments. Isolates identified as *K. deusta*, after visual analysis of macro-morphological features of the fungal colonies (Hawksworth 1972), were transferred to new 2% MEA Petri dishes and purified from potential bacterial contamination by using Raper's rings (Raper 1937). Cultures outgrowing from the rings were transferred to storage tubes containing 2% MEA and maintained at 4°C. Identification of fungal isolates as *K. deusta* was further confirmed by M-PCR. In several cases, more than one isolate of *K. deusta* (from two to five, depending on the number of wood fragments with fungal outgrowth) was obtained from a single sample and this allowed testing of the hypothesis that an area delimited by PSPs is occupied by a single genet.

Any basidiomycete growing from wood fragments was further isolated and identified through Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) analysis, after PCR amplification and sequencing of Internal Transcribed Spacers (ITS) region, as described by Guglielmo *et al*. (2007).

Prior to DNA extraction, purified cultures were grown for 2 weeks in 2% (v/w) Malt Extract liquid media, harvested by filtration, rinsed with sterilized distilled water and freeze-dried after 24hr lyophilization.

DNA extraction

DNA extraction from wood and from soil was performed using the QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) and the E.Z.N.A.™ Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA), respectively. For both types of DNA extraction we started from 100 mg of lyophilized material after a homogenization step using the FastPrep FP120 Cell Disrupter (Thermo Electron Corporation, Milford, MA, USA).

DNA extraction from lyophilized mycelia was performed by using the CTAB-based method described by Guglielmo *et al*. (2007). The concentration and purity of total DNA extracted from

mycelia was estimated using a spectrophotometer GENESYS 10 UV (Thermo Electron Scientific Instruments, Madison, WI), by analysis of absorbance at 260 and 280 nm, as well as, after a 0.8% (w/v) standard agarose (Applichem GmbH, Darmstadt, Germany) gel electrophoresis, by comparing the genomic DNA with the quantified bands of GeneRuler 100 bp DNA ladder (Fermentas GmbH, St.Leon-Rot, Germany).

Multiplex PCR analysis

Three *taxon*-specific priming M-PCRs, named M1, M2, M3, were used directly on wood, soil and mycelia DNA extracts to detect and identify *K. deusta*, as well as ten other wood decay fungal *taxa*, namely *Armillaria* spp., *Ganoderma* spp., *Hericium* spp., *Inonotus-Phellinus* spp., *Laetiporus sulphureus*, *Perenniporia fraxinea*, *Pleurotus* spp., *Schizophyllum* spp., *Stereum* spp. and *Trametes* spp. For details on M-PCR refer to Guglielmo *et al*. (2007) and Nicolotti *et al*. (2009). Each M-PCR reaction was performed in a 25 µl volume containing 1x PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs mix, 0.5 µM of each primer, 0.5 mg/ml of Bovin Serum Albumin (BSA), 0.025 $U\mu l^{-1}$ of Taq polymerase (Promega, Madison, WI, USA) and 6.25 µl of a 1/50 dilution of wood or soil DNA extract. PCR reactions were conducted using an initial denaturation at 94ºC for 5 min, followed by 35 cycles with each cycle consisting of a denaturation at 94ºC for 45 s, an annealing at a temperature ranging from 55°C to 63°C, depending on the M-PCR, for 45 s, and an extension at 72°C for 45 s, and one final cycle with a 72ºC extension for 10 min. Amplicons were visualized on a gel containing 1% (w/v) of high resolution MetaPhor (Lonza, Rockland, ME, USA) and 1% (w/v) of standard agarose, after a 2 hr-electrophoretic migration at 4 Vcm^{-1}. Estimation of amplicon size, performed by comparing it to a GeneRuler 100 bp DNA ladder, allowed identification of the corresponding *taxon*, e.g. an amplified DNA fragment of 260 base pairs (bp) after M3 indicated the presence of *K. deusta* (Nicolotti *et al*. 2009).

Random Amplified Microsatellites and somatic incompatibility assays

Three 5'-anchored RAMs primers were used: $DDB(CCA)_5$, $DHB(CGA)_5$ and $DVD(CT)_7C$, where B= C, G or T; D= A, G or T; H= A, C or T; V= A, C or G (Vainio & Hantula 1999). The PCR reactions were performed in a 25 µl volume containing 1x PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs mix, 2 µM of each primer, 0.04 $U\mu l^{-1}$ of Taq polymerase (Promega, Madison, WI, USA) and 1 ng of DNA template. PCR reactions were conducted using an initial denaturation at 94ºC for 3 min, followed by 35 cycles with each cycle consisting of a denaturation at 94ºC for 30 s, an annealing for 45 s at a temperature of 50°C, 61°C and 64°C, for CT, CGA and CCA primer, respectively, an extension at 72°C for 1 min, and one final cycle with a 72ºC extension for 10 min. Amplification products were separated and estimated by electrophoresis, as described above for M-PCR amplicons. PCR reactions on each DNA extract were repeated twice and only clear and reproducible amplification products were considered. In case of co-migrating bands, only one of these was considered for the analysis. Further, to test the repeatability of the method, 10% of the isolates were randomly chosen and re-subjected to DNA extraction and PCR reactions with the three RAMs primers. Outcomes of repeated runs were scored blindly and compared to those of the original runs to calculate RAMs error rate. The RAMs markers were named by combining the microsatellite motif of the primer with the approximate fragment length. The presence (1) or absence (0) of each clear and reliable marker was scored in a binary matrix to determine the identity or diversity among fungal isolates.

To confirm data obtained from RAMs markers, SI assays were performed by pairing isolates from the same host in all possible combinations, including self-pairings. Further, SI assays were also performed on isolates from different hosts which appeared identical after RAMs analysis. The pairings were performed by placing two agar plugs, with mycelia from the colony margin, 2 cm apart in the centre of Petri dishes filled with 2% MEA. Cultures were examined every week, for two months, to detect and examine zones of mycelial interaction characteristic of vegetative incompatibility (Anagnostakis 1977).

Data analysis

The number of different genets was determined, on the basis of RAMs profiles and SI outcomes, for isolates obtained from different samples within a section and within a host.

Genetic relationships amongst *K. deusta* isolates were computed using Jaccard's coefficient of similarity calculated on a binary matrix based on RAMs data. The resulting similarity matrix was subjected to cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA). Analysis was implemented through the utility DendroUPGMA (http://genomes.urv.es/UPGMA/index.php). Bootstrap values of branch points were generated in PAUP, version 4.0b10 (Swofford 2002) using 1000 replicates.

To test for the reproductive mode (i.e. clonal through asexual spores or recombining through ascospores), a clone-corrected RAMs binary dataset comprising all the detected genets was analyzed for Linkage Disequilibrium (LD) among polymorphic loci, as described by Haubold *et al.* (1998). This method, implemented in the LIAN 3.5 software (http://adenine.biz.fh-weihenstephan.de/cgi-bin/lian/lian.cgi.pl), tests the null hypothesis of statistical independence of alleles at all loci by calculating the observed variance (V_D) of the distribution of pairwise differences between genets within a population, and by comparing it with the variance expected under LD (V_E). The null hypothesis $V_D=V_E$ was tested by Monte Carlo simulation with 10000 iterations. A standardized index of associations (I_{AS}) and the 95% confidence limits determined by Monte Carlo simulations (L_{MC}) were also calculated (Haubold & Hudson 2000). Further, histograms of the pairwise Jaccard's coefficient of similarity were obtained using Microsoft Office Excel 2003 (Microsoft Corporation Redmond, WA, USA) to test whether their distribution was unimodal or bimodal. Test of Kolmogorov-Smirnov for normality of this distribution of values was implemented with PASW Statistics 18.0 (SPSS, Inc., Chicago, IL, USA).

To test the hypothesis that PSPs separate different genets, the correlation between the number of different genets and the density of areas delimited by PSPs was determined through the Spearman

rank order correlation test. The test was implemented with PASW Statistics 18.0 and was performed both on the average values for each tree and on data of all stem sections.

Results

Isolation assays and M-PCR analysis

A total of 387 wood samples were analyzed (Table 1). *Kretschmaria deusta* was detected through isolation assays in 85 samples and through M-PCR in further 120 samples. Most of the wood samples positive for *K. deusta* (203 out of 205) were collected from areas of stem section with evidence of decay and surrounding hollows (Table 1). *Kretschmaria deusta* was detected in only two of the 103 samples excised from discoloured areas bordering the decayed areas. None of the samples collected from branches and from sound-looking wood was positive for this fungus (Table 1). None of the 24 soil samples analyzed was positive for *K. deusta* after M-PCR. In 13 cases we obtained more than one genotype of *K. deusta* from a single wood sample (Table S1).

Random Amplified Microsatellites and somatic incompatibility assays

The analysis of *K. deusta* isolates with RAMs primers CCA, CGA and CT provided 32 reproducible RAMs markers, with size ranging from 150 bp and 1600 bp (Table S1). All the repeated runs confirmed the results of the original runs (RAMs error rate= 0%). Twenty seven of these markers were polymorphic and allowed identification, among the overall 109 isolates analyzed, of 15 genets. Each genet was present in a single tree, except one genet which was detected in both maple trees A1 and A2 (Table 2). Except for F, each tree was occupied by different genets, from three to nine (Table 2).

Pairings of isolates of *K. deusta* allowed differentiation of two types of mycelial interactions after 21-28 d: (i) intermingling mycelia between the two colonies which displayed an uniform aspect; (ii) a barrage formation, ranging from slight to strong and often displaying melanised hyphae, at sites of contact between the two colonies (Fig. 1). The former was considered as a somatic compatible

reaction, whereas the latter was interpreted as a somatic incompatible reaction. Self pairings always resulted in compatible reactions. Somatic incompatibility assays confirmed results obtained through RAMs analysis within each tree, with the exception of plane tree P, where only two of the three genets detected through RAMs were differentiated (Tables S2, S3, S4, S5). The presence of a common genet between the nearby trees A1 and A2 was further confirmed by SI assays (Table S2). *Kretzschmaria deusta* isolated from a single wood sample, for each of the 13 cases analyzed, belonged to the same genet (Table S1).

Case study 1: two maple trees along the same urban street

Trunk sections felled from the two maple trees, A1 and A2, revealed two different patterns of decay type and progress (Fig. 2). Although basal sections of both trees were extensively decayed, in A1 a central decay column surrounding a hollow persisted with similar cross-sectional extents to at least 125 cm above ground. In A2 a more eccentric decay column, following an evident longitudinal wound, was visible with decreased cross-sectional extents to 132 cm above ground, where discoloured wood was predominant (Table 1). Pseudosclerotial plates, whilst present in both trees within and at the margin of decayed wood, were more frequent in A2 (Fig. 2).

Kretzschmaria deusta was detected in all the 78 decayed wood samples collected from the four trunk sections of A1 (Table 1). In A2, *K. deusta* was detected with high frequency (91% to 95%) in the 68 decayed wood samples from the two lower trunk sections, whereas its detection frequency decreased to 33% (two out of six samples) in the third section (Table 1). *Kretzschmaria deusta* was found with a low frequency at the margin of the hollow of A1, both in the stump and in the uppermost sections (Table 1).

Eleven different genets were identified among the 54 *K. deusta* isolates obtained from the two maple trees (Table 2). Three genets, of which one predominanted in all sections, one was present with low frequency in a confined area of almost all sections (Fig. 2), and one was detected once (Table 2), were identified among the 35 isolates from A1. Nine genets resulted from the 19 isolates

of *K. deusta* obtained from the two sections of A2, one of which was common to A1 (Table 2). Four of these genets were present in both of these sections and their absolute frequency in each section ranged from one to three (Table 2 and Fig. 2).

Case study 2: a *Fagus sylvatica* tree located in a forest site

A wide central decay column, starting from the hollowed basal trunk up to at least a height of 700 cm, was evident from the stem sections from the beech tree (F) (Table 1). Pseudosclerotial plates were present only between the decayed and discoloured wood, as well as at the margin of the basal hollow (Fig. 2).

Kretzschmaria deusta was detected in all 19 decayed wood samples collected from the three trunk sections at 5, 400 and 700 cm, whereas this fungus was never found in the uppermost section, at 12 m height, where a discoloured area was still present.

RAMs analysis as well as SI tests showed that all of the 19 isolates of *K. deusta* obtained from the three trunk sections of F belonged to the same genet (Table 2).

Case study 3: a *Platanus acerifolia* tree located in an urban avenue

The plane tree P was characterized by a wide basal cavity surrounded by a restricted decayed area which extended centrally to a height of 35 cm above ground (Table 1). Stumps and branches were extensively hollowed. Discoloured wood was evident in the uppermost trunk sections. Pseudosclerotial plates were visibly present in the basal section in a decayed area restricted to one side of the cavity (Fig. 2).

Kretzschmaria deusta was detected in 16 (of 18) decayed wood samples collected from the basal trunk section, whereas it was present only in three (of 31) decayed wood samples of the two uppermost sections. Other wood decay basidiomycetes, i.e. *Rigidoporus ulmarius*, *Gloiothele lactescens* and *Pholiota aurivella*, were further isolated from decayed wood samples collected from

the stump and trunk sections (Table 1). *Kretzschmaria deusta* was never found in samples from discoloured trunk wood (Table 1).

Eight polymorphic RAMs markers allowed the identification of three genets amongst the 12 *K. deusta* isolates obtained from the three trunk sections of P (Table 2). While all three genets were present in the basal trunk section, the unique isolate of *K. deusta* obtained from each of the two uppermost sections belonged to the genet which was predominant in the basal section (Table 2 and Fig. 2).

UPGMA and population genetics analyses

The UPGMA cluster analysis based on pairwise Jaccard's similarity coefficients revealed that isolates from A1 and A2 belonged to the same cluster, distinct from another cluster comprising the isolates from F and P (Fig. 3). Several isolates from both A1 and A2 clustered together within groupings characterized by high Jaccard's similarity coefficients (> 0.9) and moderate to strong bootstrap branch support (Fig. 3). The level of Linkage Disequilibrium among polymorphic loci in the sample comprising all the detected genets was not significant (observed mismatch variance V_D= 6.9; expected mismatch variance V_E= 5.2; standardized index of association I_{AS}= 0.01; simulated 5% critical value L_{MC}= 7.2; p= 0.08). Distribution of pairwise Jaccard's similarity coefficients between genets followed an unimodal Gaussian curve (Test Kolmogorov-Smirnov for normality, p= 0.206).

Pseudosclerotial plates and number of different genets

Correlation between the number of genets of *K. deusta* populations and density of areas delimited by PSPs (Table 2) was significant, performing the test both on the average of the measurements within each tree (Spearman ρ= 1.000; p< 0.001) and on data from each trunk section (ρ= 0.945; p< 0.001).

Discussion

The three case studies conducted in this work, and focused on patterns of colonization and population structure of *K. deusta* within trees, provided evidence supporting a prevalent heart rot mode of expansion and suggested different modes of arrival and entry, presumably linked to environmental determinants. The identification of different genets in stem sections indicates PSPs can be the result of interactions between genets of *K. deusta* occupying adjacent decay columns. A common feature of all case studies was the ability of *K. deusta* to colonize the inner core of the tree, thus causing a heart rot. From the analysis of the uppermost sections, in A1 and F the central decay column progressed with similar cross-sectional extent up to several meters above ground, whereas in A2 and P its extent was much less and showed a more eccentric development. Despite these differences, a heart rot invasion mode seems the most probable colonization strategy of *K. deusta* in different host tree species. This mode of expansion is further suggested by the fact that, except for P, *K. deusta* was the unique wood decay fungus detected in each tree. Indeed, the highly selective conditions of the heartwood of standing trees, usually characterized by stringent gaseous regimes and presence of allelopathic extractives, allows the growth and establishment of only a few primary colonizers with a stress-tolerant ability typical of heart rots (Rayner & Boddy 1988). Patterns of colonization similar to those here described had been already reported in earlier studies on *Fagus* sp., *Tilia* sp. and *Ulmus* sp. infected by *K. deusta* (Wilkins 1936; 1939; 1943), indicating heart colonization might be the typical colonization pattern of *K. deusta*, irrespective of host species. These studies further reported the presence of this ascomycete on roots, suggesting that infection courts might be localized at the base of the stem and/or root system. Although no root samples were analyzed in the present study, our data support this hypothesis. Based on the number and distribution of genets within trees, a wound-mediated basal infection via airborne propagules may have occurred for A1, A2 and P; whereas a root infection through vegetative mycelium could be an explanation of the mode of arrival and entrance of *K. deusta* in F.

Concerning the first three instances, the decay columns were progressively, or drastically, curtailed extent upwards from the base, indicating a butt rot. Upward colonization from the base of the tree was further supported in P by the presence, in the second stem section, of only one of the three genets detected in the first basal section. Filtering of individuals upwards from the base was less obvious in the two maple trees. Indeed, in A1 two main genets were detected from the base to the upper edge of distinct decay columns separated by PSPs; in A2 several different genets were detected both in the first basal section and in the second upper section. The presence of multiple genets, as reported in these three instances, may thus be indicative of a mode of infection by airborne propagules (Rayner & Boddy 1986). Since *K. deusta* is reported as an unspecialized opportunist able to breach reaction zones and showing strong invasiveness in sapwood (Schwarze & Baum 2000; Deflorio *et al*. 2008b), it is likely that basal injuries exposing disrupted sapwood may have acted as preferential infection courts. The high number of different genets of *K. deusta* detected in A2 may also be explained by the presence of an extensive healed wound from the base up to the stem of this tree. Indeed, several different individuals within heart rot decay columns may reflect the combination of genetically diverse spore inoculum with large and multiple infection courts (Rayner & Boddy 1986). We can hypothesize that the difference in *K. deusta* decay column extension between A1 and A2 may be due to intraspecific competition, which was much more pronounced in the latter case. Indeed, it is reported that the simultaneous presence of multiple somatic incompatible genets can result in spatial competition for domain, often leading to inhibition of further development and resource exploitation (Boddy 2000). However, time since infection is another obvious and important factor affecting the extent of the decay in trees. The limited colonization of *K. deusta* in P, on the other hand, may be explained by spatial competition with other wood decay basidiomycetes (Table 1).

Linkage Disequilibrium, i.e. non random allelic association among loci, is one of the indirect measures used to estimate the impact of sexual and asexual reproduction in populations (Chen & McDonald 1996; Agapow & Burt 2001). The lack of Linkage Disequilibrium among the

polymorphic loci screened in this study may indicate for *K. deusta* a prevalent mode of infection through sexual spores, i.e. ascospores. Further, the distribution of pairwise Jaccard's similarity coefficients among genets followed an unimodal Gaussian curve, suggesting isolates derived from a randomly mating population (Redecker *et al.* 2001). However, these results should be considered at best preliminary and a population genetics study based on a larger number of isolates and field sites is needed to determine the contribution of sexual spores to *K. deusta* infection processes.

The strict genetic relationships among several isolates from A1 and A2 may indicate the same source of infection for both trees. Part of this source of infection may have included asexual spores, since a common genet between A1 and A2 was detected. This hypothesis is consistent with the ability of *K. deusta* to form both conidial and perithecial stromata (Sinclair *et al.* 1987).

Unlike the previous cases, in the beech tree F a single genet of *K. deusta* occupied the extensive central decay column from the base to 7 m above ground. The predominance of single genet in a butt rot may be indicative of a root infection via vegetative mycelium (Rayner & Boddy 1986). However, to fully test this hypothesis, a large number of trees surrounding the infected beech should have been analyzed. In fact, the presence of a single genet in a tree could also be related to a history of intraspecific competition resulting in only one genet surviving and colonizing the tree. Nevertheless, tree-to-tree transmission via root contacts or by hyphal growth through soil has been already suggested for *K. deusta* in tea plantations of tropical regions (Sinclair *et al.*1987).

The fact that the two different modes of infection may both operate in nature is further supported by the results of a previous inoculation experiment indicating the ability of both mycelia and ascospores to cause *K. deusta* infection in mature trees (Prljinčević 1982). As already shown in several studies on ectomycorrhizal fungi (Douhan *et al*. 2011), ecological strategies adopted by fungi can be related to environmental constraints. Species with few large genets maintained by mycelial growth can be linked to old and stable forests, whereas high number of genets established by means of sexual spores may be observed in anthropically disturbed trees, especially in urban environment (Douhan *et al*. 2011).

The analysis of population structure in each section supported the hypothesis that PSPs can be interaction zone lines between different individuals of the same species. Two main lines of evidence are reported here: (i) the highly significant correlation between the number of genets and the density of areas limited by PSPs; (ii) the presence of a single genet among isolates obtained from the same wood sample, thus presumably within an area surrounded by PSPs. It is worth noting that PSPs were further present at the boundaries between discoloured and decayed wood, supporting the other role commonly reported for PSPs, i.e. a compartmentalization barrier to maintain the most favourable growing conditions for *K. deusta* (Rayner & Boddy 1988). For more practical purposes, these results can be relevant in management strategies of urban and parkland trees. After felling a tree infected by *K. deusta*, the visual analysis of the decayed stump surface may provide indications about population diversity within the tree and, thus, on the most probable spreading strategy employed by the pathogen in the site.

In conclusion, it is worth remarking other important aspects more related to the methodological approaches employed in this study. First, M-PCR diagnostic assay proved to be a sensitive and reliable detection method allowing considerable improvement of results based on isolation assays. Whilst basic for study of the population structure, isolation assays can often fail due to contamination with other fast growing microorganisms commonly present in environmental substrata, such as wood. A somatic incompatibility system was for the first time demonstrated in *K. deusta* through the barrage formation among colonies isolated from a same tree. Interestingly, the efficiency of this approach was comparable to that based on highly polymorphic RAMs markers.

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Figure legends

Figure 1. Somatic compatibility and incompatibility reactions between *K. deusta* isolates. In **A**, **B** and **C**, plates displaying incompatibility reactions with formation of barriers differing in intensity; in **D**, plate with a compatible reaction.

Figure 2. Transverse trunk sections obtained at different heights from *Acer platanoides* A1, *Acer pseudoplatanus* A2, *Fagus sylvatica* F and *Platanus acerifolia* P. Decayed areas (De), discoloured areas (Di), hollow (Ho) and sound-looking wood (SW) are indicated in the first section of each tree. Sampling points where *K. deusta* was detected (▲) or not (Δ), as well as any wounds (W), are reported on each section. The sampling points where *K. deusta* was isolated and characterized through RAMs markers are followed by a letter. Different letters correspond to different genets. Sample bar= 10 cm.

Figure 3. Dendrogram displaying relationships among all genets of *K. deusta* obtained in this study. For each genet the corresponding tree and, within brackets, the number of the isolates are reported. The dendrogram was generated using the UPGMA method from a similarity matrix based on the percentage shared of a total of 32 RAMs markers. Numbers above branches indicate bootstrap values (over 50%) obtained from 1000 replicate analysis.

FIGURE 1

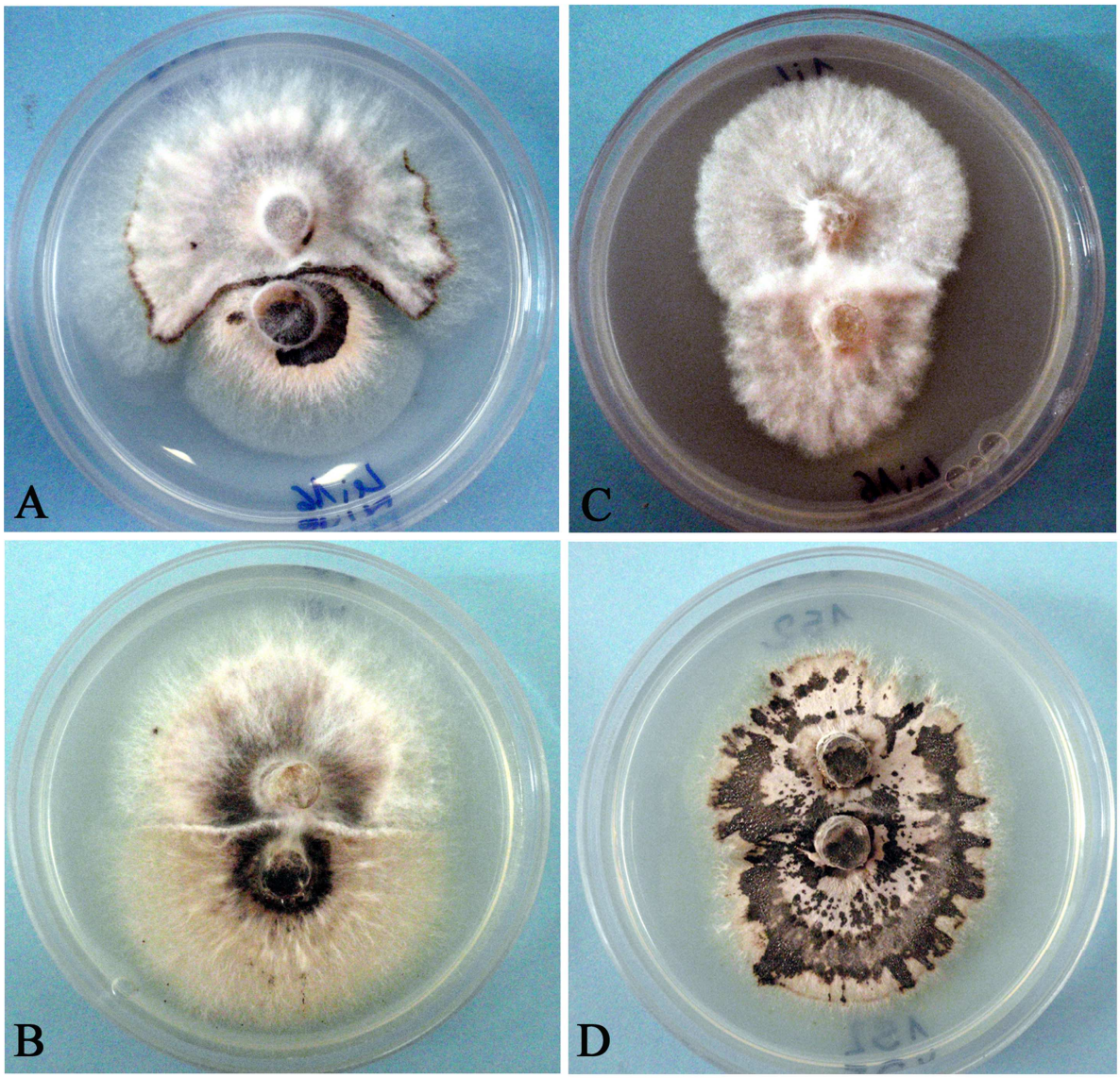

FIGURE 2

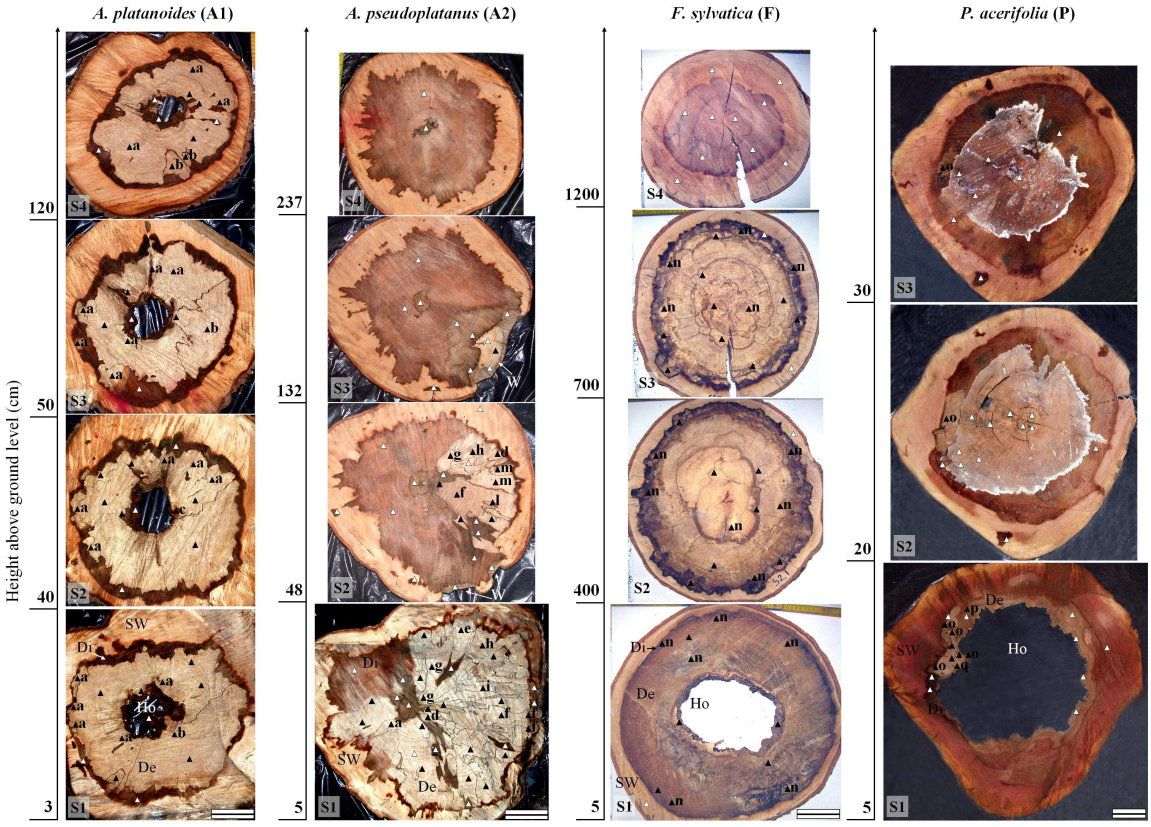
A. platanoides (A1)
A. pseudoplatanus (A2)
F. sylvatica (F)
P. acerifolia (P)
Height above ground level (cm)
120
50
40
3
237
132
48
5
1200
700
400
5
30
20
5
S4
S3
S2
S1
SW
Di
Ho
De
W

FIGURE 3

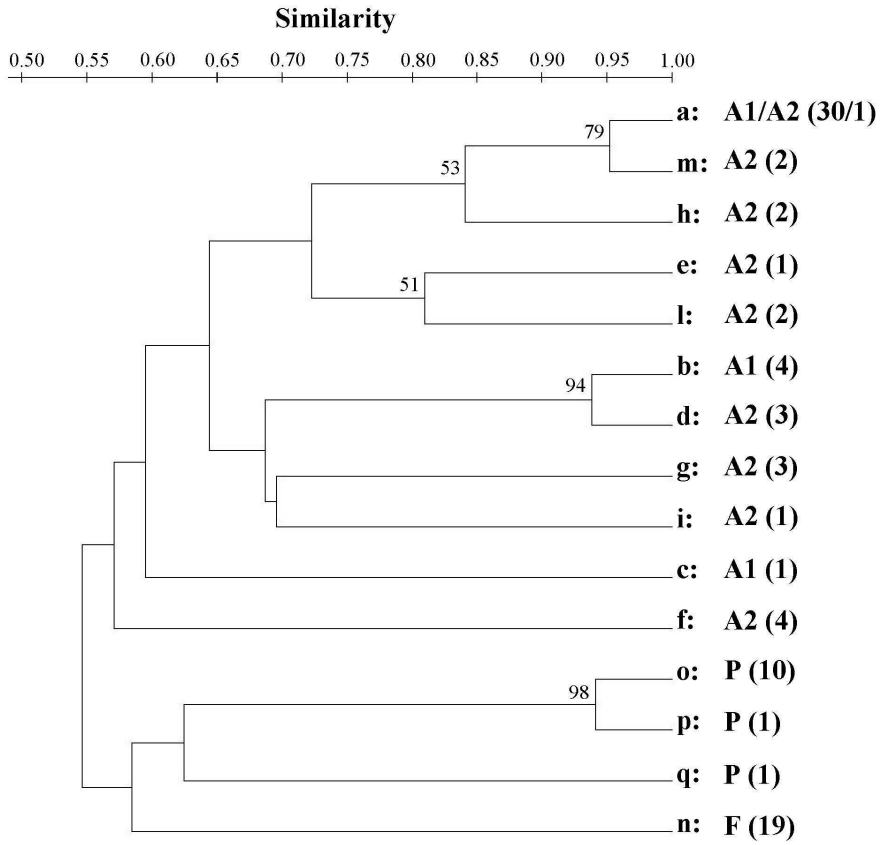
Similarity
0.50 0.55 0.60 0.65 0.70 0.75 0.80 0.85 0.90 0.95 1.00
79
53
51
94
98
a: A1/A2 (30/1)
m: A2 (2)
h: A2 (2)
e: A2 (1)
l: A2 (2)
b: A1 (4)
d: A2 (3)
g: A2 (3)
i: A2 (1)
c: A1 (1)
f: A2 (4)
o: P (10)
p: P (1)
q: P (1)
n: F (19)

TABLE 1. Types of sample analyzed per each tree and detection frequency of *K. deusta*.

Tree species (ID)	Sample source	Height above ground level (cm)[1]	Stump/ Trunk diameter (cm)[2]	Types of wood	Cross-sectional extent[3]	Frequency of *K. deusta* (№ of positive samples/ № of samples analyzed)[4]	Other basidiomycetes (№ of positive samples)
Acer platanoides (A1)	Soil	-	-	-	-	0% (0/8)	-
	Stump	0	62 ± 1	Hollow	-	33% (1/3)	-
				Decayed	35%	100% (22/22)	-
				Discoloured	9%	0% (0/1)	-
				Sound-looking	55%	0% (0/1)	-
	Trunk section 1	3 - 7	61 ± 1	Hollow	1%	33% (1/3)	-
				Decayed	37%	100% (23/23)	-
				Discoloured	11%	0% (0/5)	-
				Sound-looking	49%	-	-
	Trunk section 2	40 - 45	59 ± 1	Hollow	3%	0% (0/2)	-
				Decayed	39%	100% (18/18)	-
				Discoloured	13%	0% (0/3)	-
				Sound-looking	45%	-	-
	Trunk section 3	50 - 55	55 ± 1	Hollow	3%	0% (0/2)	-
				Decayed	41%	100% (15/15)	-
				Discoloured	12%	0% (0/5)	-
				Sound-looking	46%	-	-
	Trunk section 4	120 - 125	46 ± 1	Hollow	1%	0% (0/3)	-
	Branches	-	-	Discoloured	-	0% (0/4)	-
Acer pseudoplatanus (A2)	Soil	-	-	-	-	0% (0/8)	-
	Stump	0	59 ± 4	Decayed	-	0% (0/2)	-
				Decayed	50%	91% (43/47)	-
				Discoloured	14%	0% (0/20)	-
	Trunk section 1	5 - 10	58 ± 4	Sound-looking	36%	0% (0/1)	-
				Decayed	18%	95% (20/21)	-
				Discoloured	41%	4% (1/23)	-
	Trunk section 2	48 - 60	50 ± 3	Sound-looking	41%	0% (0/2)	-
				Decayed	6%	33% (2/6)	-
				Discoloured	34%	0% 0/14)	-
	Trunk section 3	132 - 137	47 ± 3	Sound-looking	60%	0% (0/2)	-
				Discoloured	56%	0% (0/4)	-
	Trunk section 4	237 - 242	43 ± 1	Sound-looking	44%	-	-
	Branches	-	-	Discoloured	-	0% (0/2)	-
Fagus sylvatica (F)				Decayed	68%	100% (9/9)	-
				Discoloured	5%	100% (1/1)	-
				Sound-looking	14%	0% (0/1)	-
	Trunk section 1	5	55 ± 4	Hollow	13%	100% (1/1)	-
	Trunk section 2	400	48 ± 4	Decayed	66%	100% (12/12)	-
				Discoloured	17%	100% (1/1)	-

				Sound-looking	17%	0% (0/1)	-
				Decayed	55%	100% (14/14)	-
				Discoloured	19%	0% (0/1)	-
	Trunk section 3	700	45 ± 2	Sound-looking	26%	0% (0/1)	-
				Discoloured	41%	0% (0/6)	-
	Trunk section 4	1200	43 ± 2	Sound-looking	59%	0% (0/4)	-
	Soil	-	-	-	-	0% (0/8)	-
	Stump	0	79 ± 4	Hollow	-	0% (0/2)	*R. ulmarius* (1)
				Decayed	20%	89% (16/18)	*Gloiothele lactescens* (2)
				Discoloured	2%	0% (0/6)	-
				Sound-looking	48%	0% (0/2)	-
	Trunk section 1	5 - 7	78 ± 4	Hollow	30%	33% (2/6)	-
				Decayed	34%	10% (2/21)	*Gloiothele lactescens* (4)
				Discoloured	25%	0% (0/5)	-
	Trunk section 2	20 - 22	73 ± 3	Sound-looking	41%	0% (0/2)	-
				Decayed	26%	10% (1/10)	*Pholiota aurivella* (1)
				Discoloured	35%	0% (0/4)	-
	Trunk section 3	30 - 35	71 ± 1	Sound-looking	39%	0% (0/1)	-
Platanus acerifolia (P)	Branches	-	-	Hollow	-	0% (0/4)	-

[1]The heights of both upper and lower sides are indicated for each trunk section.
[2]We reported the mean diameter ± SE.
[3]Percentage from the whole cross-sectional area.
[4]Samples in which *K. deusta* was detected with M-PCR and/or isolation assay.

TABLE 2. Number of *K. deusta* isolates sampled and related genets, detected on the basis of RAMs markers and SI tests, at each trunk section and within each tree.

Tree	Trunk section	№ *K. deusta* isolates[1]	№ genets (frequency-type)	№ of areas delimited by PSPs / m^2 of decayed area
	1	10	2 (9-a, 1-b)	57.56
	2	11	2 (10-a, 1-c)	61.55
	3	8	2 (7-a, 1-b)	62.74
	4	6	2 (4-a, 2-b)	67.74
A1	1+2+3+4	35	3 (30-a, 4-b, 1-c)	-
	1	10	7 (3-f, 2-g, 1-a, 1-d, 1-e, 1-h, 1-i)	554.80
	2	9	6 (2-d, 2-l, 2-m, 1-h, 1-f, 1-g)	763.43
A2	1+2	19	9 (4-f, 3-d, 3-g, 2-h, 2-l, 2-m, 1-a, 1-e, 1-i)	-
	1	6	1 (6-n)	5.89
	2	7	1 (7-n)	8.63
	3	6	1 (6-n)	10.67
F	1+2+3	19	1 (19-n)	-
	1	10	3 (8-o, 1-p, 1-q)	109.43
	2	1	1 (1-o)	-
	3	1	1 (1-o)	-
P	1+2+3	12	3 (10-o, 1-p, 1-q)	-

[1]Number of isolates of *K. deusta* obtained from different wood samples.