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Which came first: The disease or the pest? Is there a host mediated spread of *Beauveria bassiana* (Ascomycota: Hypocreales) by invasive palm pests?

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ABSTRACT

The red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) is threatening the palm family worldwide, causing important economic losses. Current tactics to manage the weevil are largely based on chemical control, although the use of pesticides is hampered by several environmental constraints. Since the first introduction of RPW in Spain in 1996 and during its progressive spread around the Mediterranean basin, the number of reports of natural infection of RPW populations by entomopathogenic fungi (EPF) has been rising for 15 years, and this rise could support a pest-mediated EPF spread. To challenge this hypothesis, we assessed the usefulness of the region of elongation factor 1-alpha (EF1-α), Bloc nuclear intergenic region (Bloc) and inter simple sequence repeat (ISSR) markers, alone or in combination, to infer the relationships among Mediterranean *Beauveria* and *Metarhizium* strains isolated from the RPW. Second, the effect of abiotic factors, such as temperature, humidity and UV-B radiation, on the germination and growth of these EPFs strains as a function of their genealogy and geographic origin were determined. Finally, the pathogenicity of strains from different genetic clades was evaluated against larvae and adults of *R. ferrugineus*. The phylogenetic analysis based on the EF-1α gene identified eight different sequences among 24 fungal isolates of four fungal species. Similar clades were clustered when Bloc and ISSR analyses were performed. The results showed that strains of different origins were clustered in the same clade, and this outcome could be explained by an RPW-mediated EPF spread that was also influenced by time, geographical and other RPW related factors. Neither the response to abiotic factors nor virulence to RPW larvae and adults were related to the sequence type, with all *B. bassiana* strains well adapted to Mediterraneam climatic conditions. Taken together, these findings may help to select the best strain for RPW management.

1. Introduction

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), and the moth *Paysandisia archon* (Burmeister) (Lepidoptera: Castniidae) are currently considered to be the most important pests of palms (Arecaceae) worldwide (Beaudoin-Ollivier et al., 2017). *R. ferrugineus* and *P. archon* are regulated pests in different countries and are, in some cases, quarantine pests (FAO, 2016; EPPO, 2018a,b). Both species were introduced to Europe by the trade of palms from infested areas. *R. ferrugineus* is native to southeastern Asia and Melanesia and was first reported in Europe in 1993, in southern Spain (Barranco et al., 1996; Jacas et al. 2011). It was later detected in other European countries and is currently found in all Mediterranean countries, from Portugal to Turkey and from Syria to Morocco (EPPO, 2018a). *P. archon* is native to South America and was first reported in Europe, in France and Spain, in 2001 (EPPO, 2002), but may have been introduced before 1995 on palm trees imported from Argentina (Reid and Moran, 2009). This moth is currently established in Portugal, Spain, France, Italy, Switzerland, Slovenia, Greece, Cyprus and Bulgaria, and it has also been found in the United Kingdom and Denmark, although establishment in these countries has not been confirmed (EPPO, 2018b). The larvae of both insects are endophagous and bore into the palm to feed on the succulent tissues of stipes and/or fronds of several species of Arecaceae, and are hidden throughout most of the life cycle. Damage to the meristematic tissue in the crown or to the vascular system of the palm stipe and offshoots can be so severe that it can lead

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to the collapse and death of the palm (Murphy and Briscoe, 1999; Faleiro, 2006; Dembilio and Jacas, 2012; Sarto i Monteys, 2013). Current estimates place annual losses due to these invasive species at the multimillion-euro level, including the value of the destroyed plants and their date crops, the cost of trapping and other quarantine methods, and huge budgets allocated to various chemical treatments. Infestation also restricts the movement of palms for planting (see, for example, Commission Implementing Directive (EU) 2017/1279 of 14 July 2017 amending Annexes I to V to Council Directive 2000/29/EC), especially the offshoots, resulting in drastic cuts in trading (El-Sabea et al., 2009; FERA, 2011).

Current tactics to manage these pests are largely based on chemical control with different active substances applied as either spray or trunk injection, as well as fumigation with phostoxin tablets (Faleiro, 2006; Sarto i Monteys and Aguilar, 2005; Nardi et al., 2011; Reid and Moran, 2009; Llácer and Jacas, 2010). However, several environmental hamper the use of these compounds and health concerns, including pesticide residues in fruits in date production areas, environmental pollution, and urban restrictions (i.e., EU Directive 2009/128/EC), together with non-target effects on honeybees (Palmer et al., 2013; OJEU, 2009, 2013).

In this context, entomopathogenic fungi (EPF) are biological control agents that fit well with the new requirements of environmentally friendly control products. They comprise a broad group of fungal species that infect insect and mite hosts via contact (Vega et al., 2012). The host can be infected both by direct treatment and by horizontal transmission from infected insects or cadavers to untreated insects or to subsequent developmental stages via the new generation of spores (Vega et al., 2012). Passive mechanical transmission of fungi within insect populations has been observed for various entomopathogenic fungi (Lacey et al., 1999; Quesada-Moraga et al., 2004, 2008). In addition, their relatively recently discovered role as plant endophytes and competent rhizosphere has been demonstrated to confer systemic protection to the plant against several insect pests (Resquín-Romero et al., 2016; Garrido-Jurado et al., 2017; Quesada-Moraga et al., 2009).

These unique characteristics make EPF especially useful for the control of concealed insects, for which the majority of the life cycle occurs within the plant tissues, making the pest inaccessible to direct-contact treatments (Quesada-Moraga et al., 2008; Quesada-Moraga et al., 2009). This is the case for the palm pests *R. ferrugineus* and *P. archon*, which are only exposed and can be directly targeted by the treatments after adult emergence (Murphy and Briscoe, 1999; Dembilio and Jacas, 2012; Sarto i Monteys, 2013). In recent years, the potential of EPF as microbial biological control agents of *R. ferrugineus* has been highlighted (Dembilio et al., 2010; Ortega-Garcia et al., 2017).

Natural EPF infections in *R. ferrugineus* have been extensively reported throughout the Mediterranean Basin (Ghazavi and Avand-Faghih, 2002; Shaiju-Simon Kumar and Gokulapalan, 2003; El-Sufty et al., 2009; Sewify et al., 2009; Tkaczuk et al., 2009); however, it should be stressed that natural infections of *B. bassiana* were not reported until more than a decade after the introduction of *R. ferrugineus* to Europe (Dembilio et al., 2010). Subsequently, several strains of *Beauveria* sp. and *Metarhizium* sp. have been shown to be pathogenic to *R. ferrugineus* larvae and adults in the laboratory and field (Gindin et al., 2006, El-Sufty et al., 2009; Sewify et al., 2009; Dembilio et al., 2010; Dembilio et al., 2018).

The efficacy of the EPF *Beauveria bassiana* also has been validated against *P. archon* under laboratory and field conditions with good results (Besse-Millet et al., 2008). Nevertheless, to the best of our knowledge, no natural infection with EPF has yet been detected in field populations of *P. archon*.

The use of molecular techniques to address the lack of detection of fungal infection in the field has prompted not only the identification of infective EPF strains but also the establishment of robust phylogenies from the congruence of genealogies based on appropriate polymorphic gene sequences. These genealogies can be used to test the hypothesis that the hosts *R. ferrugineus* and *P. archon* have played a key role in the spread of these EPF throughout the Mediterranean, which could explain the distribution patterns of their lineages (Rehner et al., 2006; Ghikas et al., 2010; Rehner et al., 2011; Kepler and Rehner, 2013). Moreover, even if the population genetic structure of EPF have been driven by these hosts, factors such as habitat selection and geography, which highly influence the ability of EPF to grow at high temperatures, their cold-active growth ability and resilience to ultraviolet (UV) exposure (Bidochka et al., 2001, 2002) may have influenced the evolution of these strains.

Recently, the need to use different molecular markers to identify and establish relationships among different strains of EPF found in different hosts and locations has been highlighted. Several studies have been undertaken with this purpose, focusing on the region of elongation factor 1-alpha (EF1-α) (Rehner et al., 2011; Kepler and Rehner, 2013), the nuclear intergenic region Bloc (Bloc) (Rehner et al., 2006), and inter simple sequence repeats (ISSR) or intermicrosatellite markers (Wang et al., 2005; Aquino de Muro et al., 2005; Lopes et al., 2013). In the present work, the usefulness of these molecular tools is assessed, either alone or combined, for diagnostics of EPF infecting *R. ferrugineus* and *P. archon* and to infer the relationships among the different Mediterranean strains of *Beauveria* sp. obtained from these hosts. Additionally, intraspecific variations on the thermal, RH and UV-B requirements and virulence of these strains are reported.

2. Materials and methods

2.1. Fungal isolation

Strains of entomopathogenic fungi were isolated from dead specimens of *R. ferrugineus* and *P. archon* with symptoms of natural infection. The insects were collected from different locations in the Mediterranean Basin where there were no records of prior use of EPF targeting these species.

Dead insects were individually surface sterilized with a solution of 1% sodium hypochlorite (2 min) and then rinsed twice in distilled sterile water (1 min) to eliminate sodium hypochlorite residues. Subsequently, they were transferred individually to Petri dishes on moistened filter paper and sealed with Parafilm® (Pechiney Plastic Packaging Co., Chicago, IL). Samples were incubated in complete darkness at 25 ± 2 °C to stimulate fungal growth.

When external fungal growth was observed, small amounts of conidia and mycelia were mounted in lactophenol–cotton blue and examined under 400x magnification phase microscopy (Leitz DMRB) for morphological identification to the genus level using taxonomic keys (Barnett and Hunter, 2006; Humber, 1997).

Propagules of previously identified EPF were transferred to 90-mm -diameter Petri dishes with sterile selective medium consisting of Glucose Chloramphenicol Sabouraud agar (GCSA) (Cultimed Panreac, Spain) according to the manufacturer's specifications, 500 mg/l streptomycin sulfate (Sigma Aldrich Chemie, China), 500 mg/l ampicillin (Intron biotechnology, China), and 500 mg/l dodine 65 WP (Melprex, Barcelona, Spain) with a sterile needle and incubated at 25 °C in complete darkness until colonies were visible. Then, monosporic cultures were obtained according to Goettel and Inglis (1997), lyophilized and subsequently deposited at the isolate collection of the Department of Agricultural and Forestry Sciences at the University of Cordoba (Córdoba, Spain). These isolates were then subjected to molecular analyses for identification to the species level.

2.2. Molecular identification of the strains

Isolates were grown as mycelia in 90-mm Petri plates of malt agar medium (MA) (Oxoid, Basingstoke, Hants, England) under sterile conditions. Plates were incubated at 25 °C for approximately 5 days in the dark. The mycelia then were collected and used for DNA extraction.

DNA was extracted using a modification of the method described by

Table 1
Primer sequences for amplifying and sequencing the EF1-α gene and the nuclear intergenic region Bloc.

Oligonucleotides		Reference	Amplified region
Name	Secuence 5′–3′		
Tef 1F	GTGAGCGTGGTATCACCA	O'Donnell et al. (1998)	EF-1α
1750R	GACGCATGTCACGGACGGC	Garrido-Jurado et al. (2011)	EF-1α
B51F	CGACCCGGCCAACTACTTTGA	Rehner et al. (2006)	BLOC
B31R	GTCTTCCAGTACCACTACGCC	Rehner et al. (2006)	BLOC

Raeder and Broda (1985). The quality of the DNA was checked, and concentration was estimated using a NanoDrop 2000 C Spectrophotometer (Thermo Fisher Scientific, USA). The DNA was diluted prior to PCR, and all the samples were adjusted to the same concentration.

The nuclear EF-1α gene and the intergenic region Bloc in all the isolates were amplified using the primers shown in Table 1, sequenced, and analyzed.

PCR amplifications were conducted in a total volume of 50 µL, which included 10 µL of 5 × PCR reaction buffer (MyTaq™ Red Buffer, Bioline Ltd, UK) comprisied of 5 mM of dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers, 20 mM each of opposing amplification primers, 0.5 µL of *Taq* polymerase (MyTaq™ DNA polymerase, Bioline Ltd, UK) and 1.5 µL of genomic DNA between 10 and 20 ng μL^{-1}. Finally, ultrapure water was added to a final volume of 50 µL. The reactions were carried out in a Veriti thermal cycler (Applied biosystems, USA). PCR was performed using the following thermal conditions: one cycle of denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 47 °C for EF-1α or 56 °C for Bloc for 15 s and extension at 72 °C for 10 s, with a final extension at 72 °C for 7 min.

PCR products from the EF1-α gene were visualized on 1% agarose gels in 1 × TAE, elaborated following the instructions of Sambrook et al. (1989) and stained with SYBR® Safe (Invitrogen, Paisley, UK). A 100-bp molecular weight standard ladder (Solis Biodyne, Tartu, Estonia) was used as the size marker. PCR products were purified from agarose gels using the Geneclean II kit® system (QBiogene, Inc., Carlsbad, CA) following the manufacturer's protocol. All PCR products of EF-1α and Bloc amplification were sent to Stabvida (Caparica, Portugal) for direct sequencing in both directions.

2.3. Genetic variability of the Beauveria bassiana strains

Screening for molecular diversity of the *B. bassiana* strains via ISSR-PCR was carried out using the primers developed by Ormond et al. (2010).

Each 23 µL PCR solution included 5 µL of 5× MyTaq Red Buffer (Bioline, UK), 15.8 µL sterile-distilled water, 1 µL 10 mM primer, 0.2 µL of MyTaq DNA polymerase (Bioline, UK) and 1 µL DNA. PCR was performed on a Veriti thermal cycler (Applied biosystems, USA). The amplification program included an initial denaturing cycle of 3 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, annealing at the specific annealing temperature for 15 s, 10 s at 72 °C, and a final extension step of 7 min at 72 °C. The annealing temperatures were 61 °C, 49 °C, 43 °C and 50 °C for primer 6, 7, I and D, respectively.

Amplified PCR products were analyzed using an Experion Pro260 automated electrophoresis system (Bio-Rad, Hercules, USA) using the lab-on-a-chip microfluidic technology. The virtual ISSR gels were examined to identify the different banding patterns produced by the microsatellite markers on all isolates and scored for the presence (1) or absence (0) of a band to produce binary data. Only bands that were consistent in at least two independent reactions were considered for analysis.

2.4. Phylogenetic analysis

For the EF1-α and Bloc phylogenetic analysis, sequences were edited and assembled using EditSeq (DNASTAR package, 1989–92, London, UK). Published sequences for isolates included within the genera *Beauveria*, *Metarhizium* and *Cordyceps* were retrieved from GenBank (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) and included in the alignments as representative sequences for these genera. Alignments were generated using MegAlign (DNASTAR package, London, UK) and CLUSTALX 1.81 (Thompson et al., 1997) software.

Parsimony analysis was used to infer the phylogenetic diversity and relationships among the isolates and was implemented in MEGA 4.0 (Tamura et al., 2007) using the heuristic search option close neighbor interchange. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option), and a heuristic MP bootstrap analysis consisting of 500 pseudoreplicates was performed. Clades with bootstrap values > 50% were considered to be supported by the data. The EF-1a and Bloc sequences of a *Cordyceps staphylinidicola* strain (AY883810) were used as an out group. Two phylogenetic trees were inferred: (1) EF1-α sequences of all the isolates were considered to infer relationships among them; (2) EF1-α and Bloc sequences were combined and analyzed together in a second phylogenetic tree focused only on *B. bassiana* isolates.

For ISSR phylogenetic analysis, ISSR data were converted into a binary data set (0, absence of band; 1, presence of band), and the matrices were further analyzed using FREE TREE (Hampl et al., 2001), where 1000 bootstrap replicates of trees generated via the Jaccard coefficient and neighbor joining algorithms were produced for each of the four ISSR primers. For visualization of the clusters, the TreeView package was used (Page, 1996).

2.5. Ecological characterization

Thirteen isolates identified as *B. bassiana* were selected from different genetic groups to assess their response under different abiotic variables. The effect of temperature on fungal growth and effect of temperature, osmotic potential and UV-B radiation on conidia germination were assessed.

2.5.1. Fungal preparations

To produce suspensions for experiments, slant cultures of the selected strains were subcultured on MA petri plates that were incubated at 25 °C in the dark. Conidia were harvested after 15 days of growth from the surface of these cultures directly by scraping and were suspended in sterile distilled water. Suspensions were sonicated to disrupt clumps and filtered through sterile muslin to remove mycelia and debris.

Conidia concentration was determined using a Malassez chamber (Blau Brand, Germany) at 400× magnification, and dilutions were made with distilled water to obtain the required concentration. When necessary, suspensions were stored at 4 °C overnight to prevent the germination of conidia before use in experiments.

2.5.2. Effect of temperature on in vitro radial colony growth of fungi

For each isolate, 100 µL of a 10^7 conidia/mL suspension was spread onto an MA plate (polystyrene, 90 × 15 mm). Petri dishes were held in an incubator at 25 °C under a saturated atmosphere in total darkness for 4 days.

Circular plugs (5 mm in diameter) were cut from nonsporulating mycelia of the 4-day-old culture dishes of each isolate using a cork-borer, and a single plug was placed upside down in the center of a new dish of a new Petri plate (60 mm in diameter) containing MA medium (Oxoid, Basingstoke, Hants., England).

Dishes were sealed and incubated in the dark in separate incubators at 15, 20, 25, 30, and 35 ± 1 °C. Five replicate dishes per isolate and

temperature combination were prepared.

Surface radial growth was evaluated for 2, 4, 6 and 8 days with a digital caliper, measuring two perpendicular diameters. The average diameter was then calculated and expressed in terms of real growth, subtracting the initial 8 mm of the initially sown disc plug.

Radial growth data were fitted by regression analysis using SPSS 8.0 for Windows. Because radial measurements (from the 2nd to the 8th day) fitted a linear model ($y = vt + b$), the linear regression slopes (v), which indicated the growth rates (speed in mm per day), were used as the main parameter to evaluate the influence of temperature on fungal growth (Davidson et al., 2003; Fargues et al., 1992; Ouedraogo et al., 1997; Yeo et al., 2003; Quesada-Moraga et al., 2006). To achieve this goal, a generalized β function, modified according to Bassanezi et al. (1998), was fitted to the average growth rates under different temperatures.

2.5.3. Effect of temperature on in vitro germination of fungal conidia

Considering the experiment conducted by Luz and Fargues (1997), a period of 18 h was chosen to observe the effect of temperature on conidial germination.

Conidial germination was examined for conidia incubated at temperatures of 15, 20, 25, 30 and 35 ± 1 °C on solid media. Three replicates per isolate and temperature combination were performed. Each replicate consists of a Petri dish containing water-agar medium, in which 40 µL of 1×10^5 conidia/mL suspension was spread onto each plate. The Petri dishes were then sealed with Parafilm® and held in incubators under a saturated atmosphere in complete darkness.

Dishes were scanned for germination after incubation for 18 h, and a drop of lactophenol cotton blue stain was placed into the center of each plate and covered with a glass coverslip. The plates were left open for 1–2 h to dry and then stored at 5 °C before examination. For each treatment, 100 spores were counted from several fields of view, and they were considered to be germinated if the germ-tube was two times larger than the diameter of the conidia.

The germination data were subjected to angular transformation and then were analyzed using analyses of variance (ANOVA), and all-pairwise comparisons among means were tested using Tukey's honestly significant difference (Tukey's HSD); means were considered not significantly different at $p > 0.05$.

2.5.4. Effect of osmotic potential on the in vitro germination of fungal conidia

In this assay, three petri dishes per strain and different osmotic potential (ψ), created with glycerol according to Fernández-Bravo et al. (2016), were evaluated. Each petri dish (plastic, 55 mm in diameter) was filled with 2% agar amended with various concentrations of glycerol to create different ψ, from 1 to 200 bar. Subsequently, 40 µL of 1×10^5 conidia / mL conidial suspension, prepared as indicated above, was spread on the agar. All the dishes were sealed with Parafilm and incubated at 25 °C in the dark. The rate of spore germination was assessed after an incubation time of 18 h. A drop of lactophenol cotton blue stain was deposited in the center of the plate and covered with a glass coverslip. The plates were left open for 1–2 h to dry and then stored at 5 °C before examination. For each treatment, 100 spores were counted from several fields of view, and they were considered as germinated when the germ-tube was two times larger than the diameter of the conidia.

Germination data were subjected to angular transformation prior to analysis of variance (ANOVA). Then, all-pairwise comparisons among means were tested using Tukey's HSD at $p > 0.05$.

2.5.5. Effect of ultraviolet radiation (UV-B) on conidia and their germination

Irradiation experiments were conducted in a temperature-controlled chamber (Fitoclima S600PL, ARALAB, Portugal). The temperature inside the chamber was maintained at 25 °C. The irradiated material was covered with a 0.13-mm-thick cellulose diacetate film, which reduced the radiation below 290 nm. This procedure allows the passage of most UV-B and UV-A, but it protects the samples from UV-C (< 280 nm) exposure. Control plates were covered with aluminum foil and thus physically protected from all types of radiation inside the UV chamber. These wavelengths were selected based on the fungal response to UVB-radiation, which is closer to the action spectrum for DNA damage (Paul et al., 1997). The selected irradiances were 920 mW m^{-2} for low-irradiance and 1200 mW m^{-2} for high-irradiance. These irradiances correspond in our conditions (Spain) to no sunlight for low-irradiance and 30% ozone depletion for high irradiance (Bilbao and Miguel, 2013). All UV measurements were performed with the PMA2106 UVB detector (trade mark, etc.), which provides rapid and accurate irradiance measurements in the UVB region.

UV-B radiation effect on conidial germination was evaluated using 60-mm Petri plates containing MA medium with a low concentration of 60% dodine (20 µg/mL), which slows fungal growth and permits the monitoring of germination for longer periods. Each plate corresponded to a replicate and was inoculated with 40 µL of a conidial suspension (1×10^5 conidia/mL) spread using a sterile disposable spreader. Three plates per exposure time and control were irradiated at 920 and 1200 mW m^{-2} for 2, 4 and 6 h and immediately incubated at 25 °C in the dark for 24 h (modified Braga et al., 2001a).

Subsequently, 100 conidia were selected from each exposure time, and after a 24-hour incubation, germination was observed with a Leitz DMRB optical microscope (x400/0.65PH2). The relative germination percentage after each period of incubation was calculated using the following equation:

$$\text{Relative germination } (\%) = Wt/Wc \times 100$$

where Wt is the number of germlings at exposure time t per plate and Wc is the mean number of germlings in the control plate.

Analysis of variance (ANOVA) was used to analyze the relative germination percentage, and all-pairwise comparisons among means were tested using Tukey's HSD, which was considered not significantly different at $p > 0.05$.

UV-B radiation effect on conidial culturability and colony growth was assesed using 60-mm Petri plates, which contained MA medium. Each plate corresponded to a replicate and was inoculated with 40 µL of conidial suspension (1×10^3 conidia/mL) spread as previously described. Three plates per exposure time and control were prepared per strain/treatment and irradiance combination.

Conidia were immediately exposed to irradiances of 920 and 1200 mW m^{-2} for 2, 4 and 6 h and immediately incubated at 25 °C in the dark for 48 h (modified Braga et al., 2001b).

Forty-eight hours post-inoculation, growth on the plates was halted by transferring 0.5 mL of lactophenol cotton blue onto each plate.

Conidial culturability was evaluated by counting the colony forming units (CFUs), which were observed at 40× magnification, and calculating the relative percentage of culturability after each exposure time using the following equation:

$$\text{Relative culturability } (\%) = Tt/Mc \times 100$$

where Tt is the number of CFUs of each replicate at exposure time t, and Mc is the mean number of CFUs for all control plates, regardless of the exposure time.

The effect of conidial irradiation on colony growth was determined by evaluating the size of the 48-h colonies after irradiation, recording the two orthogonal diameters of each colony and considering 10 colonies per treatment.

The analysis of colony growth was carried out by calculating the growth index after each exposure time using the following equation:

$$\text{Growth index} = (Cc - Ct)/(Cc + Ct) \times 100$$

where Ct is the mean diameter of each replicate at exposure time t, and *Cc* is the mean diameter for all control plates, regardless of the exposure

time. Values near to 100 are indicative of a very UV-susceptible isolate, as those near to 0 represent highly UV-resilient strains.

Analysis of variance (ANOVA) was used to analyze the relative culturability and growth index, and all-pairwise comparisons among means were tested using Tukey's HSD, considering no significant difference at $p > 0.05$.

2.6. Pathogenicity assay of selected Beauveria bassiana strains against Rhynchophorus ferrugineus larvae and adults

2.6.1. Rhynchophorus ferrugineus laboratory population

The *R. ferrugineus* specimens used in these assays were obtained from a stock colony established in 2012 at the Department of Agricultural and Forestry Sciences of the University of Córdoba (Spain). These insects were originally collected in the area with the most *R. ferrugineus* damage in the province of Córdoba. Beetles were attracted with traps baited with ferrugineol (the male *R. ferrugineus* aggregation pheromone) and plant kairomones (ethyl acetate and pieces of palm fronds) and the colony was periodically supplemented with the introduction of additional wild specimens. They were reared in an environmental chamber set at 26 ± 2 °C, 70 ± 5% RH, and a photoperiod of 16:8 (L:D) h.

Adult weevils were reared in plastic cages (36 × 24 × 16 cm) with small holes allowing air for circulation, with a density of approximately 30 weevils per cage. Cages contained thin apple slices over a folded piece of moistened filter paper that was used by female weevils as oviposition substrate and by both males and females as food. Apple slices were replaced three times per week (Dembilio et al., 2009)

Eggs were extracted from the oviposition substrate and transferred to a Petri dish containing a new apple slice over a moistened filter paper. Upon hatching, neonate larvae were individually transferred to 60-mm Petri dishes with 18–20 g of artificial diet (Martín and Cabello, 2006), which was replaced every 3 days. When larvae were ready for pupation (45-day-old larvae), they were moved to a 100-mL polypropylene vial, covered with muslin to allow aeration and containing artificial diet and pupation substrate used by the larvae to build a cocoon, which consisted of a mixture of coir (*Cocos nucifera* L.) and pita fiber (*Agave americana* L.) moistened with distilled water.

2.6.2. Fungal strains

R. ferrugineus is quite a difficult and expensive insect to rear in the laboratory. Therefore, the pathogenicity of all fungal strains included in this study was not assessed. Strains were selected based on the results obtained in their molecular and ecological characterizations (Table 2). Strains were selected according to the type-sequences obtained and one isolate from each type-sequence was evaluated. The geographical origin and performance under different environmental conditions (temperature, humidity and UV-B radiation) were also considered. In summary, six strains belonging to *B. bassiana*, deposited at the C.R.A.F. University of Córdoba Entomopathogenic Fungi Collection (Córdoba, Spain), were assessed (Table 3) for their pathogenicity on both adults and immature stages of *R. ferrugineus*.

2.6.3. Fungal preparations

Selected strains were cultured, conidial suspensions were prepared as described in 2.5.1., and adjusted to a final concentration of 10^8 conidia/mL.

2.6.4. Pathogenicity assay of selected B. Bassiana strains against R. Ferrugineus fourth instar larvae by topical application

Fourth instar larvae in groups of 10 were immersed for 60 s in a conidial aqueous suspension of 10^8 conidia/mL or control aqueous solution. Both treated and control solutions contained 0.01% Tween 80. Three replicates of 10 larvae per strain were treated. This concentration was selected based on previously LC_{50} results obtained by Dembilio et al. (2010).

After treatment, the larvae were individually transferred onto a Petri dish (55 mm in diameter) and were starved for 24 h. Then, they were offered artificial diet ad libitum. Feeding anomalies, motility and sublethal effects were observed, and mortality was recorded daily during the following 12 days. Dead specimens were processed as described in 2.1. In total, 210 larvae were used in this experiment.

2.6.5. Pathogenicity assay of selected B. Bassiana strains against R. Ferrugineus field-collected adults by topical application

Field-collected weevils were maintained for 15 days under rearing conditions before being used in our assays to ensure they were not infected with fungi. For each treatment, only one replicate of 10 adults, 5 males and 5 females, was evaluated due to the availability of the specimens. Groups of 5 insects were immersed for 60 s in a 20-mL aqueous suspension of 10^8 conidia/ mL or control aqueous solution. Both solutions contained 0.01% Tween 80.

After treatment, adults were transferred individually to a plastic box (6 × 8 × 8 cm) and starved for the first day. Then, apple slices were provided as food and oviposition substrate.

Feeding anomalies, motility, sublethal effects, and mortality were checked daily and oviposition was checked every other day for 30 days.

Dead specimens were processed as described previously to confirm that their death was due to fungal infection. In total, 35 males and 35 females were evaluated in this experiment.

2.6.6. Statistical analysis

The average survival time (AST) in days was calculated using Kaplan–Meier survival analysis (Kaplan and Meier, 1958) and compared by the log-rank test using SPSS 15.0® software for Windows.

Percentage data were subjected to angular transformation (Steel y Torrie, 1985) prior to analysis and checked for normality using the Shapiro–Wilk W test.

Fungal outgrowth and fecundity data were subjected to analysis of variance (ANOVA), and when necessary, all-pairwise comparisons among means were tested using Tukey's HSD using Statistix 9.0®, considering the absence of a significant difference at $p > 0.05$.

3. Results

3.1. Molecular identification of the strains: EF1-α and Bloc sequence analysis

The phylogenetic analysis based on the EF-1α gene identified eight different sequences among the 24 fungal strains (five from *B. bassiana*, one from *B. pseudobassiana*, one from *Lecanicillium attenuatum*, and one from *Metarhizium anisopliae*). These eight sequences and another 24 sequences deposited at GenBank representing different lineages of the genera *Beauveria*, *Lecanicillium* and *Metarhizium* were aligned and compared (Rehner et al., 2011; Bischoff et al., 2009). Of the 24 strains analyzed, 17 were clustered within the *B. bassiana* clade with a bootstrap support of 93%, which further grouped into four different subclades (Fig. 1). The remaining *Beauveria* isolate was placed in the *B. pseudobassiana* clade. One different genotype was identified within the *L. attenuatum* clade and another within *M. anisopliae* (Fig. 1). At least two species have been demonstrated to naturally infect *R. ferrugineus* (*B. bassiana* and *M. anisopliae*) and three species to infect *P. archon* (*B. bassiana*, *B. pseudobassiana* and *L. attenuatum*).

Five different EF1-α gene-based sequences were found for the *B. bassiana* strains. They have been named A, B, C, D, and E. The sequence of type A shows high variability in origin. In this group, different *B. bassiana* strains obtained from different stages of *R. ferrugineus* (larva, pupa and adult) found in Spain (Valencia and Ibiza), Italy (Brindisi, Lecce and Bari) and Israel (Tivon) and one *B. bassiana* strain isolated from a *P. archon* adult found in France (Montpellier) clustered together (Fig. 1, Table 2). Strains belonging to the *B. bassiana* B sequence type were found in a *R. ferrugineus* adult from Italy (Catania, in Sicily) and a

Table 2
Identity of *Rhynchophorus ferrugineus* and *Paysandisia archon* isolates from the culture collection at C.R.A.F. Department of the University of Cordoba.

Strain	Fungal species	ST	Host species	Host class: order	Year	Geographic origin			Stage	Isolated by[a]
						Locality	Province	Country		
EABb 07/06-Rf	*Beauveria bassiana*	E	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2007	Catral	Alicante	Spain	Pupa	UCO
EABb 08/05-Rf	*Beauveria bassiana*	A	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2008	Mesagne	Brindisi	Italy	Larva	UCO
EABb 08/06-Rf	*Beauveria bassiana*	A	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2008	Maglie	Lecce	Italy	Larva	UCO
EABb 08/07-Rf	*Beauveria bassiana*	A	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2008	Bari	Bari	Italy	Larva	UCO
EAMa 08/01-Rf	*Metarhizium anisopliae*	–	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2008	Maglie	Lecce	Italy	Adult	UCO
EAMa 08/02-Rf	*Metarhizium anisopliae*	–	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2008	Mesagne	Brindisi	Italy	Adult	UCO
IBB 010	*Beauveria bassiana*	C	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2008	Ellinikon	Attica	Greece	Adult	BPI
B.b.T-N	*Beauveria bassiana*	A	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2012	Tivon	Haifa	Israel	Pupa	ARO
B.b.T-A	*Beauveria bassiana*	D	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2012	Tivon	Haifa	Israel	Adult	ARO
M.a.*T*-A	*Metarhizium anisopliae*	–	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2012	Tivon	Haifa	Israel	Adult	ARO
M.a.*N*-A	*Metarhizium anisopliae*	–	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2012	Naharia	Haifa	Israel	Adult	ARO
EABb 12/04-Rf	*Beauveria bassiana*	B	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2012	Catania	Catania	Italy	Adult	UCO
EABb 12/05-Rf	*Beauveria bassiana*	C	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2012	Moncada	Valencia	Spain	Adult	UCO
EABb 13/01-Rf	*Beauveria bassiana*	A	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2013	Moncada	Valencia	Spain	Adult	UCO
EABb 13/02-Rf	*Beauveria bassiana*	A	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2013	Moncada	Valencia	Spain	Adult	UCO
EABb 13/03-Rf	*Beauveria bassiana*	A	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2013	Moncada	Valencia	Spain	Adult	UCO
EABb 13/04-Rf	*Beauveria bassiana*	A	*Rhynchophorus ferruigineus*	*Coleoptera: Curculionidae*	2013	Moncada	Valencia	Spain	Pupa	UCO
EABb 13/05-Rf	*Beauveria bassiana*	E	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2013	Sevilla	Sevilla	Spain	Adult	UCO
EABb 14/01-Rf	*Beauveria bassiana*	A	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2014	Ibiza	Ibiza	Spain	Pupa	UCO
EABb 14/02-Rf	*Beauveria bassiana*	B	*Rhynchophorus ferrugineus*	*Coleoptera: Curculionidae*	2014	–	–	Egypt	Pupa	UCO
EABps 12/02-Pa	*Beauveria pseudobassiana*	–	*Paysandisia archon*	*Lepidoptera: Castniidae*	2012	Montpellier	Languedoc-Roussillon region	France	Larva	UCO
EABb 12/03-Pa	*Beauveria bassiana*	A	*Paysandisia archon*	*Lepidoptera: Castniidae*	2012	Montpellier	Languedoc-Roussillon region	France	Adult	UCO
EALa 12/01-Pa	*Lecanicillium attenuatum*	–	*Paysandisia archon*	*Lepidoptera: Castniidae*	2012	Catania	Catania	Italy	Pupa	UCO
EALa 12/02-Pa	*Lecanicillium attenuatum*	–	*Paysandisia archon*	*Lepidoptera: Castniidae*	2012	Catania	Catania	Italy	Pupa	UCO

[a] UCO (University of Cordoba); BPI (Benaki Phytopathological Institute, Greece); ARO (Agricultural Research Organization, Volcani Center, Israel).

Table 3
Selected strains for *Rhynchophorus ferrugineus* pathogenicity assays.

Strains	Type-sequence	Host	Geographical origin	Behavior under[a]			Isolated by[b]
				Temperature	Humidity	UV-B radiation	
EABb 12/03-Pa	A	*Paysandisia archon*	Francia	+ + +	+ + +	+ +	UCO
EABb 13/04-Rf	A	*Rhynchophorus ferrugineus*	España	+ + +	+ + +	+ + +	UCO
EABb 12/04-Rf	B	*Rhynchophorus ferrugineus*	Italia	+ + +	+	+	UCO
IBB 010	C	*Rhynchophorus ferrugineus*	Grecia	+ +	+ + +	+	BPI[a]
B.b.T-A	D	*Rhynchophorus ferrugineus*	Israel	+	+ +	+	ARO[b]
EABb 07/06-Rf	E	*Rhynchophorus ferrugineus*	España	+ + +	+ +	+ +	UCO

[a] Tolerance range to environmental factors: + Low, + + Medium, + + + High.
[b] UCO (University of Cordoba) BPI (Benaki Phytopathological Institute, Greece); ARO (Agricultural Research Organization, Volcani Center, Israel).

R. ferrugineus pupa from Egypt (4). *B. bassiana* type sequence C was found in *R. ferrugineus* adults from Spain (Valencia) and Greece (Ellinikon, Attica). Conversely, *B. bassiana* type sequence D was found only in a *R. ferrugineus* adult from Israel (Tivon). Likewise, *B. bassiana* type sequence E was found only in Spain (*R. ferrugineus* pupa and adult from Alicante and Sevilla, respectively). Type sequences B and C were closely linked. Likewise, type sequences A and D were linked, and type sequence E was closer to B and C than to D and A (Fig. 1).

The second analysis focusing on the 17 *B. bassiana* isolates shed light on the relatedness among *B. bassiana* strains. Combined data for the EF1-α and Bloc partial sequences were analyzed, and the results are summarized in Fig. 2. This new analysis showed that all the strains included in the type A sequence had the same EF1-α and Bloc partial sequences, excluding two strains from Spain and Italy with different Bloc sequences. The Italian origin EABb 08/07-Rf strain showed a sequence that was more closely related to sequence type D.

Strains from sequence type B showed a different Bloc sequence. However, they were still connected to sequence type C, the strains of which also showed the same Bloc partial sequences. Strains from sequence type E showed different Bloc sequences and, in this case, were closer to sequence type A.

3.2. Genetic variability of the B. Bassiana strains: ISSR

Between 34 and 48 polymorphic bands were obtained from the isolates, while a specific band was only found in the EABb 07/06-Rf isolate. This polymorphism within isolates (Fig. 3) may indicate the existence of an evolution between them.

The phylogenetic tree showed a clear relationship between ISSR characterization and EF-1α and Bloc analyses since the same EF-1α-

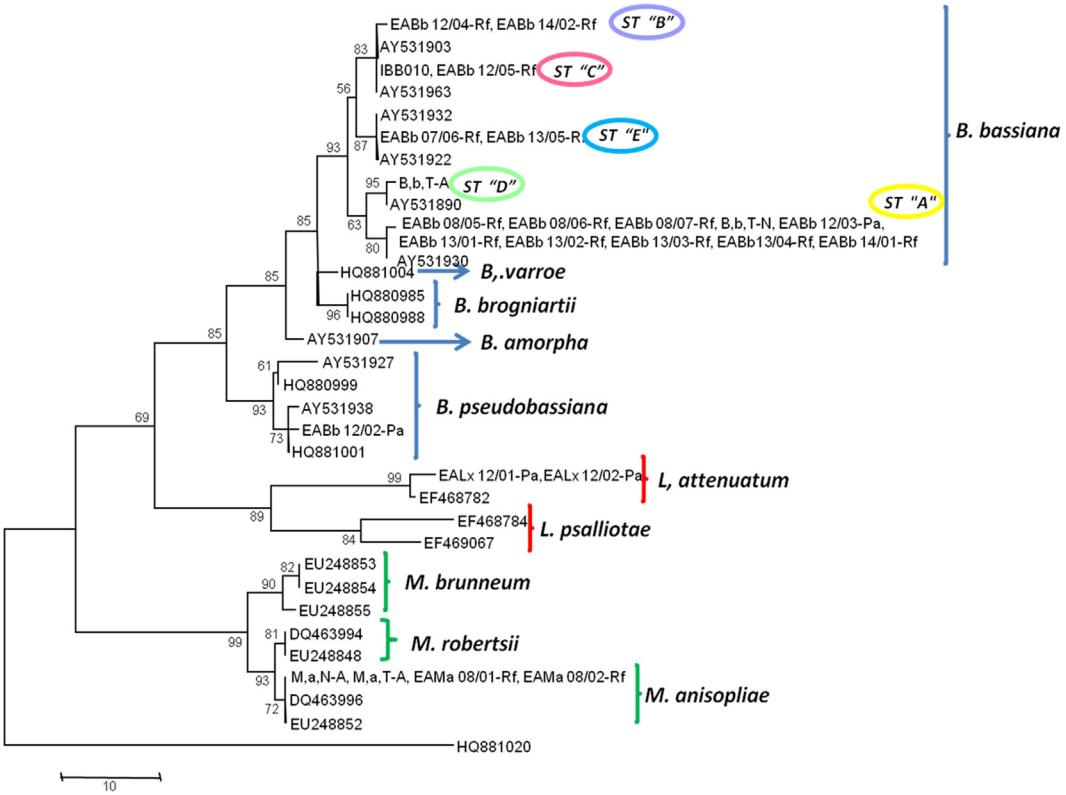

Fig. 1. Phylogenetic analysis of entomopathogenic fungi isolates obtained from *R. ferrugineus* and *P. archon* inferred from Maximum Parsimony analysis of EF1-α gene sequences. Bootstrap values (based on 500 replicates) when above 50% are indicated in the branches. Species clades are indicated by vertical bars. Type sequences of *B. bassiana* are defined with the letters "A", "B", "C", "D", and "E. Type sequence ST "A" is marked in yellow and includes isolates EABb 08/05-Rf, EABb 08/06-Rf EABb 08/07-Rf, B.b.T-N, EABb 12/03-Pa, EABb 13/01-Rf, EABb 13/02-Rf, EABb 13/03-Rf, EABb 13/04-Rf and EABb 14/01-Rf); ST "B" is marked in purple and includes the isolates EABb 12/04-Rf and EABb 14/02-Rf; ST "C" is marked in pink and includes the isolates IBB010 and EABb 12/05-Rf; ST "D" is marked in green and includes the isolate B.b.T-A; ST "E" is marked in blue and includes the isolates EABb 07/06-Rf and EABb 13/05-Rf. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

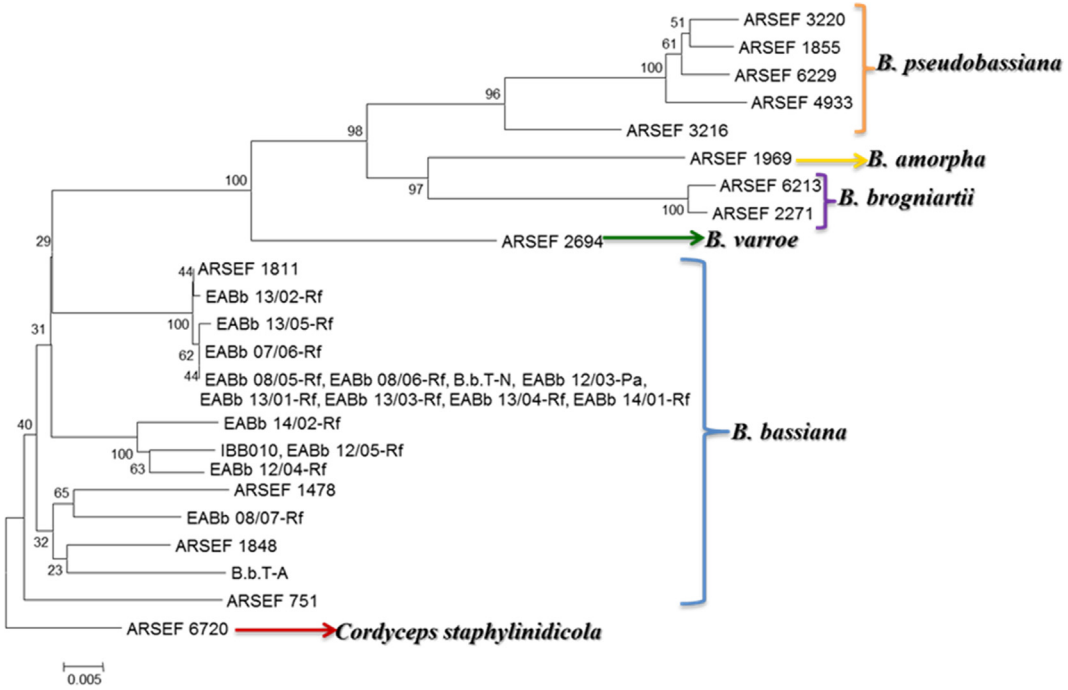

Fig. 2. Phylogenetic analysis of entomopathogenic fungi isolates obtained from *R. ferrugineus* and *P. archon* inferred from Maximum Parsimony analysis of EF1-α and BLOC partial sequences. Bootstrap values (based on 500 replicates) above 50% are indicated in the branches. Species clades are indicated by vertical bars. 0.005 represents the genetic distance.

Bloc type sequences were clustered in the same clade in the ISSR analysis. Nevertheless, there were some differences among markers that could shed light on the spread sequence of the strains under scrutiny.

3.3. Ecological characterization

3.3.1. Effect of temperature on fungal in vitro radial colony growth

Thirteen representative *B. bassiana* strains were selected to determine their thermal characteristics. Temperature had a significant effect on *in vitro* radial growth (Table 4). Although mycelial growth occurred at all temperatures, it was reduced at 15 and 35 °C. The optimum range for vegetative growth was established to be close to 25 °C for all strains and ranged from 23.6 ± 0.8 (EABb 13/02-Rf) to 26.6 ± 0.3 °C (EABb 12/04-Rf). The growth rates at the optimum temperature varied from 2.4 ± 0.1 mm day^{-1} (EABb 07/06-Rf) to 3.9 ± 0.1 mm day^{-1} (EABb 12/04-Rf) and 4.0 ± 0.1 mm day^{-1} (IBB010). There were no significant differences for upper developmental threshold temperatures. However, isolate EABb 07/06-Rf showed the highest value of 36.7 ± 1.7 °C.

The B.b.T-A and B.b.T-N strains showed similar values according to the shape parameter TB3, which describes optimum curves resulting from the effect of temperature on growth.

3.3.2. Effect of temperature on in vitro germination of fungal conidia

There were significant effects of temperature on the germination of conidia at 18 h post inoculation at 15 ($F_{12,38}$ = 32.64; p ≤ 0.0001), 20 ($F_{12,38}$ = 32.02; p ≤ 0.0001), 25 ($F_{12,38}$ = 6.50; p ≤ 0.0001), and 30 °C ($F_{12,38}$ = 5.88; p = 0.0001). None of the strains germinated at 35 °C, and in general, all the strains showed higher germination percentages at approximately 25 °C (Table 5).

3.3.3. Effect of osmotic potential on in vitro germination of fungal conidia

The results showed that conidial germination was clearly affected by the osmotic potential of the medium. In general, this percentage increased at a lower osmotic potential (Table 6). The germination tube length was also influenced by the osmotic potential and decreased with an increasing osmotic potential. After 18 h of incubation, the strains significantly differed in germination percentages depending on the medium and the osmotic potential (P < 0.05) (Table 6). For all strains, germination drastically decreased at 95.5% relative humidity. Germination at an osmotic potential of 150 and 200 bars (relative humidity values of 89.5 and 86.2%) was observed only in six strains: EABb 13/01-Rf, EABb 13/04-Rf, EABb 12/03-Pa, EABb 08/06-Rf, and IBB010.

3.3.4. Effect of ultraviolet radiation (UV-B) on conidia and their germination

There were significant differences among *B. bassiana* isolates in germination after exposure to 1200 mW m^{-2} for 2, 4, and 6 h ($F_{12,38}$ = 16.80, p < 0.001; $F_{12,38}$ = 20.47, p < 0.001; $F_{12,38}$ = 70.19, p < 0.001, respectively) (Table 7).

A delayed germination of *B. bassiana* conidia was observed after exposure to an irradiance of 1200 mW m^{-2}. Additionally, germination tubes were shorter in irradiated plates than in the control. In general, conidial germination decreased with an increasing exposure time to UV-B. The maximum germination delay was produced at 6 h of exposure, especially for EABb 08/06-Rf, B.b.T-N, and B.b.T-A isolates, for which only 2% of the conidia germinated 24 h after exposure to UV-B radiation.

In our study, strains EABb 13/02-Rf, EABb 08/06-Rf, IBB 010, B.b.T-N, and B.b.T-A showed high susceptibility to UV-B radiation, with less than 50% germination. However, strain EABb 13/04-Rf showed a germination rate above 70% for all the UV-B exposure times considered.

UV-B radiation also had a significant effect on the culturability (Table 8) at 1200 mW m^{-1} ($F_{12,59}$ = 39.25, p < 0.001; $F_{12,59}$ = 53.83, p < 0.001; $F_{12,59}$ = 26.19, p < 0.001, for 2, 4, and 6 h of exposure time, respectively). In general, after 2 h of exposure to 1200 mW m^{-2} of irradiance, the number of colonies was not affected relative to the nonirradiated conidia for all the strains. However, for some strains, after 6 h of exposition, the number of colonies dramatically decreased.

The colony size of the irradiated conidia was smaller than that of the nonirradiated ones (Table 9) ($F_{12,38}$ = 3.37; p = 0.0046; $F_{12,38}$ = 11.34; $F_{12,38}$ = 5.76; p < 0.001). There was a growth delay

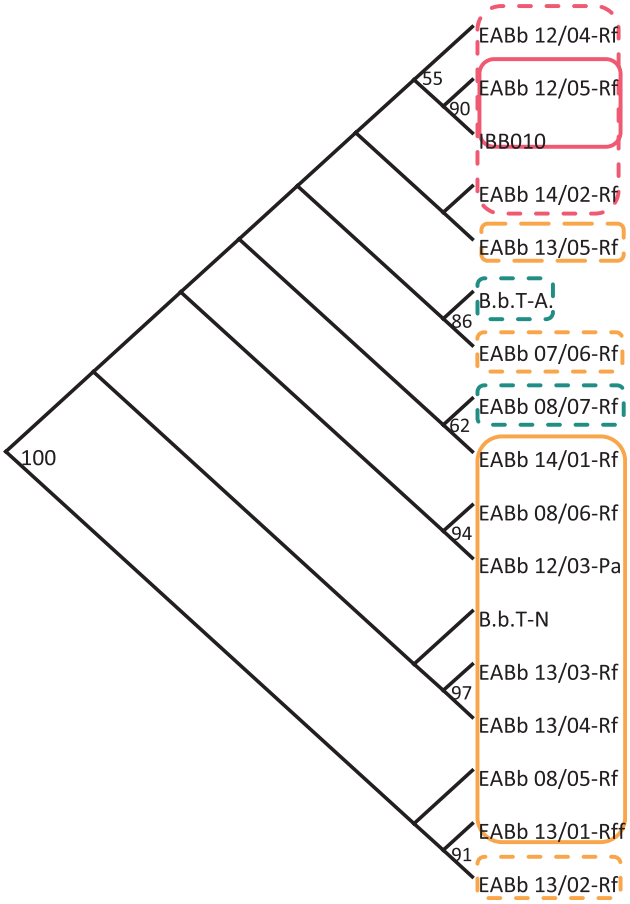

Fig. 3. Genetic relationship between the *B. bassiana* isolates obtained from *R. ferrugineus* and *P. archon* with base on ISSR makers. The resulting type sequences obtained from EF1-α and BLOC phylogenetic analysis are included in the figure as well by a color legend. Same color and continuous line indicate the same type sequence in the EF1-α and BLOC analysis. Same color and discontinuous line indicate same clade in the EF1-α and BLOC phylogenetic tree.

following UV-B exposure.

3.4. Pathogenicity assay of selected B. Bassiana strains against R. Ferrugineus fourth instar larvae by topical application

All the strains proved to be pathogenic against fourth instar larvae of *R. ferrugineus* when the larvae were immersed in a conidial suspension of 1×10^8 conidia/mL. One of the first symptoms observed in treated larvae was the onset of dark brown/black spots on the larval cuticle 2–3 days after treatment (Fig. 4A), a sign of fungal penetration through the cuticle, which sometimes became noticeable very early at natural insect openings (Fig. 4B). When the larvae died, their color sometimes changed from sandy to pink or dark brown (Fig. 4C, D). Abnormalities during molting were noted and were sometimes lethal during the actual molt (Fig. 6). As the disease progressed, the larvae lost their appetite, resulting in reduced diet consumption. Once development of the fungus inside the larvae was completed, fungal outgrowth of *B. bassiana* was observed on the surface of dead insects. This effect was enhanced when dead specimens were placed under optimal temperature and relative humidity conditions to promote fungal growth and sporulation, which required 7–10 days of incubation (Fig. 6).

Table 10 displays a summary of mortality and average survival time (AST) values recorded for each strain. All strains showed high virulence against *R. ferrugineus* larvae, resulting in significant mortality and reduction of the AST. The total mortality of *B. bassiana*-treated larvae ranged from 40.0 to 83.3%, and the greatest decrease in the cumulative survival ratio considering all the strains occurred between 4 and 6 days after treatment.

From all the tested strains, EABb 13/04-Rf caused the highest mortality (83.3%), with an AST of 8.1 days. However, the lowest AST (6.1 days) was caused by the B.b.T-A strain (70.0% mortality), which additionally led to the highest percentage of individuals presenting fungal outgrowth (46.7%, Table 10).

It should be noted that with strain EABb 07/06-Rf, which caused a mortality of 73.3% and AST of 7.7 days, a high percentage of dead insects did not present fungal outgrowth after being exposed to optimal conditions for its development.

3.5. Pathogenicity assay of selected B. Bassiana strains against R. Ferrugineus field-collected adults by topical application

As in the larval assay, treated adult specimens consumed less food

Table 4
Estimated parameters (± SE) and coefficients of determination r2 of the generalized ß function modified according to Bassanezi et al. (1998) fitted to data of the vegetative growth of different *B. bassiana* isolates from *R. ferrugineus* and *P. archon.*

Strain	Estimated parameters (a)(b)							
	T_{opt}		T_{yopt}		T_{max}		TB_3	
EABb 07/06-Rf	23.8 ± 0.6	ac	2.4 ± 0.1	ah	36.7 ± 1.7	a	1.7 ± 0.6	abd
EABb 12/05-Rf	26.3 ± 0.8	be	2.6 ± 0.1	a	35.1 ± 0.1	a	0.6 ± 0.2	a
EABb 13/01-Rf	23.7 ± 0.4	a	3.7 ± 0.1	bdf	35.9 ± 0.6	a	1.2 ± 0.3	bce
EABb 13/02-Rf	23.6 ± 0.8	ad	3.3 ± 0.2	bc	36.0 ± 1.1	a	1.2 ± 0.5	ac
EABb 13/03-Rf	25.0 ± 0.8	ab	3.1 ± 0.2	ce	35.7 ± 0.8	a	1.1 ± 0.4	ac
EABb 13/04-Rf	26.1 ± 0.9	b	3.2 ± 0.2	be	35.4 ± 0.7	a	1.0 ± 0.4	ac
EABb 12/03-Pa	25.9 ± 0.6	b	2.6 ± 0.1	ad	35.8 ± 0.8	a	1.3 ± 0.4	ac
EABb 12/04-Rf	26.6 ± 0.3	b	3.9 ± 0.1	fg	35.1 ± 0.2	a	1.1 ± 0.2	bce
EABb 08/05-Rf	26.0 ± 0.7	bgh	2.5 ± 0.1	a	35.1 ± 0.1	a	0.5 ± 0.1	a
EABb 08/06-Rf	25.3 ± 0.4	efg	3.4 ± 0.2	bc	35.8 ± 0.7	a	1.5 ± 0.4	bcf
IBB 010	24.7 ± 0.3	cdef	4.0 ± 0.1	g	36.6 ± 1.0	a	2.2 ± 0.4	defg
B.b.T-N	25.9 ± 0.3	bg	2.7 ± 0.1	h	36.0 ± 0.7	a	1.8 ± 0.4	bcg
B.b.T-A	25.9 ± 0.3	bh	2.7 ± 0.1	h	36.0 ± 0.7	a	1.8 ± 0.4	bcg

(a) The generalized βfunction is given by: P = TYopt[(T − Tmin)/(Topt − Tmin)] exp [TB3 (Topt − Tmin)/(Tmax − T)] [(Tmax − T)/(Tmax − Topt)] exp TB3, where Y(T) is the fungal growth in mm per day (dependent variable) and T is the incubation temperature (independent variable), Tmin, Tmax, and Topt are respectively the lowest, the highest, and the optimal temperature for fungal growth, TYopt is the fungal growth at the optimal temperature Topt and TB3 is the shape parameter.
(b) Means within columns with the same letter are not significantly different ($p < 0.05$) according to the least significant difference (LSD) test.

Table 5
Effect of temperature on germination (%) of *B. bassiana* isolates obtained from *R. ferrugineus* and *P. archon* at different temperatures.

Strain	Temperature							
	15 °C		20 °C		25 °C		30 °C	
EABb 07/06-Rf	32.0 ± 2.3	b	76.7 ± 3.5	ab	84.7 ± 4.7	a	79.3 ± 1.7	ab
EABb 12/05-Rf	18.1 ± 1.1	de	52.7 ± 6.4	cd	82.7 ± 1.3	a	67.3 ± 4.1	bc
EABb 13/01-Rf	27.8 ± 1.1	bc	83.3 ± 4.8	ab	96.0 ± 1.2	a	81.3 ± 0.7	ab
EABb 13/02-Rf	41.1 ± 2.4	a	88.7 ± 1.8	a	94.0 ± 2.0	a	88.0 ± 1.2	a
EABb 13/03-Rf	20.9 ± 1.4	cd	68.0 ± 4.6	bc	90.7 ± 4.4	a	76.0 ± 6.1	ab
EABb 13/04-Rf	47.2 ± 0.6	a	88.7 ± 3.3	a	94.0 ± 2.3	a	78.0 ± 3.1	ab
EABb 12/03-Pa	41.5 ± 1.1	a	87.3 ± 2.9	a	90.0 ± 4.6	a	86.7 ± 4.8	ab
EABb 12/04-Rf	25.7 ± 0.8	bcd	69.3 ± 1.8	bc	98.0 ± 2.0	a	86.7 ± 3.7	ab
EABb 08/05-Rf	28.2 ± 0.7	bc	56.0 ± 2.0	c	87.3 ± 2.4	a	75.3 ± 6.7	ab
EABb 08/06-Rf	24.8 ± 3.6	bcd	78.0 ± 1.2	ab	91.3 ± 2.4	a	85.3 ± 0.7	ab
IBB 010	21.8 ± 1.9	cd	54.7 ± 1.8	cd	82.0 ± 4.2	a	70.7 ± 4.4	abc
B.b.T-N	27.7 ± 1.4	bc	36.7 ± 2.4	de	89.3 ± 4.8	a	82.0 ± 2.0	ab
B.b.T-A	9.8 ± 1.0	e	22.0 ± 4.2	e	52.0 ± 3.1	b	51.7 ± 2.0	c

Means (± S.E.) within-column followed by the same letter and within row bearing the same letter are not significantly different by Tukey's honestly significant difference (Tukey's HSD) test ($p < 0.05$).

compared with the control specimens. After death, the specimens were maintained under optimal temperature and relative humidity conditions to promote fungal growth and sporulation for 7–10 days, when fungal outgrowth was observed (Fig. 7).

Mortality and AST values obtained for each strain against *R. ferrugineus* adults are shown in Table 11. All the *B. bassiana* strains had a significant effect on *R. ferrugineus* adult mortality, except the strain EABb 12/03-Pa, which was isolated from a *P. archon* specimen and ranged from 20.0 to 50.0%.

AST values of the treated specimens were also affected by the treatment. The highest decrease in the cumulative survival ratio, taking together all the strains observed between 9 and 18 days after treatment.

Similar to the larval assay, the strain that caused the highest mortality was EABb 13/04-Rf (50%), with an AST = 23.5 days. This strain has proven to be one of the more effective and virulent strains against *R. ferrugineus* larvae and adults. Furthermore, it showed a high conidial germination speed and germination rate under unfavorable environmental conditions (5.3% germination at 86.2% RH), which may enhance virulence due to it is direct relationship to the ability to penetrate the host.

Strain EABb 12/04-Rf caused the second highest mortality (40.0%) in *R. ferrugineus* adults among all the strains tested, and a lower AST of 22.5 days.

As in the larval assay, a great percentage of dead specimens treated with strain EABb 07/06-Rf did not show any fungal outgrowth after incubation under optimal environmental conditions.

Another important issue observed was the reduction of fecundity beginning at 14 days after treatment in *B. bassiana*-treated females in comparison to the control (Fig. 8).

4. Discussion

The complete molecular study, phylogenetic analysis based on EF-1α and Bloc partial sequences and ISSR, together with the distribution of the strains through the Mediterranean Basin (sampling locations), have led to several hypotheses to explain the spread of these fungal strains. In Fig. 9, we can observe that geographically distant strains have, by contrast, very similar sequences and are evolutionarily very close.

The finding that the *B. bassiana* sequence types A, B, C, D, and E are

Table 6
Effect of osmotic potential on *in vitro* germination of fungal conidia (%) of 17*B. bassiana* strains isolated from *R. ferrugineus* and *P. archon.*

Strain	Medium osmotic potential/Relative humidity (%)													
	1/99.9		5/99.6		20/98.5		40/97.0		60/95.5		100/92.8		150/89.5		200/86.2	
EABb 07/06-Rf	96.0 ± 2.3	ab	94.7 ± 3.5	ab	90.7 ± 1.7	ab	88.0 ± 1.2	ab	27.3 ± 3.5	bc	20.0 ± 2.0	b	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 08/05-Rf	96.0 ± 2.3	ab	89.3 ± 3.5	ab	83.3 ± 1.7	abc	35.3 ± 1.8	cde	5.3 ± 1.8	e	0.7 ± 0.7	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 08/06-Rf	91.3 ± 0.7	abc	80.7 ± 5.9	abcd	79.3 ± 3.7	bcd	36.0 ± 2.0	cde	12.7 ± 1.8	de	4.0 ± 2.0	c	2.0 ± 1.2	d	27 ± 1.3	bc
EABb 08/07-Rf	58.7 ± 1.3	e	43.3 ± 2.9	e	26.0 ± 1.2	g	22.0 ± 1.2	ef	10.0 ± 4.2	de	0.7 ± 0.7	c	0.0 ± 0.0	e	0.00 ± 0.0	d
IBB 010	96.0 ± 2.3	ab	94.0 ± 1.2	ab	93.3 ± 2.7	ab	88.0 ± 4.2	ab	55.3 ± 2.9	a	44.0 ± 4.6	a	12.0 ± 1.2	b	6.0 ± 1.2	ab
B.b.T-N	86.0 ± 3.1	bcd	77.3 ± 6.4	abcd	85.3 ± 4.4	abc	77.3 ± 2.9	b	12.7 ± 3.5	de	0.0 ± 0.0	c	0.0 ± 0.0	e	0.0 ± 0.0	d
B.b.T-A	82.0 ± 5.0	cd	86.7 ± 3.5	abc	81.3 ± 3.7	bcd	88.7 ± 3.3	ab	38.0 ± 2.0	b	1.3 ± 1.3	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 12/04-Rf	81.3 ± 3.5	cd	63.3 ± 4.8	de	58.7 ± 3.5	ef	36.7 ± 7.0	cde	6.7 ± 1.8	de	1.3 ± 1.3	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 12/05-Rf	96.0 ± 1.2	ab	96.7 ± 1.3	ab	97.3 ± 0.7	a	90.7 ± 1.3	ab	19.3 ± 4.4	cd	0.7 ± 0.7	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 13/01-Rf	96.0 ± 1.2	ab	84.0 ± 8.7	abcd	82.7 ± 3.5	abcd	74.0 ± 2.3	b	12.7 ± 1.8	de	4.0 ± 2.3	c	2.0 ± 1.2	d	0.7 ± 0.7	c
EABb 13/02-Rf	96.0 ± 0.0	ab	83.3 ± 5.2	abcd	81.3 ± 1.8	bcd	50.7 ± 7.3	c	4.0 ± 2.0	e	0.7 ± 0.7	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 13/03-Rf	74.0 ± 1.2	d	65.3 ± 1.7	cde	51.3 ± 5.8	f	39.3 ± 1.8	cd	8.0 ± 2.3	de	0.7 ± 0.7	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 13/04-Rf	100.0 ± 0.0	a	85.3 ± 2.4	abcd	70.7 ± 2.4	cde	47.3 ± 3.5	c	35.3 ± 1.8	b	32.0 ± 0.0	ab	7.3 ± 0.7	c	5.3 ± 0.7	ab
EABb 13/05-Rf	14.0 ± 2.0	g	7.3 ± 0.7	f	4.7 ± 0.7	h	1.3 ± 0.7	g	0.0 ± 0.0	e	0.0 ± 0.0	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 14/01-Rf	85.3 ± 2.4	bcd	74.7 ± 2.4	bcd	67.3 ± 3.7	de	24.7 ± 1.8	de	7.3 ± 1.8	de	0.0 ± 0.0	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 14/02-Rf	42.7 ± 5.2	f	48.7 ± 5.7	e	18.7 ± 2.4	gh	6.7 ± 1.7	fg	2.0 ± 1.2	e	0.0 ± 0.0	c	0.0 ± 0.0	e	0.0 ± 0.0	d
EABb 12/03-Pa	100.0 ± 0.0	a	98.7 ± 1.3	a	97.3 ± 1.8	a	94.7 ± 1.7	a	40.0 ± 0.0	b	22.0 ± 1.2	ab	15.3 ± 0.7	a	8.0 ± 0.0	a
	p = 0.0000 F16,50 = 87.20		*p = 0.0000* F16,50 = 31.59		*p = 0.0000* F16,50 = 88.39		*p = 0.0000* F16,50 = 91.30		*p = 0.0000* F16,50 = 42.14		*p = 0.0000* F16,50 = 25.44		*p = 0.0000* F16,50 = 64.20		*p = 0.0014* F16,50 = 10.38	

*Means in the same column followed by different letters are significantly different ($p < 0.05$), Tukey's honestly significant difference (Tukey's HSD) test.

Table 7
Relative percentage germination of *B. bassiana* isolates obtained from *R. ferrugineus* and *P. archon* at different temperatures observed at 24 h after exposure time to 1200 mW m^{-2} and irradianced for 2, 4 and 6 h of exposure time.

Strain	1200 mW m^{-2}					
	2 h		4 h		6 h	
EABb 07/06-Rf	43.6 ± 2.8	ef	66.7 ± 3.2	ab	60.4 ± 3.8	ab
EABb 12/05-Rf	69.7 ± 3.3	bc	28.0 ± 3.5	d	15.8 ± 3.0	cde
EABb 13/01-Rf	82.9 ± 4.4	b	46.8 ± 2.1	c	32.3 ± 8.0	c
EABb 13/02-Rf	30.1 ± 0.8	f	13.5 ± 2.2	de	8.6 ± 0.9	def
EABb 13/03-Rf	66.4 ± 3.7	bcd	52.6 ± 2.0	bc	29.8 ± 2.9	c
EABb 13/04-Rf	75.4 ± 4.0	bc	71.2 ± 0.8	a	74.1 ± 1.7	a
EABb 12/03-Pa	94.6 ± 3.2	a	71.6 ± 2.6	a	21.2 ± 4.2	cd
EABb 12/04-Rf	59.1 ± 2.4	cde	59.4 ± 1.6	abc	34.2 ± 1.8	bc
EABb 08/05-Rf	75.8 ± 1.8	bc	70.7 ± 2.2	abc	71.6 ± 2.8	a
EABb 08/06-Rf	43.0 ± 2.7	ef	5.4 ± 1.1	e	2.9 ± 1.5	ef
IBB 010	45.0 ± 4.3	ef	24.7 ± 2.4	d	12.2 ± 1.1	cdef
B.b.T-N	47.2 ± 1.4	def	10.9 ± 4.3	e	2.0 ± 2.0	f
B.b.T-A	38.3 ± 3.3	f	16.7 ± 4.0	de	2.6 ± 2.6	f

The percentage of germination after each period was calculated in relation to non-irradiated controls. Standard errors are after each mean. Means in the same column followed by different letters are significantly different ($p < 0.05$), Tukey's honestly significant difference (Tukey's HSD) test.

Table 8
Relative culturability of *B. bassiana* isolates obtained from *R. ferrugineus* and *P. archon* at different temperatures observed at 48 h after the exposition to 1200 mW m^{-2} irradiance for 2, 4 and 6 h of exposure time.

Strain	1200 mW m^{-2}					
	2 h		4 h		6 h	
EABb 07/06-Rf	91.7 ± 8.3	De	90.5 ± 4.8	ab	100.0 ± 0.0	a
EABb 12/05-Rf	100.0 ± 0.0	E	73.9 ± 1.6	abc	43.9 ± 8.2	ef
EABb 13/01-Rf	98.7 ± 1.1	Bc	91.2 ± 1.9	ab	95.3 ± 4.7	ab
EABb 13/02-Rf	86.2 ± 4.5	A	100.0 ± 0.0	a	92.9 ± 6.2	ab
EABb 13/03-Rf	90.9 ± 4.6	De	91.9 ± 3.2	ab	85.4 ± 0.5	abc
EABb 13/04-Rf	92.4 ± 4.3	De	81.5 ± 3.0	abc	85.4 ± 0.5	bcd
EABb 12/03-Pa	100.0 ± 0.0	De	95.4 ± 2.5	ab	73.2 ± 6.7	cde
EABb 12/04-Rf	86.2 ± 6.5	B	85.5 ± 3.0	abc	61.2 ± 0.8	efg
EABb 08/05-Rf	84.8 ± 7.9	Cd	59.1 ± 9.7	c	38.6 ± 6.0	ab
EABb 08/06-Rf	100.0 ± 0.0	Bc	60.6 ± 6.1	c	90.6 ± 5.9	g
IBB 010	99.6 ± 0.4	E	83.7 ± 8.3	abc	16.6 ± 1.9	ab
B.b.T-N	100.0 ± 0.0	Bc	71.2 ± 2.7	bc	89.2 ± 6.2	def
B.b.T-A	92.8 ± 6.1	E	71.8 ± 9.1	bc	54.6 ± 3.3	fg

The percentage of culturability after each period was calculated in relation to non-irradiated controls. Standard errors are after each mean. Means in the same column followed by different letters are significantly different ($p < 0.05$), Tukey's honestly significant difference (Tukey's HSD) test.

Table 9
Growth index observed for *B. bassiana* isolates obtained from *R. ferrugineus* and *P. archon* at different temperatures at 48 h after the exposition to 1200 mW m^{-2} irradiance for 2, 4 and 6 h of exposure time.

Strain	1200 mW m^{-2}					
	2 h		4 h		6 h	
EABb 07/06-Rf	1.2 ± 0.7	b	18.8 ± 3.3	ab	30.8 ± 2.9	a
EABb 12/05-Rf	7.6 ± 2.2	ab	15.5 ± 4.2	ab	5.2 ± 1.5	d
EABb 13/01-Rf	6.8 ± 5.2	ab	25.7 ± 2.8	a	25.3 ± 0.8	a
EABb 13/02-Rf	15.3 ± 1.4	a	26.0 ± 1.4	a	20.4 ± 2.6	abc
EABb 13/03-Rf	1.6 ± 0.7	ab	10.8 ± 0.6	ab	24.4 ± 2.4	abc
EABb 13/04-Rf	7.2 ± 2.6	ab	21.8 ± 1.1	a	31.1 ± 1.2	a
EABb 12/03-Pa	5.8 ± 2.5	ab	22.9 ± 1.9	a	10.8 ± 2.6	cd
EABb 12/04-Rf	15.5 ± 0.3	a	23.1 ± 2.8	a	21.1 ± 3.6	abc
EABb 08/05-Rf	10.7 ± 6.1	ab	18.2 ± 2.7	ab	12.7 ± 3.4	bcd
EABb 08/06-Rf	5.4 ± 2.9	ab	19.1 ± 2.7	ab	22.0 ± 2.4	abc
IBB 010	0.9 ± 0.6	b	24.9 ± 0.5	a	16.9 ± 0.6	abc
B.b.T-N	7.7 ± 1.0	ab	20.4 ± 1.2	ab	19.1 ± 1.2	abc
B.b.T-A	4.2 ± 1.1	ab	0.0 ± 0.0	c	22.1 ± 5.9	abc

The growth index after each period was calculated in relation to the non-irradiated controls. Standard errors are after each mean. Means in the same column followed by different letters are significantly different ($p < 0.05$), Tukey's honestly significant difference (Tukey's HSD) test.

apparently polyphyletic suggests a temporal heterogeneity between these subclades. These data concur with those from other studies in which it was not possible to generate phylogenetic relationships with different clusters according to the sample geographical origin (Berreta et al., 1998; Wang et al., 2003).

The cluster relationship could be explained by the dispersion of the same fungal strain carried by the insect host, *R. ferrugineus*, reaching even the *P. archon* populations. This is an important phenomenon to consider since, to the best of our knowledge, this is the first time that *B. bassiana*, *B. pseudobassiana* and *L. attenuatum* EPF have been found naturally infecting *P. archon* specimens. This dispersion could be connected to the intercontinental palm trade, as observed for *R. ferrugineus* and *P. archon* pests, which have spread through human assistance following the trade routes associated with palm plants for planting shipped from infested areas, and even for entomopathogenic fungi that endophytically colonize palms (Mahmoud et al., 2017).

It is currently important to note that Spain was the first European country invaded by *R. ferrugineus* (EPPO, 1996), where the first natural infection of *R. ferrugineus* pupae by *B. bassiana* was observed in Europe in 2007 (Dembilio et al., 2010). That same year, the first *P. archon* adults infected by *B. pseudobassiana* and *L. lecanii* were detected in France and Italy. This moth was first detected in Europe (France and Spain) in 2001, although this detection is believed to lag several years behind the actual introduction date (Reid and Moran, 2009).

Furthermore, this fungal spread could have been affected by geographical and temporal factors. Thus, we found different type sequences in more isolated areas, such as islands (Sicily) or peninsulas (Spain, Italy and Greece). It was precisely in Spain where the first infective strain (i.e., EABb 07/06-Rf, the oldest one) was isolated, and therefore, this isolate may have its origin in the first *R. ferrugineus* populations that reached Europe. *R. ferrugineus* could have been infected by endemic *B. bassiana* strains in regions where this insect pest has been introduced, for example, Greece, where the endemic palm species *Phoenix theoprasthi Greuter* (Arecaceae) is located.

In addition, *B. bassiana* strain evolution may result from its adaptation to other insect developmental stages, as could have occurred, for example, for strains EABb 08/05-Rf, B.b T-N and EABb 13/01-Rf, all of which belong to the A type sequence and have been isolated from larvae, pupa and adults of *R. ferrugineus*. The observation that specimens infected by the same *B. bassiana* strain were likely identified 7 years prior (Ex. EABb 07/06-Rf) indicates the temporal and geographical stability of that inoculum and its infective capacity.

The *M. anisopliae* strains EAMa 08/01-Rf and EAMa 08/02-Rf isolated from dead specimens from southern Italy belong to the same type sequence of the strains found in Israel. Therefore, spread of the inoculum similar to that observed for *B. bassiana* strains could have occurred.

It should be noted that the number of *M. anisopliae* strains isolated was significantly lower than that of *B. bassiana*. Additionally, *M. anisopliae* was found only in *R. ferrugineus* adults (Table 2). *Metarhizium* sp. are commonly found in soil habitats and distributed along temperate regions (Quesada-Moraga et al., 2007), in agreement with their isolation during terrestrial developmental stages only. Conversely, *B. bassiana* has been recorded frequently in Mediterranean habitats, forming plant associations both as an epiphyte and an endophyte (Meyling and Eilenberg, 2007; Garrido-Jurado et al., 2015). Indeed, the *B. bassiana* strains considered in this study were isolated from larvae and pupa of *R. ferrugineus* and an adult of *P. archon*, all of which were detected in developmental stages occurring in above-ground habitats. Other studies have also reported that *B. bassiana* is able to infect pupae

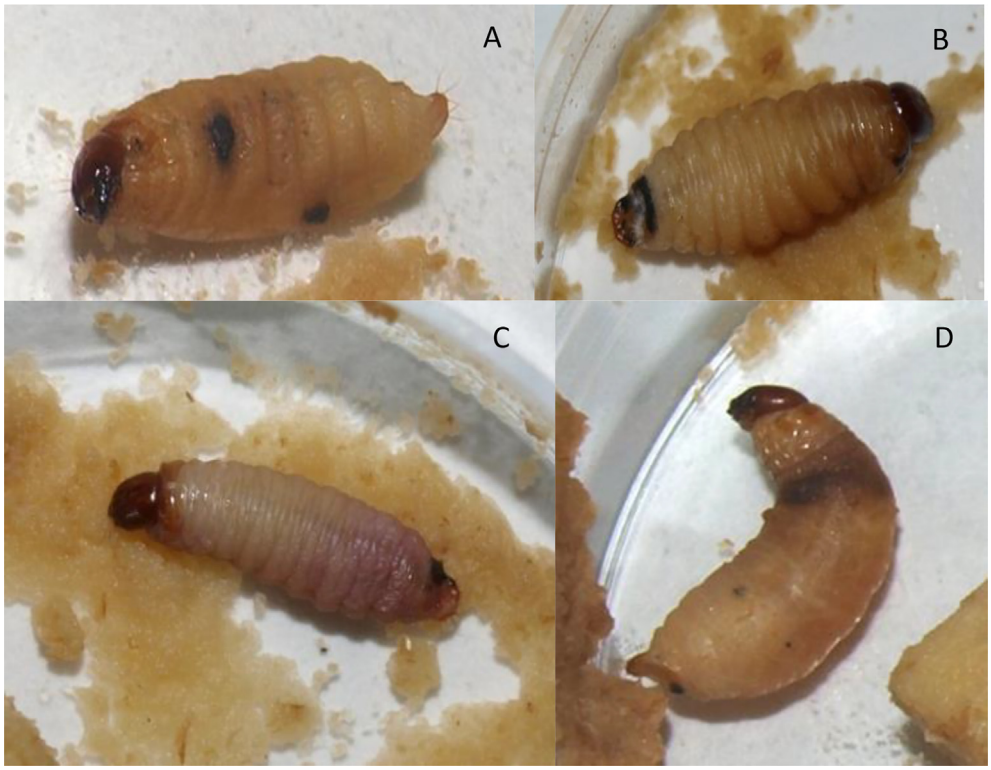

Fig. 4. *R. ferrugineus* larval infection 2–3 days after immersion in a *B. bassiana* conidial suspension at 1.0×10^8 conidias/mL. (A) Dark brown/black spots, sign of fungal penetration through the cuticle by EF. (B). Starting fungal outgrowth. (C) Pink coloration and darkening (D) of 4th instar *R. ferrugineus* larvae. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Dead 4th instar larvae of *R. ferrugineus* through the moulting process as a consequence of the EF treatment.

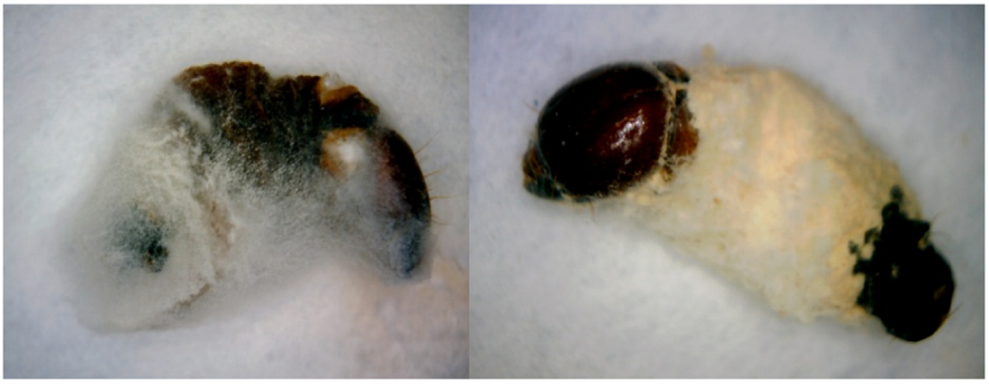

Fig. 6. Dead 4th instar *R. ferrugineus* larvae that showed fungal outgrowth after being treated by immersion with a *B. bassiana* conidial suspension at 1.0×10^8 conidias/mL of the strains IBB010 and B.b.T-A.

or to associate with the cocoons of *R. ferrugineus* and infect the emerging adults (Ghazavi and Avand-Faghih, 2002; Shaiju-Simon Kumar and Gokulapalan, 2003). Thus, *B. bassiana* could have been associated with the palm tree, providing protection in its natural habitat. At this point, *B. bassiana* is considered the best suited EPF for Mediterranean palm growth environmental conditions and could be the most appropriate EPF species to control these pests.

When ecological parameters were analyzed, all the *B. bassiana* strains responded positively to Mediterranean environmental conditions. Since palms are found mainly in tropical and warm temperate regions (Dransfield, 1978), these *B. bassiana* strains seem to be well adapted to the weevil distribution area. Thermal requirements for

Table 10
Susceptibility of fourth instar *Rhynchophorus ferrugineus* larvae to 6 *Beauveria bassiana* selected strains by immersion in a conidial suspension.

Treatment*	Mortality (%)			Kaplan-Meier Survival analysis		
	Mean (± SE)**	Fungal outgrowth	Other causes	AST*** (± SE; days)	Upper limit	Lower limit
Control	1.1 ± 1.1 C	0	1.1	11.7 ± 0.3 a	12.3	11.2
EABb 07/06-Rf	73.3 ± 1.1 AB	10.0	63.3	7.7 ± 0.5 bd	8.7	6.6
EABb 12/03-Pa	60.0 ± 3.3 AB	33.3	26.7	9.0 ± 0.5 bc	10.1	7.9
IBB010	40.0 ± 1.9 B	16.7	23.3	7.9 ± 0.6 b	9.0	6.8
B.b.T-A	70.0 ± 1.9 AB	46.7	23.3	6.1 ± 0.5 d	7.2	5.1
EABb 13/04-Rf	83.3 ± 2.9 A	40.0	43.3	8.1 ± 0.6 b	9.2	6.9
EABb 12/04-Rf	66.7 ± 2.2 AB	30.0	36.7	9.5 ± 0.6 c	10.7	8.3

* Immersion treatment was performed in a 1×10^8 conidia/ml suspension.
** Means within columns with the same uppercase letter are not significantly different according to the Tukey's honestly significant difference (Tukey's HSD) test ($p < 0.05$).
*** AST: Average survival time. Means within columns with the same lowercase letter are not significantly different according log rank test ($p < 0.05$). Average survival time limited at 12 days.

Fig. 7. Dead adult of *R. ferrugineus* showing fungal outgrowth after being treated by immersion with a *B. bassiana* conidial suspension at 1.0×10^8 conidias/mL of the strain EABb-12/04-Rf.

vegetative growth were established for all strains, with an optimum range close to 25 °C. Similar results were reported in other studies addressing *B. bassiana* thermal requirements (Fargues et al., 1997; Yeo et al., 2003; Quesada-Moraga et al., 2006), in which the optimum temperature for fungal growth was between 20 and 30 °C.

Although positive relationships between relative *in vitro* growth rates and geoclimatic origin have been found for some mitosporic ascomycetes (Roberts and Campbell, 1977), in our case and in agreement with Fargues et al. (1997), a relationship between upper threshold temperatures or growth rates at different temperatures and origins could not be established. We note that strains originally isolated in Israel (B.b.T-A and B.b.T-N) showed similar values according to the shape parameter TB3 (which describes optimum curves resulting from the effect of temperature), although molecular analysis showed that they did not belong to the same type sequence. Thus, this coincidence could be explained by their shared geographical origin. However, there was no apparent relationship between the fungal growth rate and the geographical origin or molecular analysis.

Although all strains reached higher germination percentage rates at approximately 25 °C, the optimum temperature for germination was variable for A sequence strains, ranging from 20 to 30 °C. The observed differences may be due to small latitudinal differences between the locations in the Mediterranean Basin where the isolates were found. However, B and C type sequences had an optimum temperature for germination fixed at 25 °C, showing a relationship with the genetic analysis in which both sequences were closely linked. Generally, all strains exceeding 80% germination at 30 °C belonged to the type A sequence and were closely clustered in the phylogenetic tree performed with ISSR, similar to the results reported by Kryukov et al (2012), who observed similarity between the ISSR spectra of *B. bassiana* thermotolerant strains despite their geographic origin.

Otherwise, strains B.b.T-N and B.b.T-A, both from Tivon (Israel) and linked in the phylogenetic tree resulting from EF1-α evaluation, showed an optimum temperature for germination in the range from 25 to 30 °C. However, the germination percentage was lower for strain B.b.T-A (D sequence), suggesting an evolution of the strain in response to temperature to improve the ability of the fungus to infect the host, as pathogenicity assays against both larvae and adults revealed superior virulence of A sequence strains. This kind of evolution has been reported previously for strains of *Beauveria* sp. isolated from locusts (Acrididae) in desert areas, resulting in more thermotolerance than the cultures isolated from insects in other orders (Fargues et al., 1997).

It is known that the thermotolerance of EPF increases with a decreasing original latitude (Vidal et al., 1997; Bidochka et al., 2002). However, the present study was performed with thirteen strains from the Mediterranean Basin that were isolated in a narrow range of

Table 11
Susceptibility of *Rhynchophorus ferrugineus* field-collected adults to 6 *Beauveria bassiana* selected strains by immersion in a conidial suspension.

Treatment*	Mortality (%)					Kaplan-Meier Survival analysis		
	Total	Males	Females	Fungal outgrowth	Other causes	AST** (± SE; días)	Upper limit	Lower limit
Control	0.0	0.0	0.0	0.0	0.0	30.0 ± 0.0 a	30.0	30.0
EABb 07/06-Rf	20.0	10.0	10.0	0.0	20.0	25.9 ± 2.6 ab	31.0	20.8
EABb 12/03-Pa	10.0	10.0	0.0	0.0	10.0	29.5 ± 0.5 a	30.4	28.6
IBB010	10.0	0.0	10.0	0.0	10.0	28.3 ± 1.6 ab	31.5	25.1
B.b.T-A	20.0	20.0	0.0	20.0	0.0	27.9 ± 1.4 ab	30.7	25.1
EABb 13/04-Rf	50.0	10.0	40.0	50.0	0.0	23.5 ± 2.6 b	28.6	18.5
EABb 12/04-Rf	40.0	10.0	30.0	40.0	0.0	22.5 ± 3.0 ab	28.4	16.6

* Immersion treatment was performed in a 1×10^8 conidia/ml suspension.
** AST: Average survival time. Means within columns with the same letter are not significantly different according log rank test ($p < 0.05$). Average survival time limited at 30 days.

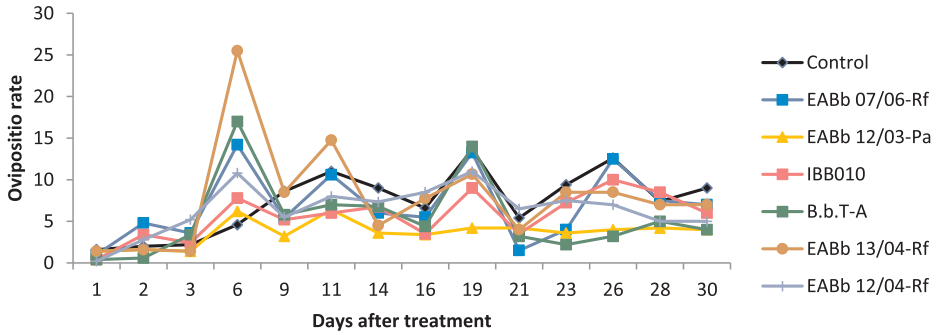

Fig. 8. Oviposition rate (mean number of eggs per female) of *R. ferrugineus* females treated by immersion in a conidial suspension (concentration 1.0×10^8 conidia/mL) of different *B. bassiana* strains and fed on apple slices.

latitude, and in general, all the strains showed a similar response.

In relation to the osmotic potential of the culture medium, the germination tube length decreased as the osmotic potential increased. This may ocurr because the germination speed increased with the relative humidity, and at relative humidity values below 95% RH, the latent period of spore germination was much longer (Luz and Fargues, 1997). In addition, after 24 h of incubation at 25 °C, no conidial germination was observed at relative humidity values below 92.5% (Walstad et al., 1970).

For all strains, the percentage of germinated conidia drastically decreased at 95.5% relative humidity. This phenomenon is usually the case for *B. bassiana* (Luz and Fargues, 1997) because the relative humidity for optimal development of most entomopathogenic hyphomycetes is greater than 97% (Gillespie and Crawford, 1986).

However, germination at an osmotic potential of 150 and 200 bars (relative humidity of 89.5 and 86.2%) was observed in five strains only, namely, EABb 13/01-Rf, EABb 13/04-Rf, EABb 12/03-Pa and EABb 08/06-Rf. These strains belong to sequence type A according to EF-1α characterization, which grouped most of the strains, with the exception of strain IBB010 belonging to type sequence C. Luz and Fargues (1997) observed conidial swelling at 86% relative humidity, so these strains may have developed an adaptive advantage and germinated faster under low relative humidity conditions, which may have influenced the spread of the strain.

A significant effect of UV-B radiation on the germination and culturability of *B. bassiana* conidia after exposure to 1200 mW m^{-2} of irradiance was also noted.

Interestingly, the exposure to UV-B radiation resulted in a delay of germination for *B. bassiana*, and this effect was not linked to either the type of sequence or the geographic origin. This effect was already reported by other authors (Braga et al., 2001b; Fernandes et al., 2007; Fernandez-Bravo et al., 2017). They observed that longer periods of exposure delayed germination for days. Furthermore, depending on the dose, the time required to recover and to resume germination could increase. However, the molecular basis of this process has not yet been studied. In our study, strains EABb 13/02-Rf, EABb 08/06-Rf, IBB 010, B.b.T-N and B.b.T-A showed high susceptibility to UV-B radiation, with less than 50% conidia germination. All of them belonged to the A sequence type, excluding B.b.T-A belonging to the D type. Nevertheless, strain EABb 13/04-Rf, which also belongs to the A sequence type, showed more than 70% germination at 2, 4, and 6 h after exposure to UV-B radiation.

UV-B radiation also had a significant effect on the culturability of the 13 strains (Table 8). In general, after 2 h of exposure to 1200 mW m^{-2} of irradiance, the number of colonies was not affected relative to the control for all the strains. This finding can be considered as indicative that UV-B does not kill conidia but delays their germination. However, for some strains, after 6 h of exposition, the number of colonies dramatically decreased. Remarkably, all these strains belonged to the B, C and D sequences. In general, colonies from strains belonging to the A type (except EABb 08/05-Rf) showed greater resilience to UV-B exposure. These strains also showed a lower percentage of conidial germination at 24 h after exposure to UV-B radiation, which this is probably indicative of an adaptation to UV. Although the molecular process governing the response of EPF to UV-B is not yet clear, it is known that tolerance to UV irradiation depends on enzyme regulation, which provides suitable genes (e.g., production of melanins, thioredoxins) to increase conidial tolerance to UV irradiation (Xie et al, 2012; Wang and Feng, 2014).

Finally, there was a significant effect of UV-B exposure on colony

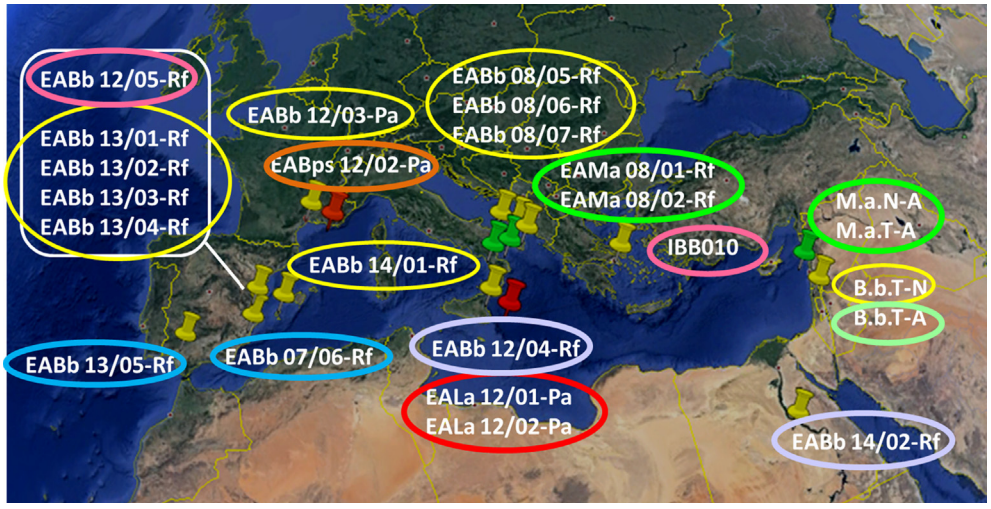

Fig. 9. Distribution of fungal species and type sequences as a function on their origin. Pointed marked in the map signal the origin of the isolate. Yellow pushpin icons indicates *B. bassiana* strains, orange are used for *B. pseudobassiana*, green for *M. anisopliae* and red for *L. attenuatum*. Yellow circles indicate that the strain belongs to type sequence A, purple to sequence B, green to sequence D, pink to sequence C and blue to sequence E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

size (Table 9). The colony size of irradiated conidia was smaller than that of nonirradiated conidia. These smaller colonies also showed a growth delay in response to UV-B irradiance exposure. Braga et al. (2001b) observed a greater delay and heterogeneity of colony development after exposition to 1200 mW m^{-1}. We could not find any relationship between the growth index and the origin or genetic characteristics of the strains.

Despite their differences at molecular and ecological concerns, all the strains showed high virulence against fourth instar *R. ferrugineus* larvae, resulting in significant mortality and a reduction of AST. These values are in agreement with previous results (Dembilio et al., 2010; Francardi et al., 2012). In our case, all the *B. bassiana* strains had a significant effect on *R. ferrugineus* adult mortality, excluding strain EABb 12/03-Pa, which was isolated from the moth *P. archon*. Conversely, the relatively stronger cuticle and exoskeleton of the adult stage was the most likely cause for the lower mortality rates observed for the adult stage compared with 4th instar larvae, although they were treated with the same strain and conidial concentration. Efficacy may be enhanced with a proper formulation. A stronger effect of *B. bassiana* has been observed when applied as a solid formulation in comparison to an aqueous suspension (Gindin et al., 2006; Ricaño et al., 2013). This phenomenon could be due to higher penetration of conidia through insect natural openings, avoiding the cuticular pathway.

Strain EABb 13/04-Rf, which belongs to the sequence type A, has been shown to be one of the more effective and virulent strains against *R. ferrugineus* larvae and adults. It showed a high conidial germination speed and germination rate under unfavorable environmental conditions (5.3% germination at 86.2% de RH), which may enhance virulence due to its capacity to penetrate the host.

Regardless, mortality is not the only indicator of EPF efficacy since several sublethal effects on food intake, reproduction and later development of the insect were observed, in addition to horizontal transmission of the inoculum to nontreated individuals (Quesada Moraga et al. 2004, 2006, 2008; Dembilio et al., 2010), which could provide an additional reduction of palm damage.

Some characteristic symptoms of EF infection previously observed by other authors were identified. Treated *R. ferrugineus* larvae and adults consumed less food compared with control specimens. This decrease in food intake has been previously described by Dembilio et al. (2010) and observed in different insects treated with EF (Ekesi, 2001; Tefera and Pringle, 2003; Thomas et al., 1997).

Abnormalities during the molting process were noted, resulting in a lethal molt (Fig. 5), which has been observed in other insects (Fargues and Vey, 1974).

Some larvae turned pink or dark brown after treatment, as previously observed by Gindin et al. (2006), who related this change to the secretion of oosporein by the EF inside the insect body. EPF have different modes of action, including the production of insecticidal compounds, either high-molecular-weight products such as proteins or secondary metabolites with a low molecular weight (Quesada-Moraga and Santiago-Álvarez, 2008). Indeed, a large percentage of dead larvae and adults treated with strain EABb 07/06-Rf did not show any fungal outgrowth after incubation under optimal conditions. This strain produces some metabolites, including oosporein and beauvericin (data not published). Therefore, the low percentage of fungal outgrowth recorded could be because its main mode of action consists of the production of these insecticidal compounds within the insect.

Despite a high variation among individuals of the *Rhynchophorus* genus (Wattanapongsiri, 1966), in the present study we observed that from the 14th day after treatment onwards, fecundity was reduced in females treated with *B. bassiana* compared with the control females. This finding confirmed the previous findings of Dembilio et al. (2010): fecundity in control females increased (3.5 eggs/day), while that of females exposed to EPF decreased (1.5 eggs/day). In contrast, Gindin et al. (2006) did not observe any difference between treated and control females, but egg hatching and the duration of the oviposition period in treated females decreased when EPF were applied via a solid formulation.

The results support a key role of *R. ferrugineus* as a passive carrier of EPF during its colonization of the Mediterranean Basin. Our results also emphasize the key role of EPF, especially *B. bassiana*, as natural enemies of red palm weevil and *P. archon* in the Mediterranean basin, their ecological competence in this region and their promising role as microbiological control agents, either naturally occurring or used in inundative biological control methods against *R. ferrugineus*.

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References

Aquino De Muro, M., Elliott, S., Moore, D., Parker, B.L., Skinner, M., Reid, W., El Bouhssini, M., 2005. Molecular characterisation of *Beauveria bassiana* isolates obtained from overwintering sites of Sunn Pests (*Eurygaster* and *Aelia* species). Mycol. Res. 109, 294–306.

Barnett, H.L., Hunter, B.B., 2006. Illustrated Genera of Imperfect Fungi, fourth ed. APS Press, St. Paul, MN, pp. 218.

Barranco, P., de la Peña, J.A., Cabello, T., 1996. The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera, Curculionidae), a new pest in Europe. Phytoma España 67, 36–40 (in Spanish).

Bassanezi, R.B., Amorim, L., Bergamin Filho, A., Hau, B., 1998. Effects of bean line pattern mosaic virus on the monocyclic components of rust and angular leaf spot of *Phaseolus* bean at different temperatures. Plant. Pathol. 47, 289–298.

Beaudoin-Ollivier, L., Isidoro, N., Jaques, J.A., Riolo, P., Kamal, M., Rochat, D., 2017. Some representative palm pests: ecological and practical data. In: Soroker, V., Colazza, S. (Eds.), Handbook of Major Palm Pests: Biology and Management. John Wiley & Sons Ltd., United Kingdom, pp. 1–38.

Berretta, M.F., Lecuona, R.E., Zandommeni, R.O., Grau, O., 1998. Genotyping isolates of the enthomopathogenic fungus *Beauveria bassiana* by RAPD with fluorescent label. J. Invertebr. Pathol. 71, 145–150.

Besse-Millet, S., Bonhomme, A., Panchaud, K., 2008. Efficacy of a *Beauveria bassiana*. (Vuillemin) strain against the Castniid palm borer *Paysandisia archon* (Burmeister, 1880) under laboratory and natural conditions. From Lab. Field: Key Points 31, 215–219.

Bidochka, M.J., Kamp, A.M., Lavender, M., Dekoning, J., Amritha de Croos, J.N., 2001. Habitat association in two genetic groups of the insect- pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? Appl. Environ. Microbiol. 67, 1335–1342.

Bidochka, M.J., Menzies, F.V., Kamp, A.M., 2002. Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. Microbiology 178, 531–537.

Bilbao, J., Miguel, A., 2013. Contribution to the study of UV-B solar radiation in Central Spain. Renew. Energy 53, 79–85.

Bischoff, J.F., Rehner, S.A., Humber, R.A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101, 512–530.

Braga, G.U.L., Flint, S.D., Messias, C.L., Anderson, A.J., Roberts, D.W., 2001a. Effect of UV-B on conidia and germlings of the entomopathogenic hyphomycete *Metarhizium anisopliae*. Mycol. Res. 105, 874–882.

Braga, G.U.L., Flint, S.D., Miller, C.D., Anderson, A.J., Roberts, D.W., 2001b. Both solar UVA and UVB radiation impair conidial culturability and delay germination in the entomopathogenic fungus *Metarhizium anisopliae*. Photochem. Photobiol. 74, 734–739.

Davidson, G., Phelps, K., Sunderland, K.D., Pell, J.K., Ball, B.V., Shaw, K.E., Chandler, D., 2003. Study of temperature–growth interactions of entomopathogenic fungi with potential for control of *Varroa destructor* (*Acari*: *Mesostigmata*) using a nonlinear model of poikilotherm development. J. Appl. Microbiol. 94, 816–825.

Dembilio, O., Jacas, J.A., Llácer, E., 2009. Are the palms *Washingtonia filifera* and *Chamaerops humilis* suitable hosts for the red palm weevil, *Rhynchophorus ferrugineus* (Col. Curculionidae)? J. Appl. Entomol. 133, 565–567.

Dembilio, O., Quesada-Moraga, E., Santiago-Álvarez, C., Jacas, J.A., 2010. Biocontrol potential of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* (Ascomycota; Hypocreales) against the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae). J. Invertebr. Pathol. 104, 214–221.

Dembilio, O., Jacas, J.A., 2012. Bio-ecology and integrated management of the red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), in the region of Valencia (Spain). HPPJ 5, 1–12.

Dembilio, O., Moya, P., Vacas, S., Ortega-García, L., Quesada-Moraga, E., Jaques, J.A., Navarro-Llopis, V., 2018. Development of an attract-and-infect device to control Rhynchophorus ferrugineus with the entomopathogenic fungus Beauveria bassiana. Pest Manage. Sci. https://doi.org/10.1002/ps.4888.

Dransfield, J., 1978. The growth forms of rain forest palms. In: Tomlinson, P.B., Zimmermann, M.H. (Eds.), Tropical Trees as Living Systems. Cambridge Univ. Press, Cambridge, pp. 247–268.

Ekesi, S., 2001. Pathogenicity and antifeedant activity of entomopathogenic hyphomycetes to the cowpea leaf beetle, *Ootheca mutabilis* Shalberg. Insect Sci. Appl. 21, 55–60.

El-Sabea, A.M.R., Faleiro, J.R., Abo-El-Saad, M.M., 2009. The threat of red palm weevil *Rhynchophorus ferrugineus* to date plantations of the Gulf region in the Middle-East: An economic perspective. Outlooks Pest Manage. 20, 131–134.

El-Sufty, R., Al-Awash, S.A., Al-Bgham, S., Shahdad, A.S., Al-Bathra, A.H., 2009. Pathogenicity of the fungus *Beauveria bassiana* (Bals.) Vuill to the Red Palm Weevil, *Rhynchophorus ferrugineus* (Oliv.) (Col.: Curculionidae) under laboratory and field conditions. Egypt J. Biol. Pest Control 19, 81–85.

EPPO Reporting Service, 1996. Introduction of the Asiatic palm weevil. Rhynchophorus ferrugineus into Spain, 1996/096, No 05.

EPPO, 2018a. EPPO Global database Rhynchophorus ferrugineus: < https://gd.eppo.int/taxon/RHYCFE/categorization > (accessed 20.03.18).

EPPO, 2018b. EPPO Global database Paysandisia. archon: < https://gd.eppo.int/taxon/PAYSAR/categorization > (accessed 20.03.18).

EPPO Reporting Service, 2002. Further details on the finding of *Paysandisia archon* in France. 2002/096, No 06.

FAO, 2016. ISPM 5. Glossary of phytosanitary terms.

Fargues, J., Vey, A., 1974. Modalités d'infection des larves de *Leptinotarsa decemlineata* par *Beauveria bassiana* au cours de la mue. Entomophaga 19, 311–323.

Fargues, J., Maniania, N.K., Delmas, J.C., Smits, N., 1992. Influence de la temperature sur la croissance *in vitro* d'hyphomycetes entomopathogénes. Agronomie 12, 557–564.

Fargues, J., Goettel, M., Ouedraogo, A., Rougier, M., 1997. Effect of temperature on vegetative growth of *B. bassiana* isolates from different origins. Mycologia 89, 383–392.

Faleiro, J.R., 2006. A review of the issues and management of the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Rhynchophoridae) in coconut and date palm during the last one hundred years. Int. J. Trop. Insect Sci. 26, 135–154.

FERA (Federal Emergency Relief Administration), 2011. News. August 22, 2011. < http://www.fera.defra.gov.uk/showNews.cfm?id=485 > (accessed 29.09.13).

Fernandes, E.K.K., Rangel, D.E.N., Moraes, A.M.L., Bittencourt, V.R.E.P., Roberts, D.W., 2007. Variability in tolerance to UV-B radiation among *Beauveria* spp. isolates. J. Invertebr. Pathol. 96, 237–243.

Fernández-Bravo, M.C., Garrido-Jurado, I., Valverde-García, P., Enkerli, J., Quesada-Moraga, E., 2016. Responses to abiotic environmental stresses among phylloplane and soil isolates of *Beauveria bassiana* from two holm oak ecosystems. J. Invertebr. Pathol. 144, 6–17.

Fernández-Bravo, M., Flores-León, A., Calero-López, S., Gutiérrez-Sánchez, F., Valverde-García, P., Quesada-Moraga, E., 2017. UV-B radiation-related effects on conidial inactivation and virulence against *Ceratitis capitata* (Wiedemann) (Diptera; Tephritidae) of phylloplane and soil *Metarhizium* sp. Strains. J. Invertebr. Pathol. 148, 142–151.

Francardi, V., Benvenuti, C., Roversi, P.F., Rumine, P., Barzanti, G., 2012. Entomopathogenicity of *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorokin isolated from different sources in the control of *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae). REDIA J. Zool. 95, 49–55.

Garrido-Jurado, I., Márquez, M., Ortiz-Urquiza, A., Santiago-Álvarez, C., Iturriaga, E.A., Quesada-Moraga, E., Monte, E., Hermosa, R., 2011. Genetic analyses place most Spanish isolates of *Beauveria bassiana* in a molecular group with word-wide distribution. BMC Microbiol. 11, 84.

Garrido-Jurado, I., Fernández-Bravo, M., Campos, C., Quesada-Moraga, E., 2015. Diversity of entomopathogenic Hypocreales in soil and phylloplanes of five Mediterranean cropping systems. J. Invertebr. Pathol. 130, 97–106.

Garrido-Jurado, I., Resquín-Romero, G., Amarilla, S.P., Ríos-Moreno, A., Carrasco, L., Quesada-Moraga, E., 2017. Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae). J. Pest Sci. 90, 319–330.

Ghazavi, M., Avand-Faghih, A., 2002. Isolation of two entomopathogenic fungi on red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Col. Curculionidae) in Iran. Appl. Entomol. Phytopathol. 9, 44–45.

Ghikas, D.V., Kouvelis, V.N., Typas, M.A., 2010. Phylogenetic and biogeographic implications inferred by mitochondrial intergenic region analyses and ITS1-5.8S-ITS2 of the entomopathogenic fungi *Beauveria bassiana* and *B. brongniartii*. BMC Microbiol. 10, 174–189.

Gindin, G., Levski, S., Glazer, I., Soroker, V., 2006. Evaluation of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* against the Red Palm Weevil *Rhynchophorus ferrugineus*. Phytoparasitica 34, 370–379.

Gillespie, A.T., Crawford, E., 1986. Effect of water activity on conidial germination and mycelial growth of Beauveria bassiana, Metarhizium anisopliae, Paecilomyces spp., and Verticillium lecanii. In: Samson, R.A., Vlak, J.M., Peters, D. (Eds.), Fundamental and Applied Aspects of Invertebrate Pathology, Proceedings of the Fourth International Colloquium of Invertebrate Pathology. Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen, Netherlands, pp. 254.

Goettel, M.S., Inglis, G.D., 1997. Fungi: hyphomycetes. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, London, pp. 213–249.

Hampl, V., Pavlícek, A., Flegr, J., 2001. Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with a freeware program FreeTree: application to trichomonad parasites. Int. J. Syst. Evol. Microbiol. 51, 731–735.

Humber, R.A., 1997. Fungi: identification. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, San Diego, California, pp. 153–186.

Jacas, J.A., Dembilio, O., Llacer, E., 2011. Research activities focused on management of red palm weevil at the UJI-IVIA associated unit (region of Valencia, Spain). Bull. OEPP/EPPO Bull. 41, 122–127.

Kaplan, E.L., Meier, P., 1958. Nonparametric estimation from incomplete observations. I. Am. Statist. Assn. 53, 457–481.

Kepler, R.M., Rehner, S.A., 2013. Genome-assisted development of nuclear intergenic sequence markers for entomopathogenic fungi of the *Metarhizium anisopliae* species complex. Mol. Ecol. Resour. 13, 210–217.

Kryukov, V.Yu., Yaroslavtseva, O.N., Elisaphenko, E.A., Mitkovets, P.V., Lednev, G.R., Duisembekov, B.A., Zakian, S.M., Glupov, V.V., 2012. Change in the temperature preferences of *Beauveria bassiana* sensu lato isolates in the Latitude Gradient of Siberia and Kazakhstan. Microbiology 81, 453–459.

Lacey, L.A., Kirk, A.A., Millar, L., Mereadier, G., Vidal, C., 1999. Ovicidal and larvicidal activity of conidia and blastospores of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae) with a description of a bioassay system allowing prolonged survival of control insects. Biocontrol Sci. Technol. 9, 9–18.

Llácer, E., Jacas, J.A., 2010. Short communication. Efficacy of phosphine as a fumigant against *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) in palms. Span. J. Agric. Res. 8, 775–779.

Lopes, R.B., Mesquita, A.L.M., Tigano, M.S., Souza, D.A., Martins, I., Faria, M., 2013. Diversity of indigenous *Beauveria* and *Metarhizium* spp. in a commercial banana field and their virulence toward Cosmopolites sordidus (Coleoptera: Curculionidae). Fungal Ecol 6, 356–364.

Luz, C., Fargues, J., 1997. Temperature and moisture requirements for conidial germination of an isolate of *Beauveria bassiana*, pathogenic to *Rhodnius prolixus*. Mycopathologia 138, 117–125.

Mahmoud, F.M., Krimi, Z., Maciá-Vicente, J.G., Errahmani, M.B., Lopez-Llorca, L.V., 2017. Endophytic fungi associated with roots of date palm (*Phoenix dactylifera*) in coastal dunes. Revista Iberoamericana de Micología 34, 116–120.

Martín, M.M., Cabello, T., 2006. Manejo de la cría del picudo rojo de la palmera, *Rhynchophorus ferrrugineus* (Olivier, 1790) (Coleoptera, Dryophthoridae), en dieta artificial y efectos en su biometría y biología. Boletín de Sanidad Vegetal de Plagas 32, 631–641.

Meyling, N.V., Eilenberg, J., 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. Biol. Control 43, 145–155.

Murphy, S.T., Briscoe, B.R., 1999. The red palm weevil as an alien invasive: biology and the prospects for biological control as a component of IPM. Biocontrol. News Inform. 20, 35–46.

Nardi, S., Ricci, E., Lozzi, R., Marozzi, F., Ladurner, E., Chiabrando, F., Granchelli, L., Verdolini, E., Isidoro, N., Riolo, P., 2011. Control of *Rhynchophorus ferrugineus* (Olivier, 1790) according to EU Decision 2007/365/EC in the Marche region (Central-Eastern Italy). EPPO Bull. 41, 103–115.

O'Donnell, K., Cigelnik, E., Niremberg, H.I., 1998. Molecular systematics and phylogeography of the *Giberella fujikuroi* species complex. Mycologia 90, 465–493.

OJEU, 2009. Directive 2009/128/ED of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides. Off. J. Eur. Union L 309, 71–86.

OJEU, 2013. Comission Implementing Regulation (EU) n° 485/2013 (2013) Implementing Regulation (EU) No 540/2011, as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds treated with plant protection products containing those active substances. Off. J. Eur. Union L 139, 12–26.

Ormond, E.L., Thomas, A.P.M., Pugh, P.J.A., Pell, J.K., Roy, H.E., 2010. A fungal pathogen in time and space: the population dynamics of *Beauveria bassiana* in a conifer forest. FEMS Microbiol. Ecol. 74, 146–154.

Ortega-García, L., Tabone, E., Beaudoin-Ollivier, L., Ment, D., Buradino, M., Jaques, J.A., Garrido-Jurado, I., Dembilio, O., Quesada-Moraga, E., 2017. Natural Enemies of *Rhynchophorus ferrugineus* and *Paysandisia archon*. In: Soroker, V., Colazza, S. (Eds.), Handbook of Major Palm Pests: Biology and Management. John Wiley & Sons Ltd., United Kingdom, pp. 171–186.

Ouedraogo, A., Fargues, J., Goettel, M.S., Lomer, C.J., 1997. Effect of temperature on vegetative growth among isolates of *Metarhizium anisopliae* and *M. flavoviride*. Mycopathologia 137, 37–43.

Palmer, M., Moffat, C., Saranzewa, N., Harvey, J., Wright, G.A., Connolly, C.N., 2013. Cholinergic pesticides cause mushroom body neuronal inactivation in honeybees. Nat. Commun. 4, 1634.

Page, R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Paul, N.D., Rasanayagam, S., Moody, S.A., Hatcher, P.E., Ayres, P.G., 1997. The role of interactions between trophic levels in determining the effects of UV-B on terrestrial ecosystems. Plant Ecol. 128, 296–308.

Quesada-Moraga, E., Santiago-Álvarez, C., 2008. Hongos entomopatógenos. In: Urbaneja, A., Jacas, J. (Eds.), Control biológico de plagas. Phytoma y Publicaciones de la

Universidad Pública de Navarra, Navarra, España, pp. 98–120.
Quesada-Moraga, E., Maranhao, E.A.A., Valverde-García, P., Santiago-Álvarez, C., 2006. Selection of *Beauveria bassiana* isolates for control of the whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* on the basis of their virulence, thermal requirements, and toxicogenic activity. Biol. Control 36, 274–287.
Quesada-Moraga, E., Navas-Cortes, J.A., Maranhao, E.A.A., Ortiz-Urquiza, A., Santiago-Alvarez, C., 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. Mycol. Res. 111, 947–966.
Quesada-Moraga, E., Martín-Carballo, I., Garrido-Jurado, I., Santiago-Álvarez, C., 2008. Horizontal transmission of *Metarhizium anisopliae* among laboratory populations of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Biol. Control 47, 115–124.
Quesada-Moraga, E., Muñoz-Ledesma, F.J., Santiago-Álvarez, C., 2009. Systemic protection of the opium poppy, *Papaver somniferum* L., against *Iraella luteipes* (Hymenoptera; Cynipedae) by an endophytic strain of *Beauveria bassiana* (Ascomycota:Hypocreales). Environ. Entomol. 38, 723–730.
Quesada-Moraga, E., Santos-Quirós, R., Valverde-García, P., Santiago-Álvarez, C., 2004. Virulence, horizontal transmission, and sublethal reproductive effects of *Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). J. Invertebr. Pathol. 87, 51–58.
Raeder, U., Broda, P., 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1, 17–20.
Rehner, S.A., Posada, F., Buckley, E.P., Infante, F., Castillo, A., Vega, F.E., 2006. Phylogenetic origins of African and Neotropical *Beauveria bassiana* s.l. pathogens of the coffee berry borer, *Hypothenemus hampei*. J. Invertebr. Pathol. 93, 11–21.
Rehner, S.A., Minnis, A.M., Sung, G.H., Luangsa-ard, J.J., Devotto, L., Humber, R.A., 2011. Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. Mycologia 103, 1055–1073.
Reid, S., Moran, H., 2009. Palm borer *Paysandisia archon*. Plant Health Fact Sheet. The Food and Environment Research Agency, UK www.defra.gov.uk/fera.
Resquín-Romero, G., Garrido-jurado, I., Delso, C., Ríos-moreno, A., Quesada-moraga, E., 2016. Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. J. Invertebr. Pathol. 136, 23–31.
Ricaño, J., Guerri-Agullo, B., Serna-Sarrias, M.J., Rubio-Llorca, G., Asensio, L., Barranco, P., Lopez-Llorca, L.V., 2013. Evaluation of the pathogenicity of multiple isolates of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) for the assessment of a solid formulation under simulated field conditions. Fla. Entomol. 96, 1311–1324.
Roberts, D.W., Campbell, A.S., 1977. Stability of entomopathogenic fungi. Misc. Publ. Entomol. Soc. Amer. 10, 19–76.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
Sarto i Monteys, V., Aguilar, L., 2005. The Castniid Palm Borer, *Paysandisia archon* (Burmeister, 1880) in Europe: Comparative biology, pest status and possible control methods (Lepidoptera: Castniidae). Nach Entomol Ver Apollo NF.26, pp. 61–94.
Sarto i Monteys, V., 2013. *Paysandisia archon* (Castniidae): Description, biological cycle, behaviour, host plants, symptoms and damages. AFPP – Palm Pest Mediterranean Conference. Nice – 16, 17 and 18 January 2013.
Sewify, G.H., Belal, M.H., Al-Awash, S.A., 2009. Use of the Entomopathogenic Fungus, *Beauveria bassiana* for the Biological Control of the Red Palm Weevil, *Rhynchophorus ferrugineus* Olivier. Egypt J. Biol. Pest Control 19, 157.
Shaiju-Simon Kumar, R.K., Gokulapalan, C., 2003. Occurrence of *Beauveria* sp. on red palm weevil, *Rhynchophorus ferrugineus* (Oliv.) of coconut. Insect Environ. 9, 66–67.
Steel, R.G.D., Torrie, J.H., 1985. Bioestadística: Principios y Procedimientos. McGraw-Hill, Bogotá, Colombia.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Tefera, T., Pringle, K.L., 2003. Food consumption by *Chilo partellus* (Lepidoptera: Pyralidae) larvae infected with *Beauveria bassiana* and *Metarhizium anisopliae* and effects of feeding natural versus artificial diets on mortality and mycosis. J. Invertebr. Pathol. 84, 220–225.
Thompson, J.D., Gibso, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. (Online) 25, 4876–4882.
Thomas, M.B., Blanford, S., Lomer, C.J., 1997. Reduction of feeding by the variegated grasshopper, *Zonocerus variegatus*, following infection by the fungal pathogen *Metarhizium flavoviride*. Biocontrol Sci. Technol. 3, 327–334.
Tkaczuk, C., Kontodimas, D.C., Martinou, A., Mantzoukas, S. and Giannouri, D., 2009. Isolation of entomopathogenic fungi from different habitats in Greece using Galeria bait method, semiselective media and sampling cadavers. In: Proceeding of 13th Pan-Hellenic Entomological Congress, Alexandroupoli, 3–6 November 2009, pp. 251–255.
Vega, F.E., Meyling, N.V., Luangsa-ard, J., Blackwell, M., 2012. Fungal entomopathogens. In: Vega, F.E., Kaya, H.K. (Eds.), Insect Pathology, second edition. Academic Press, San Diego, pp. 171–220.
Vidal, C., Lacey, L.A., Fargues, J., 1997. Pathogenicity of *Paecilomyces fumosoroseus* (*Deuteromycotina*: *Hyphomycetes*) against *Bemisia argentifolii* (*Homoptera*: *Aleyrodidae*) with a description of a bioassay method. J. Econ. Entomol. 90, 765–772.
Walstad, J.D., Anderson, R.F., Stambaugh, W.J., 1970. Effects of environmental conditions on two species of muscardine fungi (*Beauveria bassiana* and *Metarhizium anisopliae*). J. Invertebr. Pathol. 16, 221–226.
Wang, C., Li, Z., Typas, M.A., Butt, T.M., 2003. Nuclear large subunit rDNA group I intron distribution in a population of *Beauveria bassiana* strains: phylogenetic implications. Mycol. Res. 107, 1189–1200.
Wang, S., Miao, X., Zhao, W., Huang, B., Fan, M., Li, Z., Huang, Y., 2005. Genetic diversity and population structure among strains of the entomopathogenic fungus, *Beauveria bassiana,* as revealed by inter-simple sequence repeats (ISSR) Mycol. Res. 109, 1364–1372.
Wang, C., Feng, M.G., 2014. Advances in fundamental and applied studies in China of fungal biocontrol agents for use against arthropod pests. Biol. Control 68, 129–135.
Wattanapongsiri, A., 1966. A revision of the genera *Rhynchophorus* and *Dynamis* (Coleoptera: Curculionidae). Department of Agriculture Science Bulletin, Bangkok, Thailand.
Xie, X.Q., Li, F., Ying, S.H., Feng, M.G., 2012. Additive Contributions of Two Manganese-Cored Superoxide Dismutases (MnSODs) to Antioxidation, UV Tolerance and Virulence of *Beauveria bassiana*. PLoS ONE 7, e30298.
Yeo, H., Pell, J.K., Alderson, P.G., Clark, S.J., Pye, B.J., 2003. Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species. Pest Manage. Sci. 59, 156–165.