

## Characterization of Italian isolates of *Inonotus rickii*

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**Summary.** Thirty-seven isolates of *Inonotus rickii*, a pathogenic fungus causing white rot and cankers, were collected from diseased boxelder trees lining boulevards in Rome and from other hosts in Rome and Sicily. During the survey, it was observed that this fungus occasionally produced basidiomes, but more frequently it had anamorphic structures that released a brown powdery mass of chlamydo-spores, presumably acting as asexual propagules. All isolates were characterized using random amplified microsatellite analysis and somatic incompatibility tests in order to investigate the diversity of genotypes within and between the different disease centers surveyed in Italy. The results suggest that both sexual and asexual reproduction play an important role in the spread of this disease, with important epidemiological implications.

**Key words:** *Ptychogaster cubensis*, RAMS, somatic incompatibility, wood decay.

### Introduction

*Inonotus rickii* (Pat.) Reid is a pathogenic fungus that infects the branches and stems of standing hardwood trees and causes wood decay. As it invades the sapwood and the cambium, it causes a decline in the crown with sparse foliage and die-back of twigs and branches.

The fungus was first reported in the early 1900s in tropical and subtropical countries (Davidson *et al.*, 1942), and since 1970 it has also been reported in the Mediterranean area (Malençon, 1970). In Italy it was first reported by Jaquenoud (1985) and, more recently, it has been found in public and botanical gardens in Sicily (Annesi *et al.*, 2005) and in boulevards in Rome (Annesi *et al.*, 2003; Mazza *et al.*, 2008), where it has caused severe damage. Many infected boxelder (*Acer negundo* L.) and European hackberry (*Celtis australis* L.) trees have also been observed in Spain and Portugal respec-

tively (Intini, 2002; Ramos *et al.*, 2008). This confirms that the fungus is now well established in the Mediterranean region and on different hosts.

A knowledge of pathogen biology is the basis for studying its epidemiology and thus defining the most suitable strategies for controlling the disease. *I. rickii* produces both annual basidiomes and conspicuous fungal structures with a soft zonate inner part that release brown powdery masses of chlamydo-spores, which characterize its anamorphic stage: *Ptychogaster cubensis* Pat. (Jaquenoud, 1985; Melo *et al.*, 2002; Bernicchia, 2005). The connection between *I. rickii* and *P. cubensis* is corroborated by the analysis and comparison of nucleotide sequences in Argentina (Gottlieb *et al.*, 2002).

The objective of this work was to investigate the diversity of *I. rickii* genotypes within and between surveyed disease centers in Italy, through a somatic incompatibility test and random amplified microsatellite (RAMS) analysis, in order to elucidate the possible mechanisms of disease establishment and the role of sexual and asexual reproduction in the spread of this fungus.

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## Materials and methods

### Fungal isolates

The study was carried out in four boulevards (sites A, B, C, D) with declining *A. negundo*, located in Rome in different areas within a radius of 6 km. Boxelder trees were spaced 8–10 m apart on both sides of the street. The pathogen had previously been recorded at two of these sites (B and D) (Annesi *et al.*, 2003).

During 2004–2006, a total of 350 healthy and declining trees (110, 55, 140, 45 at sites A, B, C and D respectively) were carefully and repeatedly examined, at different periods, for signs of *I. rickii*. All the reproductive structures found were collected and the fungus was isolated in the laboratory by plating fragments, removed from chlamydospore masses, basidiome, or decayed wood on 2% malt extract agar (MEA). Teleomorph and anamorph structures and mycelial cultures were identified using the keys of Bernicchia (2005) and Stalpers (1978, 2000). Other isolates previously collected from anamorphic structures were also included in the study. Two had been collected on other tree species in Rome [site E: *C. australis* (Annesi *et al.*, 2003); site F: *Koelreuteria paniculata* Laxm. (Annesi *et al.*, 2007)]; five had been found in Sicily [Palermo: tree G.1: *Quercus cerris* L., tree G.2: *A. negundo*; Catania: tree H.1: *Sambucus nigra* L., tree H.2: *Aberia caffra* Hook. F. & Harv. (= *Dovyalis caffra* Warb.)] (Annesi *et al.*, 2005) (Table 1). Isolates of *Inocutis tamaricis* (Pat.) Flasson & Niemelä (CRA-PAV PF 210: *Tamarix gallica* L.) and *Inonotus hispidus* (Bull.:Fr.) (PF 46: *Sophora japonica* L.) were used as the outgroup.

### RAMS and M13 analyses

All isolates were grown on potato dextrose agar and fungal DNA was extracted according to Cenis (1992). Micro and minisatellite M13 markers (Meyer *et al.*, 1991; Hantula *et al.*, 1996) were amplified by PCR using anchored trinucleotide repeat primers. The RAMS primers used in this study were: DBH (CGA)<sub>5</sub>, VHV(GT)<sub>7</sub>G, VDV(CT)<sub>7</sub>C, where B=G/T/C, D=G/A/T, H=A/T/C and V=A/C/G. The minisatellite M13 primer was GAGGGTGGCGTTCT. PCR cycling conditions were: denaturation at 95°C for 10 min, followed by 37 cycles consisting of: 94°C for 45 s, 45 s annealing at a temperature depending on the RAMS

primer, 2 min primer extension at 72°C, followed by a final extension of 7 min at 72°C. The annealing temperatures were: 61°C for the CGA primer, 58°C for the GT primer, 50°C for the CT primer and 48°C for the M13 primer. The amplification products were separated by electrophoresis on a gel containing 1% (w:v) of both agarose (FMC BioProducts, Rockland, ME, USA) and SynerGel (Diversified Biotech, Boston, MA, USA). At least three independent PCR amplifications were carried out on each isolate. The amplification products were scored manually for the presence or absence of bands ('1' or '0' respectively), and only distinct and reproducible bands, visible in all the three amplifications, were included in the analysis (Figure 1). A similarity matrix based on Jaccard's similarity coefficient (Sneath and Sokal, 1973) was used to construct a dendrogram using the unweighted pair-group method algorithm (UPGMA) clustering method of the NTSYS software package (2.02) (Rohlf, 1997).

### Somatic incompatibility studies

All the isolates collected were paired, in four replications, in all possible combinations by placing mycelial disks (4 mm diameter) 1.5 cm apart on MEA. All the isolates were also paired against themselves as a control. Paired isolates were incubated in the dark at 30°C and reactions inspected weekly. Reactions were assessed as incompatible when a barrage formed with deposition of brown pigment between paired isolates. Pairings were considered compatible when mycelia coalesced with no barrage formation (Holmer *et al.*, 1994; Miller *et al.*, 1999).

## Results

### Fungal isolates

During the survey, *I. rickii* was detected on 21 out of 350 boxelder trees. Thirty isolates were collected: 27 from anamorphic structures, one from a basidiome (site C) and two from decayed wood (site B). Up to four isolates were obtained from different locations on the same tree (Table 1). At sites A, C, and D, no two trees presenting reproductive structures were adjacent, and the minimum distance between trees showing signs of the pathogen was 100 m at site A (A.2, A.6), 30 m at site C (C.5, C.6), and

Table 1. Isolates of *Inonotus rickii* collected in four boulevards on *Acer negundo* (A, B, C, D), on different sites and hosts in Roma (E.1, *Celtis australis*; F.1, *Koelreuteria paniculata*) and in Sicily (Palermo [(G.1, *Quercus cerris*, G.2, *A. negundo*); Catania [H.1, *Sambucus nigra*, H.2, *Aberia caffra*]). a, anamorph; w, decayed wood; s, sporocarp.

Stock No.	Sample code <sup>a</sup>	Isolate	Source
PF 213	A.1	1	a
PF 214/215/216	A.2	1/2/3	a/a/a
PF 217/218	A.3	1/2	a/a
PF 219/220	A.4	1/2	a/a
PF 221	A.5	1	a
PF 222	A.6	1	a
PF 53	B.1	1	a
PF 54	B.2	1	a
PF 55/223	B.3	1/2	a /w
PF 224	B.4	1	w
PF 225	B.5	1	a
PF 226	B.6	1	a
PF 227	C.1	1	a
PF 228	C.2	1	a
PF 229	C.3	1	a
PF 230	C.4	1	a
PF 231/232/233/234	C.5	1/2/3/4	a/s/a/a
PF 235/236	C.6	1/2	a/a
PF 56	D.1	1	a
PF 237	D.2	1	a
PF 238	D.3	1	a
PF 57	E.1	1	a
PF 239	F.1	1	a
PF 40/43	G.1	1/2	a/a
PF 44	G.2	1	a
PF 42	H.1	1	a
PF 41	H.2	1	a

<sup>a</sup> Capital letters and numbers refer to the site and the host tree respectively, as in the table caption.

25 m at site D (D.1, D.3). Only at site B, two out of six infected trees were contiguous (8 m apart).

#### RAMS and M13 analyses

The dendrogram revealed 16 distinct genotypes among the 32 isolates collected in Rome (Figure 2). Each infected boxelder tree located at sites C and D and the other two different hosts sampled in Rome (E and F) contained distinct genotypes. Site B was the only site in which a single clone infected all the trees within a distance of 250 m. It is worth noting that the genotype detected in

boulevard B was also found in three trees (A.3.1, A.4.1, A.4.2, and A.6.1) in boulevard A, located approximately 5 km away, along with four different genotypes (A.1.1, A.2.1/2/3, A.3.2, A.5.1). There were two further cases, in Sicily, when a single genotype was detected in individual trees growing 100–200 m from each other and belonging to different species (*S. nigra* and *A. caffra* in Catania [H.1.1 and H.2.1]; *Q. cerris* and *A. negundo* in Palermo [G.1.1/2 and G.2.1]). On six boxelder trees in Rome (A.2, A.3, A.4, B.3, C.5, C.6) and on the Turkey oak in Palermo (G.1), multiple isolates (up

to four) were recovered. In a case (A.3) the analyses recognized two different fungal strains, whilst in the other six cases it was shown that each tree was infected by one strain only.

#### Somatic incompatibility studies

When an incompatible reaction became macroscopically visible after three or four weeks, with a dark-brown barrage between the paired colonies,

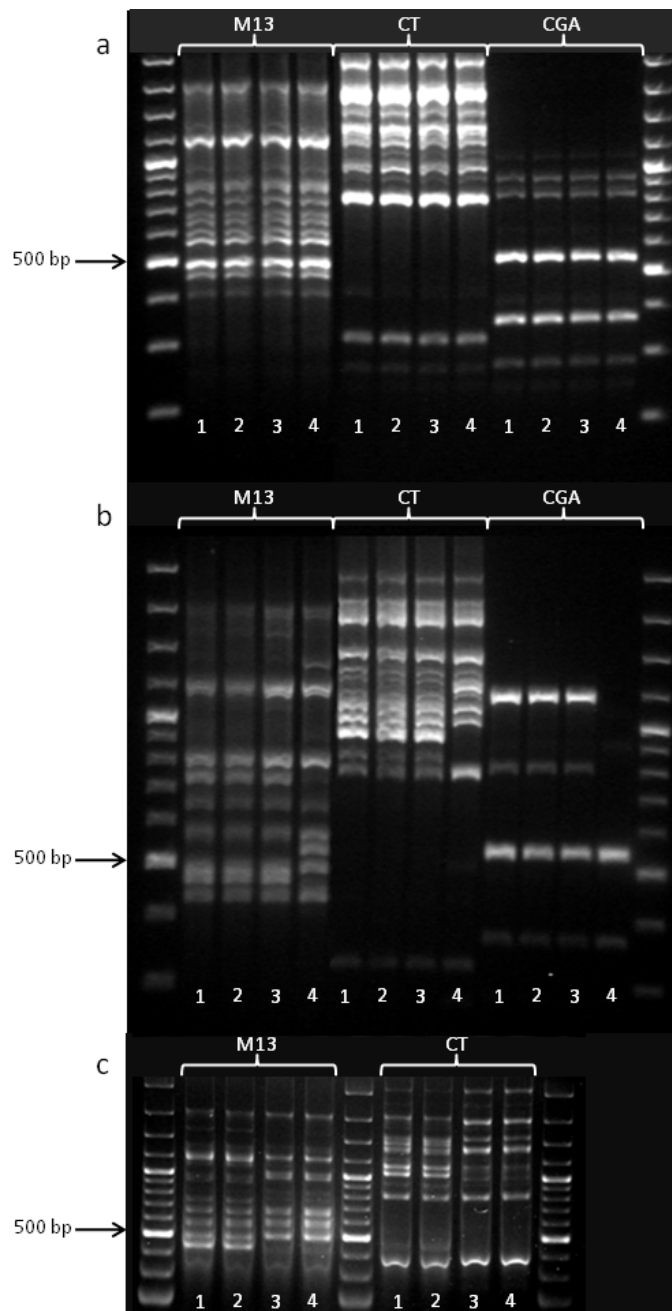


Figure 1. Examples of M13 and RAMS patterns of *Inonotus rickii* isolates. a) Lane 1 - C.5.1; Lane 2 - C.5.2; Lane 3 - C.5.3; Lane 4 - C.2.1. b) Lane 1 - B.1.1; Lane 2 - B.2.1; Lane 3 - B.3.1; Lane 4 - B.4.1. c) Lane 1 - G.1.1; Lane 2 - G.2.1; Lane 3 - H.1.1; Lane 4 - H.2.1.

the isolates were deemed to belong to different genotypes. Self-paired isolates always showed a fully compatible reaction. When the compatible reaction was observed in pairings performed with isolates from a different origin, they were considered the same genotype (Figure 3). Replications always gave the same compatible or incompatible reactions. All results were in complete accordance with the molecular analysis.

### Discussion

*Inonotus rickii* produces both sexual and asexual reproductive structures, although fruiting bodies occur only occasionally (Mazza *et al.*, 2008; Ramos *et al.*, 2008). During the present survey, only one basidiome was detected in the four boulevards examined in Rome. Since sexual reproduction is the most important way to in-

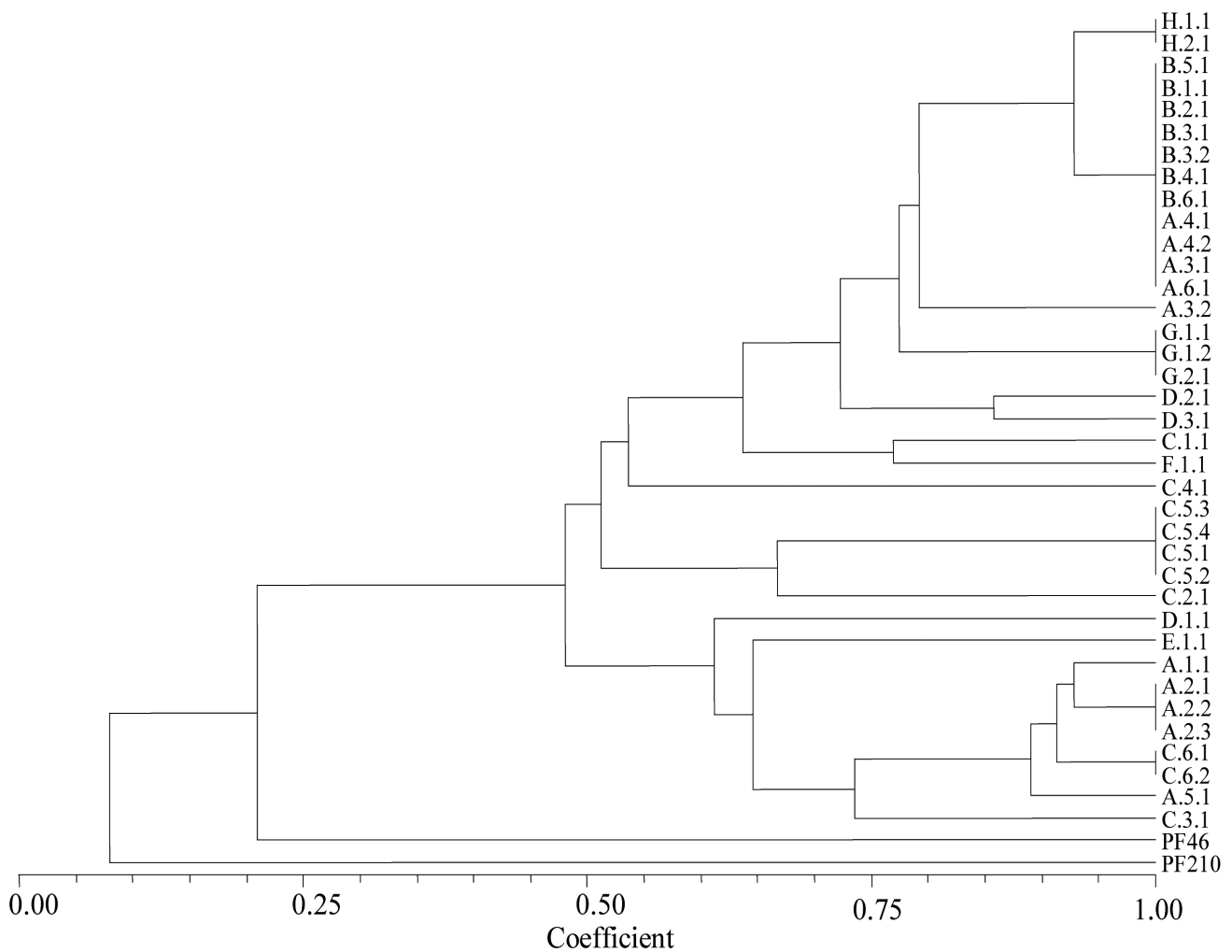


Figure 2. Dendrogram of RAMS and M13 profiles generated by UPGMA analysis, using Jaccard's similarity coefficient, from isolates of *Inonotus rickii*. Tested isolates are reported in Table 1. *I. hispidus* (PF 46) and *Inocutis tamaricis* (PF 210) were used as outgroup.

crease genetic variability, a high predominance of clones would be expected. A previous study, conducted on a few isolates from boulevard B (the first disease centre detected in Rome), suggested that chlamydospores were the only propagules involved in dissemination along a city street (Annesi *et al.*, 2003). However, the somatic incompatibility test and RAMS analysis of a larger number of fungal isolates collected from the different disease centres in this study suggest a mixed dispersal mode, in which both basidiospores and asexual propagules play a role. The occurrence of a single clone in nine boxelder trees growing in two boulevards 5 km apart confirmed that large bursting anamorph structures, releasing brown powdery chlamydospore masses, make this fungus efficient in asexual dispersal. Numerous carriers (pruning tools, used on branches and epicormic shoots, and other maintenance activities, such as lawn

mowing at the base of the trees, parked cars, wind, insects, and other animals) may also play an important role in dispersing the asexual propagules of the fungus. Its asexual fructifications were seen both along the trunk, near wounds, and on the branches of infected trees, and these trees were apparently randomly located along the boulevards. That the fungal spreads through root contact seems unlikely; however, a role of root contact in the spread of the fungus cannot be ruled out in the absence of studies specifically investigating this particular dispersal route.

At the same time, the contribution of basidiospores in disease spread was supported by the occurrence of multiple genotypes in the disease centres, both in the boulevard where the single basidiome was found and in those boulevards where only the anamorph of the fungus was detected. Although novel genotypes can arise through iso-

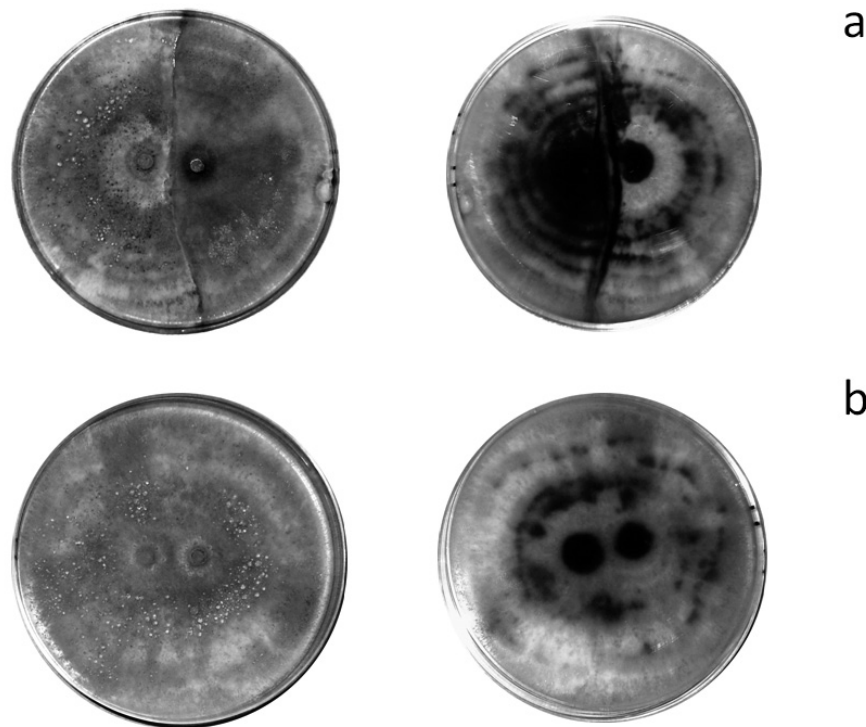


Figure 3. Examples of somatically incompatible (a: A.3.1 vs. A.3.2) and compatible (b: A.3.1 vs. B.4.1) reactions between paired isolates of *Inonotus rickii*.

lated mutations or parasexual recombinations (Milgroom, 1996), it is more likely that the presence of basidiomes, albeit sporadic, over the years, produces airborne sexual spores that facilitate the long-distance dispersal of different fungal genotypes.

Wounds on trunks or branches seem to be necessary to enable the pathogen to penetrate the host with both its basidiospores and its asexual propagules (Mazza *et al.*, 2008; Ramos *et al.*, 2008). Once the infection is established, the fungus is able to grow extensively through the woody tissues inside the tree upwards and downwards from the first infection point, with the result that often only one fungal genotype is detected within the same infected tree.

The same genotype was twice found in trees belonging to different species in Sicily, suggesting that *I. rickii* lacks host specificity. It has already been shown (Barnard, 1993) that *I. rickii* has a large host range. In Italy this fungus was first recorded on boxelder and European hackberry trees (Annesi *et al.*, 2003) and in further surveys (Annesi *et al.*, 2005; Annesi *et al.*, 2007; Mazza *et al.*, 2008) it was also detected, although with different disease incidence and symptoms, on several other hosts: *A. caffra*, *Albizia julibrissin* Durazz., *K. paniculata*, *Q. cerris*, *S. nigra*, *Platanus acerifolia* Willd., *Robinia pseudoacacia* L. and *Gleditsia triacanthos* L. Many other host genera have been recorded in several countries (Melo *et al.*, 2002; Dai *et al.*, 2010).

In conclusion, two points have been highlighted in this study, which are of particular interest because of their epidemiological implications. First, both sexual and asexual reproduction play an important role in the spread of the pathogen. Secondly, the same clone infects different host species: in urban plantations, a large number of trees species are grown, and most of these are hosts of *I. rickii*; therefore, they all can form effective biological corridors. These two aspects make this fungus particularly invasive along city boulevards.

Based on the findings, well-known control practices entailing the prompt removal of inoculum sources and sanitation, a careful choice of a suitable tree species when replacing a tree, and the disinfection of pruning tools, are recommended for controlling this pathogen, particularly within a disease centre.

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