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RESEARCH LETTER

Characterization and comparison of *Metarhizium* strains isolated from *Rhynchophorus ferrugineus*

Annarita Cito[1], Giuseppe Mazza[1], Agostino Strangi[1], Claudia Benvenuti[1], Gian P. Barzanti[1], Elena Dreassi[2], Tullio Turchetti[3], Valeria Francardi[1] & Pio F. Roversi[1]

[1]Consiglio per la ricerca e la sperimentazione in agricoltura, Research Centre for Agrobiology and Pedology, Firenze, Italy; [2]Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Siena, Italy; and [3]CNR, Consiglio Nazionale delle Ricerche, Sesto Fiorentino, Italy

Correspondence: Annarita Cito, Consiglio per la ricerca e la sperimentazione in agricoltura, Research Centre for Agrobiology and Pedology, Cascine del Riccio, via di Lanciola 12/a, 50125 – Firenze, Italy.
Tel.: +39 055 2492224;
fax: +39 055 209177;
e-mail: annarita.cito@isza.it

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Abstract

Rhynchophorus ferrugineus is considered the worst pest of palm species, and few natural enemies are reported for this parasite in its area of origin. Here, we report the first recovery of the entomopathogenic fungus *Metarhizium pingshaense* associated with *R. ferrugineus* from Vietnam. The morphological, biochemical, and toxicological features of this strain were studied and compared with those of another *Metarhizium* strain associated with this weevil in Sicily (Italy), an area of recent introduction. The potential use of these fungi as biocontrol agents was tested against adult insects in laboratory trials and a similar mortality rate was found. Both strains were able to produce toxins and cuticle-degrading proteases, but they showed dissimilar enzymatic and toxicological profiles, suggesting a different virulence activity.

Introduction

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier, 1790) (*Coleoptera*, *Dryophthoridae*), is considered the worst pest of palm species and it has invaded all continents (reviewed in Fiaboe *et al.* (2012). Several studies have been carried out on strategies to manage this weevil, due to its economic and ecological impacts. The current management approach involves integrated pest management (IPM) consisting of monitoring, mass trapping, insecticide application and early detection (Murphy & Briscoe, 1999; Faleiro, 2006). Although recent attempts have been made to improve the efficacy of these procedures and to reduce the application costs and toxicity, current research is mainly focused on long-term sustainable and environmentally friendly control strategies.

Few investigations have been conducted on natural enemies of *Rhynchophorus* species. Some of them were found to be effective in laboratory conditions, but the control of *R. ferrugineus* in areas of new introduction proved to be unsustainable (G. Mazza, V. Francardi, C. Benvenuti, R. Cervo, J.R. Faleiro, E. Llacer, S. Longo, R. Nannelli, E. Tarasco & P.F. Roversi, pers. comm.). The use of entomopathogenic fungi, in particular indigenous strains of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin, obtained from naturally infected weevils, should be seriously considered for biological control because both have provided encouraging results for the control of certain economic pests (Jaronski, 2010). Entomopathogenic fungi have sublethal effects such as alteration of feeding behavior (e.g. Tefera & Pringle, 2003) and survival and reproductive potential of the progeny (e.g. Gindin *et al.*, 2006). Moreover, as the RPW is highly promiscuous and adults live in aggregation, the fungi could spread in the population, infecting healthy insects by horizontal transmission, as suggested also by Llácer *et al.* (2013) and Francardi *et al.* (2013).

Entomopathogenic fungi from RPW have been isolated mainly in the newly invaded areas (e.g. Spain, Italy, Iran, Egypt), whereas few records concern the native area (G. Mazza, V. Francardi, C. Benvenuti, R. Cervo, J.R. Faleiro, E. Llacer, S. Longo, R. Nannelli, E. Tarasco & P.F. Roversi, pers. comm.). *Metarhizium anisopliae* was isolated from *R. bilineatus* in New Guinea, but this was probably due to an accidental infection during treatment against the scarabaeid *Scapanes australis* with a formulation based on *M. anisopliae* spores (Prior & Arura, 1985).

In the present paper, we report the recovery of entomopathogenic fungi isolated directly from *R. ferrugineus* in the native area and in an area of recent introduction. The aim of the study was to characterize and compare these strains, because biological control approaches require enhanced knowledge of the biology of the control agent. For this purpose, toxins and cuticle-degrading proteases were investigated to assess their role in virulence (Schrank & Vainstein, 2010). Laboratory trials against RPW adults were performed to assess the potential use of these fungi as candidates for biocontrol.

Materials and methods

Isolation and identification of fungal strains

Sampling and isolation

The fungal strain from the native range was isolated from an adult male of RPW found dead in Vietnam in June 2013 during an entomological expedition (Van Vu *et al.*, 2013). The precise provenience is Hoa Binh Province, Mai Chau District, N 20°43 12″ E 104°59′44″, altitude ca. 700 m a.s.l.

The fungal strain from the area of recent introduction was isolated from a dead *R. ferrugineus* adult found in Italy, precisely in Catania, Sicily, N 37°30′ E 15°04′, altitude ca. 50 m a.s.l. The two adults were stored in sterile 50 mL tubes until fungal isolation.

These two isolates were subcultured in Petri dishes on Sabouraud Dextrose Agar (SDA) plus 0.25% (w/v) yeast extract (SDAY) and maintained in a climatic chamber at 25 ± 1 °C. Both strains were isolated and stored as frozen dried cultures in the entomopathogenic micro-organisms collection of the C.R.A. – Research Centre for Agrobiology and Pedology, Florence (Italy).

Morphological observations

Agar plugs (7 mm diameter) from 5-day-old cultures, grown as described above, were placed in the center of Petri dishes with SDAY and Potato Dextrose Agar (PDA) and incubated at 25 ± 1 °C for 18 days. Radial growth (mm day^{-1}), conidia production (conidia mL^{-1}) and germination rate (%) were measured in three replicates for each strain according to Petlamul & Prasertsan (2012).

Scanning electron microscopy (SEM)

Both fungi were cultured on SDAY Petri dishes at 25 ± 1 °C in the dark until sporulation. Conidia were fixed in 2.5% glutaraldehyde with 1.8% sucrose overnight at 4 °C in phosphate buffer 0.1 M pH 7.2 and postfixed in 1% osmium tetroxide for 1 h at 4 °C in the same buffer. Finally, samples were dehydrated in an ethanol series (30%, 50%, 70%, 90%, and 100%), fixed on a stub and sputter-coated with gold. Conidia were observed by SEM (JCM-5000, Jeol Neoscope). The maximum length (L) and width (W) ±SD of 60 conidia were measured for both species.

DNA extraction, amplification, and sequencing

Aliquots of mycelium and conidia (about 10 mg maximum) were frozen in liquid nitrogen and pulverized with Precellys 24. Genomic DNA was extracted using the DNeasy Plant Mini Kit (QUIAGEN) according to the manufacturer's instructions. Amplification and sequencing of *loci* 18S-ITS1-5.8S-ITS2-28S and EF1-α were performed according to Sevim *et al.* (2010) and Rehner & Buckley (2005), respectively.

PCR products were sequenced at the Centro di Servizi per le Biotecnologie di Interesse Agrario Chimico ed Industriale (CIBIACI), Università degli Studi di Firenze, Italy. The sequences were submitted to GenBank (accession nos. KJ588065, KJ588066, KJ588067 and KJ588068). BLAST similarity searches were used with ITS *loci* to identify fungal strains at genus level.

Subsequent analyses were limited to *Metarhizium* PARB clade (*Metarhizium anisopliae*, *M. pingshaense*, *M. brunneum* and *M. robertsii*) identified by Bischoff *et al.* (2009) on EF1-α locus.

Maximum Likelihood, Maximum Parsimony, and Neighbor-Joining trees were constructed using MEGA 6 (Tamura *et al.*, 2013). Each tree was tested with 500 bootstrap replicates.

Bayesian analyses were conducted using BEAST 1.8.0 (Drummond & Rambaut, 2007) on the three introns inside EF1-α *locus* assuming uncorrelated mutational frequencies (HKY substitution model). Lognormal molecular clock model was assumed to take account of small number of taxa considered (Lepage *et al.*, 2007). Analyses were run for 10 million generations, sampling every 1000 generations (first million discarded as burn-in). Four independent Markov chain Monte Carlo (MCMC) analyses were performed starting from a randomly chosen tree. Maximum clade credibility tree was summarized.

Enzymatic activity assays

Culture conditions

To determine the activity of total proteases, subtilisin type (Pr 1) and trypsin type (Pr 2), we inoculated conidia from both strains at a concentration of 1×10^7 conidia mL^{-1} into a basal salts medium (1 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 0.5 g L^{-1} NaCl) containing 1 g L^{-1} casein, followed by incubation at 25 ± 1 °C for 72 h. Mycelia were harvested by centrifugation and the supernatants were used to determine enzymatic activity in UV-VIS spectrophotometer assays (SmartSpec™ Plus, Bio-Rad). Measurements were made in five replicates for both strains.

Total protease activity assay

A total protease activity assay was performed according to the method described by Bhagya Lakshmi *et al.* (2010). Briefly, 0.4 mL of casein substrate (1 g casein/10 mL of 0.01 M Tris buffer at pH 8.0), 0.2 mL of supernatant and 0.2 mL of 0.01 M Tris buffer at pH 8.0 were incubated for 10 min at 37 °C. The reaction was stopped by adding 0.7 mL of 1.2 M trichloroacetic acid (TCA). The samples were centrifuged at 90 ***g*** for 5 min, and the resulting supernatants were used for absorbance determination at 280 nm. One unit of total protease activity was defined as the amount of enzyme that produced 1.0 mM of tyrosine per minute under the above conditions.

Pr 1 and Pr 2 enzymatic activity assays

Pr 1 and Pr 2 enzymatic activity was assayed by a modified St Leger *et al.* (1987) method. N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide was used as the specific synthetic substrate for Pr 1, while N-benzoyl-Phe-Val-Arg-p-nitroanilide was used as the specific substrate for Pr 2. In detail, 0.05 mL of both substrates was mixed with 0.85 mL of Tris buffer 0.05 M pH 8.0 and 0.025 mL of culture supernatant. The reaction mixture was incubated for 45 min at 30 °C, and the reaction was terminated using 0.25 mL of 30% acetic acid. Samples were centrifuged at 1400 ***g*** for 10 min and placed in ice for 15 min before spectrophotometric determination. Absorbance of para-nitro aniline produced by the reaction was observed at 410 nm. One unit of Pr1 and Pr2 enzymatic activity was defined as the amount of enzyme that produced 0.001 mM of para-nitro aniline per minute under the above conditions.

Destruxins detection

Liquid chromatography-UV-mass spectrometry (LC-UV-MS) was used to quantify the amount of the six principal destruxins (Dtxs) produced by the two strains. Fungal culture and toxin extraction were performed according to the method described by Wang *et al.* (2003).

Quantitative analysis and metabolite recognition and separation were performed by means of the LC-UV-MS method according to Hu *et al.* (2006). LC analysis was carried out with an Agilent 1100 LC/MSD VL system (G1946C; Agilent Technologies) consisting of a vacuum solvent degassing unit, a binary high-pressure gradient pump, a 1100 series UV detector and a 1100 MSD model VL benchtop mass spectrometer. UV detection was monitored at 205 nm. The Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument was equipped with API-ES orthogonal spray (Agilent Technologies). Nitrogen was used as the nebulizing and drying gas. The pressure of the nebulizing gas, the flow of the drying gas, the capillary voltage and the vaporization temperature were set at 40 psi, 9 L min^{-1}, 3000 V and 350 °C, respectively. The various Dtxs were identified by a fragmentation study with the fragmentor voltage set in the range 70–200 V. The LC-ESI-MS determination was performed by operating the MSD in the positive ion mode. Spectra were acquired over the scan range *m/z* 50–1500 using a step size of 0.1 μ.

Chromatographic analysis was performed using a Phenomenex Jupiter 4u Proteo 90A column (150 × 2 mm, 4 μm particle size) at room temperature. The analysis was carried out with a gradient elution of a binary solution; eluent A was MeOH, while eluent B consisted of water. The analysis started at 10% A for two min, then increased to 90% in 13 min, and remained at that level until 20 min. The flow rate was 0.3 mL min^{-1}, and the injection volume was 20 μL. The quantitative analysis was performed on the basis of UV signal using calibration curves realized with destruxin A standard (90% purity). Dtxs identification was further confirmed by retention times and fragmentation patterns obtained using higher fragmentation energies.

Pathogenicity assays

Insect rearing

RPW unmated and mite-free adults were provided by UTAGRI ECO ENEA CR Casaccia, Rome, Italy, in September 2013. Adults were individually maintained in a climatic room (27 °C, 70–80% RH with a photoperiod of 12:12) for 2 weeks and fed on apples in plastic boxes.

Bioassays

Fungal strains were grown on sterilized rice according to Gindin *et al.* (2006). For each fungal strain, 30 adults (with a balanced sex ratio) were treated for 30 min with sporulated rice (about 3×10^7 conidia mL^{-1}) according

to Francardi *et al.* (2013). The control group was treated with sterilized rice. Treated and control adults were individually placed in plastic containers in the same conditions described above.

Following Francardi *et al.* (2013), mortality was recorded daily for 28 days after treatment. Dead weevils were surface sterilized with 1% sodium hypochlorite, rinsed three times with sterile distilled water, and placed on moistened filter paper in Petri dishes at 25 ± 1 °C to determine the presence of external sporulation of both strains according to Llácer *et al.* (2013).

Statistical analysis

Data were checked for normality and homogeneity of variance using Shapiro–Wilk and Levene tests, respectively. Data from the virulence bioassays were used for a Kaplan–Meier survival analysis, and comparisons were carried out by Log-rank test (statistic: χ^2). Results from the other experiments were analyzed by one-way analysis of variance (ANOVA; statistic: F). All tests were performed with SPSS v. 15.0 software.

Results

Isolation and identification of fungal strains

The morphological features of the colonies are shown in Fig. 1. Radial growth rate, conidia production, and germination rate are summarized in Table 1. Conidia observed by SEM were morphologically similar in both species, but with significantly different sizes ($L_{MET\ 13/I68}$: 5.41 ± 0.7 μm; $L_{MET\ 08/I05}$: 4.96 ± 0.47 μm; $F = 16.92$, d.f. = 119, $P < 0.01$ and $W_{MET\ 13/I68}$: 1.61 ± 0.18 μm; $W_{MET\ 08/I05}$: 1.52 ± 0.2 μm; $F = 5.86$, d.f. = 119, $P < 0.05$; Fig. 2). On the basis of molecular data, the fungal strain from the native area of *R. ferrugineus* was identified as *Metarhizium pingshaense* Q.T. Chen & H.L. Guo (MET 13/I68). The Italian strain was identified as *M. anisopliae* (MET 08/I05). For further details see supporting information, Fig. S1.

Enzymatic activity assays

Total protease and Pr 1 enzymatic activity were significantly higher in the MET 13/I68 strain than in MET 08/I05 ($F = 179.94$, d.f. = 1, $P < 0.01$; $F = 64.99$, d.f. = 1, $P < 0.01$, respectively), while no difference was found in Pr 2 enzymatic activity ($F = 0.37$, d.f. = 1, $P = 0.561$). The data are shown in Fig. 3.

Destruxins detection

Data from the LC-UV-MS analysis of toxin release are shown in Fig. 4. Both strains were able to produce Dtxs, but Dtxs E, DA, and A were significantly higher in MET 13/I68 than in MET 08/I05 (E: $F = 24.18$, d.f. = 1, $P < 0.05$; DA: $F = 4154.96$, d.f. = 1, $P < 0.01$; A: $F = 76.89$, d.f. = 1, $P < 0.05$).

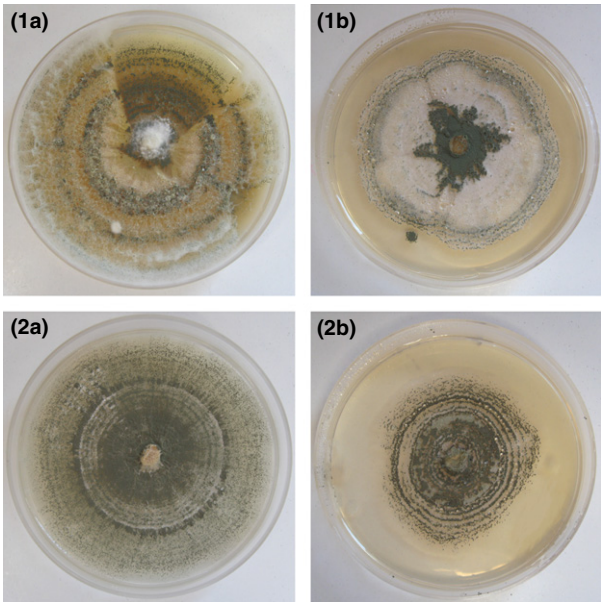

Fig. 1. Colony features of *Metarhizium anisopliae* MET 08/I05 and *M. pingshaense* MET 13/I68 on PDA and SDAY. The MET 08/I05 colony is dark green while MET 13/I68 is lighter grayish-green on both PDA and SDAY. On PDA, MET 08/I05 produces some aerial mycelium (1a) not present in MET 13/I68 (1b), which has a diffuse conidia production denser at the center of the colony and organized in a dense pattern of concentric circles. The MET 13/I68 colony shows the same circular arrangement on SDAY (2b) while MET 08/I05 exhibits a rosette-like pattern of growth, an irregular thick crust of conidia in the center, a whitish, almost sterile zone of mycelium and a light marginal strip of sporulation (2a).

Pathogenicity assays

After 4 days, in both treatments, individuals started to die and there was no difference in the mortality caused by the two fungal strains. Instead, significant differences were found between both treatments (MET 13/I68 and MET 08/I05) and the control ($\chi^2 = 60.90$, d.f. = 1, $P < 0.01$; $\chi^2 = 49.86$, d.f. = 1, $P < 0.01$, respectively). Comparison of the effectiveness using the Abbott method showed 100% and 93% for MET 13/I68 and MET 08/I05, respectively (Table 2 and Fig. S2).

Discussion

Metarhizium spp. are widespread in nature as saprophytes and they parasitize a broad range of insects and ticks (Schrank & Vainstein, 2010). Like *B. bassiana*, this ento-

Table 1. Growth rate (mm day^{-1}), conidia production (conidia mL^{-1}) and germination rate (%) of the two strains (*Metarhizium anisopliae* MET 08/I05 and *M. pingshaense* MET 13/I68) cultured on two different substrates (values are mean ± SD; * significance $P < 0.01$). Germination rate data were processed after ArcSin transformation

	PDA			SDAY		
Strain	Growth rate	Conidia production ($\times 10^7$)	Germination rate	Growth rate	Conidia production ($\times 10^7$)	Germination rate
MET 08/I05	2.12 ± 0.04*	8 ± 6.1	96.67 ± 2.93	1.22 ± 0.01*	10.23 ± 8.18	83.5 ± 14.6
MET 13/I68	1.70 ± 0.08	18 ± 5	94.08 ± 2.08	1.07 ± 0.05	37.33 ± 5.69*	86.75 ± 4.77

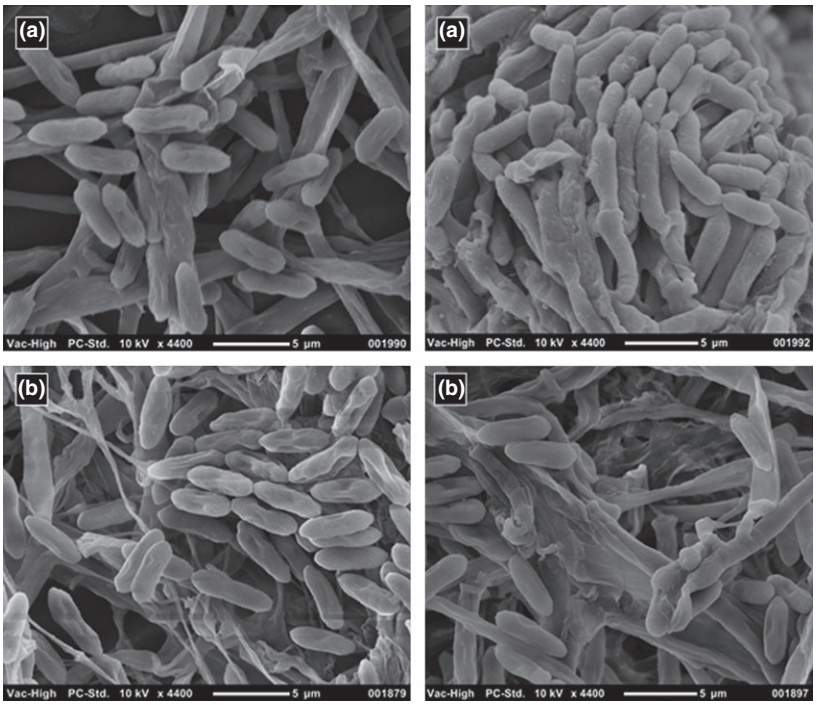

Fig. 2. Conidia and phialides of (a) *Metarhizium anisopliae* MET 08/I05 and (b) *M. pingshaense* MET 13/I68 observed by SEM.

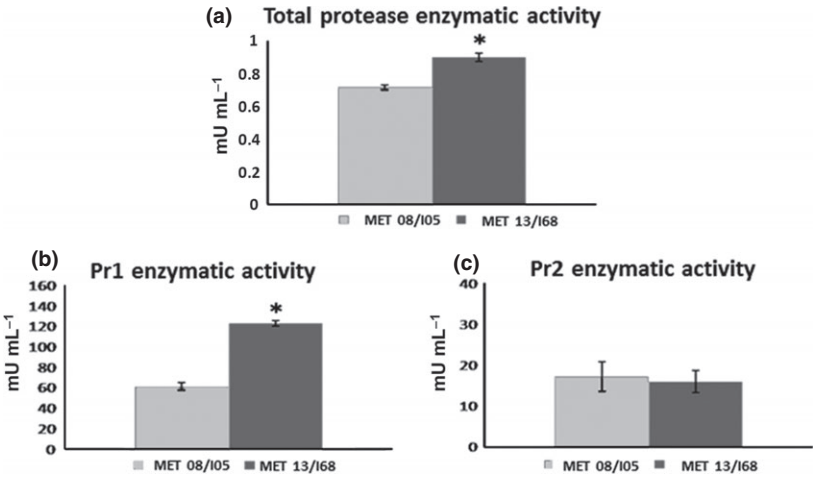

Fig. 3. *Metarhizium anisopliae* MET 08/I05 and *M. pingshaense* MET 13/I68 total protease (a), Pr 1 (b) and Pr 2 (c) enzymatic activity (values are mean ± SD; * significance $P < 0.01$).

mopathogenic fungus could provide an alternative for the management of insect pests difficult to control.

Although some natural enemies of the invasive weevil *R. ferrugineus* have been found, particularly in areas of recent introduction, none has proved effective in reducing its spread. Moreover, few antagonists have been identified in the native area where this pest is normally an uncommon insect (G. Mazza, V. Francardi, C. Benvenuti, R. Cervo, J.R.

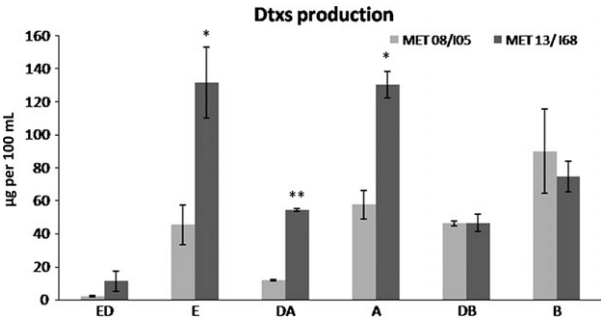

Fig. 4. Principal Dtxs production by the two fungal strains: Dtx E-diol (ED), Dtx E (E), desmethyl Dtx A (DA), Dtx A (A), desmethyl Dtx B (DB) and Dtx B (B) (values are mean ± SD; * significance $P < 0.05$, ** significance $P < 0.01$).

Faleiro, E. Llacer, S. Longo, R. Nannelli, E. Tarasco & P.F. Roversi, pers. comm.). We report the first recovery of an indigenous strain of *M. pingshaense* MET 13/I68 in the native area of *R. ferrugineus*. In contrast, several well-known entomopathogenic fungi have been isolated from RPW in newly invaded areas (reviewed in G. Mazza, V. Francardi, C. Benvenuti, R. Cervo, J.R. Faleiro, E. Llacer, S. Longo, R. Nannelli, E. Tarasco & P.F. Roversi, pers. comm.), including *M. anisopliae* MET 08/I05 in Sicily (Italy).

The morphological features of the MET 13/I68 and MET 08/I05 colonies fit with the previous descriptions for *M. pingshaense* and *M. anisopliae*, respectively (Bischoff *et al.*, 2009). Conidial width and length are within the ranges reported in Bischoff *et al.* (2009), even if MET 13/I68 conidia are significantly bigger than those of MET 08/I05. MET 08/I05 exhibited a significantly higher radial growth rate than MET 13/I68 on both substrates.

Proteases, especially Pr 1, and Dtxs were detected in both strains as important virulence factors. Like most entomopathogenic fungal species, *Metarhizium* uses a combination of mechanical force and metabolites such as cuticle-degrading enzymes and toxins to penetrate the host cuticle, weaken the insect's immune defenses, and access the nutrient-rich hemolymph (Schrank & Vainstein, 2010). As protein, not chitin, is the predominant matrix polymer of the cuticle, it has been suggested that proteases (particularly Pr 1) play a key role in insect penetration and subsequent pathogenicity (St Leger *et al.*, 1988; Shah *et al.*, 2005). Pr 2 seems to be involved in Pr 1 induction (St Leger *et al.*, 1996; Gillespie *et al.*, 1998). Dtxs are the most prevalent of the secondary metabolites produced by *Metarhizium* spp. and showed insecticidal activity (Wang *et al.*, 2012).

Our data confirmed that both MET 08/I05 and MET 13/I68 are able to produce Pr 1 and Dtxs in laboratory conditions. However, the protease and Pr1 enzymatic activity and production of some Dtxs were significantly higher in MET 13/I68 than in MET 08/I05. Both MET 08/I05 and MET 13/I68 showed a mortality rate > 90% against *R. ferrugineus* adults, suggesting that different mechanisms are involved in overcoming the host insect's immune defense system and causing death (e.g. Mazza *et al.*, 2011). Although the Italian strain MET 08/I05 is able to produce proteases and toxins, its virulence seems to be supported mainly by greater growth ability than MET 13/I68. MET 08/I05 could cause the host's death by a copious growth of propagules in the insect's hemolymph, as suggested by Valadares-Inglis & Peberdy (1998). The virulence of MET 13/I68 is assured mainly by more efficient protease activity and toxin production than in MET 08/I05. However, it is known that the pathogenic mechanism of *Metarhizium* is complex and the balance among factors is strain-dependent. Wang and coauthors found that a spontaneous Pr1 and Dtxs gene-deficient mutant demonstrated the same ability to infect the host as the corresponding wild-type strain (Wang *et al.*, 2002, 2003). Our data confirm the idea of two different virulence abilities ('toxin' and 'growth') as reported by Valadares-Inglis & Peberdy (1998). Further studies are necessary to verify whether the mechanism observed *in vitro* occurs also *in vivo*.

Entomopathogenic fungi are one of the most promising biological agent in *R. ferrugineus* pest management (G. Mazza, V. Francardi, C. Benvenuti, R. Cervo, J.R. Faleiro, E. Llacer, S. Longo, R. Nannelli, E. Tarasco & P.F. Roversi, pers. comm.), but more in-depth knowledge of the features that seem to influence fungal strains virulence is necessary to improve their potential applications.

Table 2. Pathogenicity assays of *Rhynchophorus ferrugineus* adults performed with the two fungal strains (*Metarhizium anisopliae* MET 08/I05 and *M. pingshaense* MET 13/I68). Within columns, means followed by the same letter are not significantly different from each other (Log-rank test)

						Kaplan–Meier survival analysis		
							95% Confidence interval	
Treatment	LT_{50} days	LT_{90} days	Abbott%	% survival	% sporulation	AST (mean ± SE)	Lower	Upper
Control	0	0	–	90	0	27.20 ± 0.92[a]	25.39	29.90
MET 13/I68	7	13	100	0	97	8.63 ± 0.62[b]	7.42	9.84
MET 08/I05	6	21	93	6.7	97	9.80 ± 1.19[b]	7.45	12.14

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Authors' contribution

A.C. and G.M. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. A) Maximum Likelihood phylogenetic tree of strains *Metarhizium anisopliae* MET 08/I05 and *M. pingshaense* MET 13/I68 based on EF1-α locus. Support values are shown. B) Bayesian inference of phylogenetic tree. Posterior Probability of clades is shown.

Fig. S2. Cumulative survival of *Rhynchophorus ferrugineus* adults treated with *Metarhizium anisopliae* MET 08/I05 and *M. pingshaense* MET 13/I68.