Fungal Ecology 60 (2022) 101190

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Comparative gene expression and genomics reflect geographical divergence in the plant symbiotic and entomopathogenic fungal genus *Metarhizium*

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ARTICLE INFO

Corresponding Editor: Duur Aanen

Keywords:
Metarhizium
Phylogenomic
Adhesin
Mad1
Mad2
Gene expression

ABSTRACT

Several species within the fungal genus *Metarhizium* can both infect insects and colonize plant roots. In Brazil, a specific subgroup within *Metarhizium anisopliae* s.str. named "subclade Mani 2" is frequently observed infecting above-ground insects, whereas sympatric *M. robertsii* and *M. brunneum* predominantly occur in the soil environment. Genotypic variability within the genus may be linked to adaptations to these different habitats. We present a comparative analysis of the complete genomes and the adhesin genes *Mad1* and *Mad2* of 14 *Metarhizium* isolates representing *M. anisopliae* Mani 2 (n = 6), *M. robertsii* (n = 5) and *M. brunneum* (n = 3). In addition, the relative gene expression of six selected target genes was compared in root exudate solution and insect cuticle suspension. We hypothesized that *M. anisopliae* Mani 2 is adapted to insect-pathogenicity in the above-ground environment, reflected by higher relative expression of pathogenicity-related genes. In contrast, *M. robertsii* and *M. brunneum* are adapted to the soil environment, hence hypothesized to have a higher expression of genes related to plant associations. Phylogenomic and adhesin phylogenetic trees revealed species differences but also intraspecific variability associated with the geographic origin of isolates. Differences in relative gene expression were observed, with one pathogenicity-related gene (*Pr1*) being higher expressed in *M. anisopliae*. The insect adhesion *Mad1* gene was more conserved than the plant adhesion *Mad2* and similarly expressed in exudate solution, while *Mad2* was highly expressed by all Brazilian isolates in both exudate and cuticle conditions. The variabilities observed correlated with different habitats and lifestyles, demonstrating the importance of selecting a diverse collection of isolates in genomic and gene expression studies.

1. Introduction

Entomopathogenic fungi in the genus *Metarhizium* (Ascomycota: Hypocreales) can naturally infect and kill insects and are widely used as biological control agents (St. Leger and Wang, 2020). These fungi infect their hosts through the cuticle and present a low risk to natural enemies, other non-host arthropods and vertebrates (Zimmermann, 2007a, 2007b).

Besides infecting insects, many entomopathogenic fungi of the order Hypocreales can associate intimately with plants in the rhizosphere or inside tissues as endophytes (Vega, 2008; Vega et al., 2008). The plant association of *Metarhizium* spp. was first reported for *Metarhizium anisopliae* by Hu and St. Leger (2002), who coined *M. anisopliae* as rhizosphere competent, and later other species of *Metarhizium* were reported to possess the ability to colonize plants (Wyrebek et al., 2011; Behie et al., 2015). During the interaction with plant roots, it has been demonstrated that *Metarhizium* can supply nitrogen derived from infected insects in the soil to the plants and, in return, obtain carbohydrates exuded from the plant roots (Behie et al., 2012, 2017; Behie and Bidochka, 2014; Barelli et al., 2016). The fungus can also promote plant growth (Sasan and Bidochka, 2012; Jaber and Enkerli, 2016a, 2016b) and reduce above-ground herbivore populations feeding on fungal colonized plants (Canassa et al., 2020).

The fungal species *M. anisopliae*, *Metarhizium robertsii* and *Metarhizium brunneum* can generally be found in soils of natural and managed ecosystems worldwide (Lacey et al., 2015). Although the three species can function as both entomopathogens and plant associates, an essential distinction regarding their ecological role in Brazil is emerging. While

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https://doi.org/10.1016/j.funeco.2022.101190
Received 2 September 2021; Received in revised form 25 July 2022; Accepted 27 July 2022
Available online 20 August 2022

M. anisopliae is very abundant in the Cerrado biome in Brazil (Rocha et al., 2013), phylogenetic data of the 5' EF-1α region of isolates from different areas of Brazil showed that *M. anisopliae* s.str. can be split into three subclades, namely Mani 1, Mani 2 and Mani 3, with isolates of Mani 2 usually being the only representatives infecting insects above ground under field conditions, and Mani 1 and Mani 3 only found in the soil (Rezende et al., 2015). This indicates that Mani 2 isolates are mainly adapted for utilization of insects as resources. Overall, *M. robertsii* is the most prevalent *Metarhizium* species in Brazil, widely present in native and agricultural soils of different biomes throughout the country (Botelho et al., 2019). Under laboratory conditions, isolates of *M. robertsii* can effectively kill various species of insects, yet natural infections of insects by *M. robertsii* are rarely observed in Brazil (Lopes et al., 2013). Instead, *M. robertsii* is frequently reported to form associations with plant roots, indicating that isolates of this species are primarily adapted to activities in the soil environment, including root colonization (Sasan and Bidochka, 2012; Rezende et al., 2015). While *M. brunneum* is abundant in agricultural soils in temperate regions of Europe, the species is rarely isolated in Brazil (Steinwender et al., 2014, 2015; Botelho et al., 2019; Brunner-Mendoza et al., 2019). Similar to *M. robertsii*, *M. brunneum* is mainly associated with the soil environment and, at least in temperate regions, is rarely observed infecting insects above ground in field conditions (Meyling et al., 2011; Steinwender et al., 2014, 2015).

The dual lifestyle of *Metarhizium* spp. exploiting both insects and plants as host organisms is possible partly because of adhesion proteins specific to different hosts. Wang et al. (2005) detected a high frequency of two expressed sequence tags (ESTs), later categorized as the genes *Mad1* (*Metarhizium* adhesin-like protein 1) and *Mad2* (*Metarhizium* adhesin-like protein 2), when an isolate of *M. anisopliae* was grown in culture medium containing hemolymph of *Manduca sexta* or bean root exudate, respectively. Experiments with knockout mutants of *M. anisopliae* and mutant yeasts verified the role of these two genes in the adhesion of conidia to either insect or plant host surfaces and, in consequence, the ability of these fungi to infect insects or to associate with plants (Wang and St. Leger, 2007).

While genes (and their encoded proteins) involved in the endophytic ability of *Metarhizium* species are still mostly unknown (Branine et al., 2019), the functions of proteins relevant in the insect pathogenicity process, such as subtilisins and hydrophobins, are well characterized (St. Leger et al., 1988a; Goettel et al., 1989; Small and Bidochka, 2005; Sevim et al., 2012). The protein Pr1A, a subtilisin-like serine protease member of the family Pr1 (Freimoser et al., 2003; Bagga et al., 2004), is the main protein produced during penetration of the host cuticle and is a determinant factor of pathogenicity against insects (Goettel et al., 1989; St. Leger et al., 1988a; 1989). The gene *Pr1A* is upregulated under nutrient-deprived conditions and downregulated in nutrient-rich conditions, such as in the insect hemolymph (St. Leger et al., 1988b; 1989; Small and Bidochka, 2005).

Hydrophobins are amphiphile proteins exclusive to the kingdom Fungi, with an important role related to surface forces (Linder et al., 2005; Bayry et al., 2012). Hydrophobins form rodlet layers which cover fungal aerial structures, such as conidia, rendering their surface hydrophobic, and in *Metarhizium* spp., these proteins have a role in adherence to the host cuticle, together with MAD1 (St. Leger et al., 1992a; St. Leger and Wang, 2020).

Studies of gene expression and genomics allow us to understand how genes are regulated depending on the growth conditions of an organism and to identify relevant intra- and interspecific variations in the genomes of individuals. A comparative genomic study showed that *M. robertsii* (reported as *M. anisopliae* at the time) encodes more subtilisins, trypsins, chitinases, dehydrogenases and cytochrome P450s than *Metarhizium acridum*, which was related to their presumed lifestyles: *M. robertsii* is a generalist species with a broad host range, thus the higher number of these proteins in the genome allows this species to deal with multiple types of hosts (Gao et al., 2011). On the other hand, the genome of *M. acridum* contains a reduced number of proteins involved in cuticle degradation and detoxification due to its limited host range as a specialist pathogen of locusts (Gao et al., 2011). Investigating differential gene expression of *M. robertsii* under several conditions, Barelli et al. (2011) reported that *M. robertsii* up- or downregulated the expression of *Mad1*, *Mad2* and other selected genes according to the composition, temperature and pH of the culture medium.

Genomic studies have spearheaded the identification of relevant protein families (and their quantity) in the genus *Metarhizium* (St. Leger and Wang, 2020). Suitable examples include: cytochrome P450s, which are involved in the general metabolism of organisms, including detoxification, and are also related to the production of secondary metabolites and pathogenicity in fungi (Chen et al., 2014); MRT, a raffinose transporter fundamental for the rhizosphere competency of *M. robertsii* (Fang and St. Leger, 2010); and the modular enzymes polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) (Dutta et al., 2014; Miller and Gulick, 2016), involved in the production of secondary metabolites which may act during infection in hosts or against other microorganisms (Zimmermann, 2007a; Molnár et al., 2010).

However, species of the genus *Metarhizium* exhibit significant genotypic variation, and even isolates of the same species show genetic variability, potentially linked to their adaptation to different habitats (Bidochka et al., 2001). Nevertheless, much of the current knowledge about genomic variation and gene expression within and between *Metarhizium* species are based on studies of a limited number of isolates (*M. robertsii* ARSEF 23 and ARSEF 2575, *M. anisopliae* ARSEF 549, *M. acridum* CQMa 102). Therefore, it may not be straightforward to extrapolate results obtained from a single isolate to the population level or conclude about adaptations of particular species. For example, Wyrebek and Bidochka (2013) showed that *Mad2* had diverged more than *Mad1* among *Metarhizium* spp. when studying 14 isolates mostly of North American origin. The authors suggested that this divergence represented selection for plant association rather than for insect hosts as a main adaptive trait within individual species. If so, we should expect *M. anisopliae* Mani 2, which potentially is more adapted to explore insect resources, to have more variation in *Mad1*, while Brazilian isolates of *M. brunneum* and *M. robertsii*, which are considered to be more adapted to the soil/rhizosphere environment, would exhibit higher variation in *Mad2*, similar to what Wyrebek and Bidochka (2013) reported. Although these authors found low intraspecific variability for both *Mad1* and *Mad2* in *M. brunneum* and *M. robertsii*, a limited number of isolates was tested (two and five, respectively), and all but one had the same origin (Ontario, Canada). The higher variation found for *Mad1* and *Mad2* in *M. guizhouense* (two isolates from Ontario and one from China) indicates that sampling from different locations may reveal intraspecific variation not yet reported (Wyrebek and Bidochka, 2013).

In the present study, we therefore selected twelve isolates of Brazilian origin belonging to *M. anisopliae* Mani 2 (n = 6), *M. brunneum* (n = 3) and *M. robertsii* (n = 3), and compared their genomes and specific gene sequences against two well-described reference isolates of *M. robertsii* from the USA. In addition, the expression of selected genes for the 14 isolates was evaluated. First, we sequenced the genomes of all isolates to provide comparative information on their genetic distances and abundance of selected protein families between and within the three species. Then, because of their relevance in the adhesion of conidia to host surfaces, we sanger-sequenced the coding sequences of genes *Mad1* and *Mad2* to verify their genetic sequences and assess the variation among all isolates through the construction of gene phylogenies. Finally, the variation in expression of six target genes was evaluated after inoculating the isolates into a root exudate solution or an insect cuticle suspension. We hypothesized that: (i) pathogenicity-related genes would be highly expressed in *M. anisopliae* Mani 2 compared to *M. brunneum* and *M. robertsii* since isolates of Mani 2 are more frequently isolated from insects in field conditions; (ii) the expression of an endophytism-related gene would be higher in *M. brunneum* and *M. robertsii* than in *M. anisopliae* Mani 2, as they are usually associated to plants in the soil; and (iii) the expression of constitutive genes would be similar in the

three species. Knowledge of variation in genome composition and gene expression provides insight into the adaptation of entomopathogenic fungi to their environment, identification of potential constraints in the application of fungal isolates for biological control, as well as for the identification of target genes that could be used for future bioengineering approaches.

2. Materials and methods

2.1. Fungal isolates

Fourteen isolates of *Metarhizium* spp. (six of *M. anisopliae* subclade Mani 2, five of *M. robertsii* and three of *M. brunneum*) were selected: 12 from the Entomopathogen Collection "Prof. Sérgio Batista Alves", of the Laboratory of Pathology and Microbial Control of Insects (Luiz de Queiroz College of Agriculture – University of São Paulo, Piracicaba, SP, Brazil), and two from the USDA Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA) (Table 1, Fig. 1). Isolates have previously been identified by Rezende et al. (2015) (*M. robertsii* ESALQ 1426 and ESALQ 1635, all isolates of *M. anisopliae*), Iwanicki et al. (2019) (*M. robertsii* ESALQ 5168, all isolates of *M. brunneum*), and by Bischoff et al. (2009) and Gao et al. (2011) (ARSEF 23, ARSEF 2575).

2.2. Whole-genome Re-sequencing

2.2.1. DNA extraction and sequencing

Isolates were cultivated in culture media Sabouraud Dextrose Agar (SDA) for 7–10 d, and then conidia were harvested and inoculated in Erlenmeyer flasks containing 20 mL of YPD broth (0.2% yeast extract, 2% peptone, 3% dextrose), which were incubated on a rotary shaker for three days (0.4 *g*, 20–23 °C). The resulting fungal material was vacuum filtered and lyophilized overnight.

The fresh, dried material was crushed into a powder and put in Eppendorf tubes, in which 500 μL of CTAB buffer, 1 μL of 2-mercaptoethanol, 3 μL of RNase (diluted to 10 mg ml^{-1}) and 3 μL of proteinase K were added per sample, then the tubes were placed in a heat block at 60 °C for 1 h. Next, 500 μL of phenol/chloroform/isoamyl alcohol (25:24:1) were added to each sample, shaking the tubes slightly and centrifuging at 11,363 *g* and 4 °C for 10 min. Approximately 400 μL (2 x 200 μL) of the aqueous upper phase were transferred to new tubes, in which 500 μL of chloroform/isoamyl alcohol (24:1) were added, followed by centrifugation at 11,363 *g* and 4 °C for 10 min. After this, 300 μL of isopropanol were added to the samples, which were mixed and put in a freezer (−20 °C) overnight. The following day, samples were centrifuged at 11,363 *g* and 4 °C for 10 min before supernatants were discarded, and the pellets were washed with 500 μL of absolute ethanol and centrifuged at 11,363 *g* and 4 °C for 10 min. The ethanol was discarded, and the samples were washed twice with 70% ethanol. The tubes were air-dried, and lastly, DNA was dissolved in 40–50 μL of 1xTE buffer (pre-heated at 60 °C). The material was quantified on a Qubit 4 (Thermo Fisher Scientific) and sent to BGI Europe A/S (Copenhagen, Denmark), which performed short insert fragment library preparation and PE150 sequencing on BGISEQ (DNBseq technology), as well as quality control (removing adaptors and low-quality readings). Raw reads are deposited in the Sequence Read Archives, under the BioProject PRJNA746571, and their accession numbers are shown in Supplemental Table S1.

Table 1
List of isolates used in the study. ESALQ isolates were obtained from the Entomopathogen Collection "Prof. Sérgio Batista Alves" (ESALQ/USP, Piracicaba, São Paulo, Brazil), and ARSEF isolates from the USDA Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA).

Species	Isolate code	Origin	Collection site (state, country)
Metarhizium anisopliae s.str. subclade Mani 2	ESALQ 43	Hemiptera: Cercopidae	Alagoas, Brazil
	ESALQ 1116	Coleoptera: Scarabaeidae	São Paulo, Brazil
	ESALQ 1641	Hemiptera: Cercopidae	Alagoas, Brazil
	ESALQ 1076	Meadow soil	Paraná, Brazil
	ESALQ 1175	Meadow soil	São Paulo, Brazil
	ESALQ 1604	Biotech G, Biotech® Controle Biológico (commercial isolate)	–
Metarhizium brunneum	ESALQ 5022	Sugarcane soil	São Paulo, Brazil
	ESALQ 5286	Sugarcane soil	São Paulo, Brazil
	ESALQ 5181	Sugarcane root	São Paulo, Brazil
Metarhizium robertsii	ESALQ 1426	Soybean soil	Paraná, Brazil
	ESALQ 1635	Native forest soil	Alagoas, Brazil
	ESALQ 5168	Coleoptera: Scarabaeidae	São Paulo, Brazil
	ARSEF 23	Coleoptera: Elateridae	North Carolina, USA
	ARSEF 2575	Coleoptera: Curculionidae	South Carolina, USA

2.2.2. Mapping of the reads

The reference genomes selected for mapping were ARSEF 23 for *M. robertsii* (GenBank accession number ADNJ00000000), ARSEF 549 for *M. anisopliae* (GenBank accession number AZNF00000000) and ARSEF 3297 for *M. brunneum* (GenBank accession number AZNG00000000). Alignment of reads to the reference genomes was performed using BWA version 0.7.17, applying the algorithm BWA-MEM on paired-end mode (Li and Durbin, 2009; Li, 2013). SAMtools version 1.11 was used to sort and remove duplicate reads (Li et al., 2009), and the quality of mapped reads was assessed with Qualimap version 2.2.2 (Okonechnikov et al., 2016). A consensus sequence for each isolate was obtained using RGAAT version 1.0 (Liu et al., 2018), and genome sizes were determined by assembly-stats version 1.0.1 (https://github.com/sanger-pathogens/assembly-stats). Estimation of genome completeness was assessed using BUSCO version 4.1.3 (Seppey et al., 2019), selecting the dataset for Hypocreales version odb10.2019-11-20 as a reference.

2.2.3. Annotation

Genomes were annotated with AUGUSTUS version 3.3.3, trained with the reference genomes aforementioned (Stanke and Morgenstern, 2005; Hoff and Stanke, 2013). Functional annotation of proteins was performed using InterProScan 5.46–81.0, running the Pfam analysis (Mitchell et al., 2019).

2.2.4. Assessment of orthologues and phylogenomic analysis

Orthology inference was performed using OrthoFinder version 2.3.12 (Emms and Kelly, 2019). A maximum-likelihood phylogenomic tree was constructed with the sequences of the orthogroups, utilizing the software IQ-TREE version 2.0.3 (Nguyen et al., 2015) implementing the substitution model JTT + F + R5, determined as the best-fit by ModelFinder (option -m MFP) (Kalyaanamoorthy et al., 2017), based on AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion), and with branch supports using the ultrafast bootstrap (Hoang et al., 2018) with 1,000 bootstrap replicates. The *M. acridum* isolate CQMa 102 was used as an outgroup. FigTree version 1.4.4 (https://github.com/rambaut/figtree/releases) was used for editing the tree.

2.2.5. Comparison between genome alignments

Whole genome pairwise comparisons were performed using D-

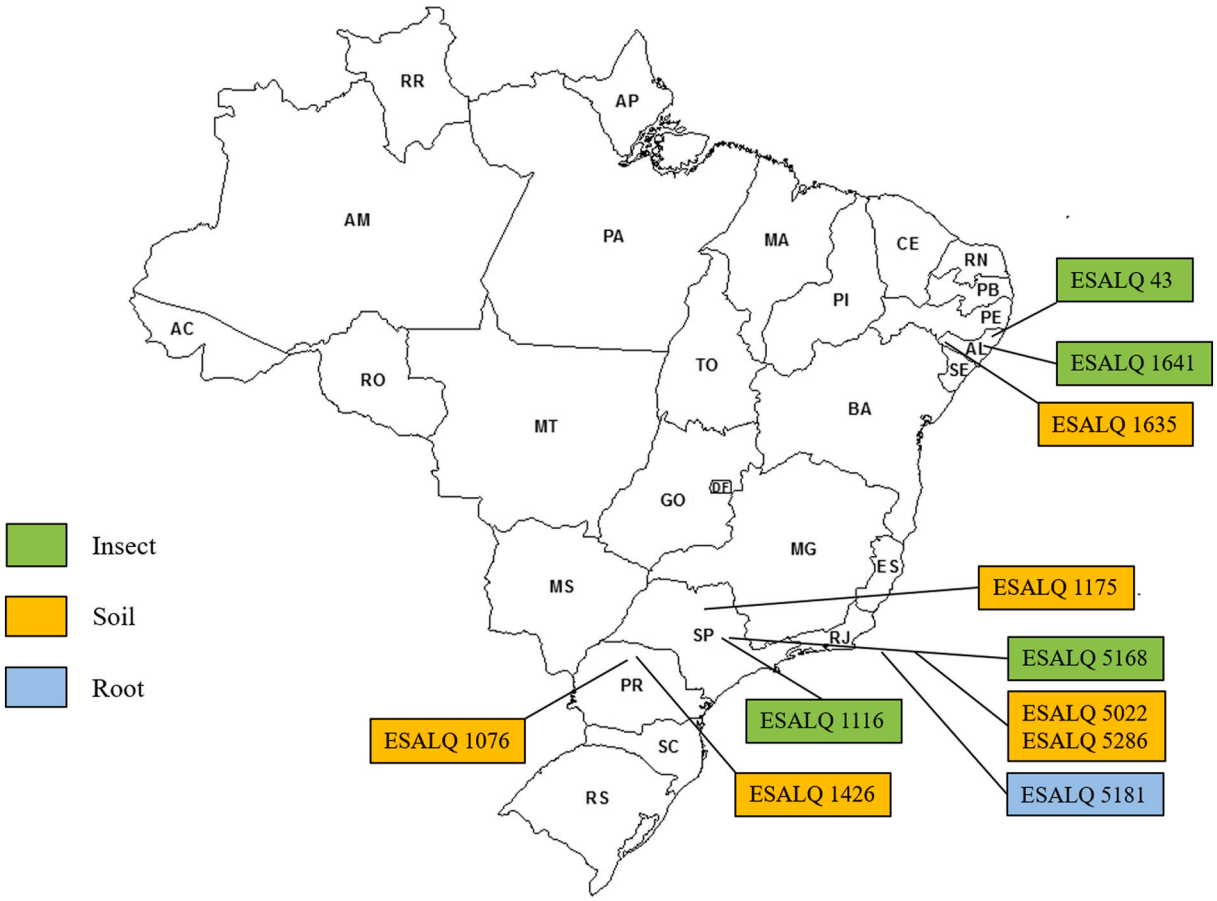

Fig. 1. Location of the collection sites in Brazil of ESALQ isolates of *Metarhizium anisopliae* s.str. Mani 2 (ESALQ 43, ESALQ 1076, ESALQ 1116, ESALQ 1175 and ESALQ 1641), *M. brunneum* (ESALQ 5022, ESALQ 5181 and ESALQ 5286) and *M. robertsii* (ESALQ 1426, ESALQ 1635 and ESALQ 5168) used in the present study. Green boxes indicate isolates obtained from insect hosts, orange boxes indicate isolates obtained from soil samples, and the blue box indicates isolate obtained from root sample. Isolate ESALQ 1604 (*M. anisopliae*) is a commercial isolate with an unknown collection site, thus it is not represented in the map.

GENIES (Cabanettes and Klopp, 2018). Genomes were aligned using minimap2 (Li, 2018). The resulting pairwise mapping format (PAF) file was parsed and plotted into dot-plots generated by D-GENIES, sorting the contigs of one isolate along the sequence of the other isolate.

2.3. Coding sequences of genes Mad1 and Mad2

Fungal pellets were obtained as described under 2.2.1. Small pellets of each isolate were put in Eppendorf tubes and crushed into a powder. Next, 500 μL of CTAB mixture (500 μL of CTAB buffer + 1 μL of 2-mercaptoethanol per sample; pre-heated in a water bath at 60 °C) were added to the tubes, which were shaken and placed in a heat block at 60 °C for 1 h (slightly shaking them each 15 min). After this, 500 μL of chloroform/isoamyl alcohol (24:1) were added, and the tubes were centrifuged at 11,363 *g* and 4 °C for 10 min. Then, 350 μL of the aqueous upper phase of each sample were transferred to new Eppendorf tubes containing 1 μL of RNase (stock: 100 mg mL^{-1}) and incubated in a heating cabinet at 37 °C for 30 min, after which 210 μL of isopropanol were added, with the samples being mixed and placed in a freezer (−20 °C) overnight. The following day, the samples were centrifuged at 11,363 *g* and 4 °C for 10 min so that the DNA would stick to the bottom of the tubes. The supernatants were discarded, and 500 μL of 70% ethanol were added to the samples, which were again centrifuged at 11,363 *g* and 4 °C for 10 min. The ethanol was discarded, the material was rinsed twice with 70% ethanol, and the tubes were air-dried. Lastly, 100 μL of 1xTE buffer were added to dissolve the DNA. Quantification of the material was performed on 0.8% agarose gel and on mySPEC (VWR).

For all 14 isolates, polymerase chain reactions (PCR) were performed for the coding sequences (CDS) of insect adhesin gene *Mad1* and plant adhesin gene *Mad2*. Primers were designed using CLC Main Workbench 8.1 (QIAGEN). Reactions were conducted in a thermocycler (T100, Bio-Rad Laboratories) and performed in volumes of 51 μL (*Mad2*) or 56 μL (*Mad1*), consisting of: 37.5 μL of Milli-Q water, 5 μL of 10X DreamTaq buffer (Fisher Scientific), 2 μL of dNTP 2.5 μM, 0.5 μL of DreamTaq DNA polymerase (Fisher Scientific), 2.5 μL of each forward and reverse primers (TAG Copenhagen A/S) and 1 μL of genomic DNA (ca. 25 ng). For *Mad1*, Mad1_F3 (5′-CCTGACATCCAACAACACACT) and Mad1_R3 (5′-CGCCGCAGCTCAATTCAT) were used as end primers in PCR reactions, while Mad1_F4i (5′-AGCAGACCACTCCCAGCAA) and Mad1_R4i (5′-AGGCAGAATAACAGTCGTAGGT) were used as internal primers for sanger sequencing. A one-step PCR was performed with the following conditions: 10 min at 94 °C for initial denaturation; 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 60 °C and extension for 3 min at 72 °C; and 10 min at 72 °C for the final extension. For *Mad2*, primers used were Mad2_F2 (5′-CGTCCACTCTTTTTCACATT) and Mad2_R2 (5′-GGATATATGCTGTGCGGT), and PCR conditions were: 1 min at 94 °C for initial denaturation; 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 58 °C and extension for 3 min at 72 °C; and 10 min at 72 °C for the final extension. The presence of PCR products was evaluated on 0.8% agarose gel. Samples were purified using illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) according to the manufacturer's instructions and sequenced by Macrogen Inc. (Amsterdam, the Netherlands).

CLC Main Workbench 8.1 was used to edit and assemble the sequences using the default settings. The sequences were deposited in GenBank and their accession numbers are shown in Supplemental

Table S1. For each gene, multiple alignments of the 14 CDS sequences were constructed in CLC Main Workbench 8.1, including the reference sequence of *M. robertsii* ARSEF 2575 [(accession numbers DQ338437 (*Mad1*) and DQ338439 (*Mad2*)]. Maximum likelihood phylogenetic trees were constructed with MEGA X (Kumar et al., 2018), implementing the nucleotide substitution model HKY + G for both genes, based on the AIC score of the optimal substitution model, with 1,000 bootstrap replicates. The phylogenies also included nine other isolates representing five species of *Metarhizium* (accession numbers can be found in Supplemental Table S2).

2.4. Analysis of in vitro gene expression

2.4.1. Preparation of fungal growth substrates

Wheat seeds were surface sterilized in a 4% sodium hypochlorite solution for 2 h, washed three times in sterile distilled water and placed in Petri dishes containing YPD agar (0.2% yeast extract, 1% peptone, 2% dextrose, 1.5% agar) to germinate. Four days later, germinated seeds were transferred to Schott glass bottles (Sigma-Aldrich) filled with 50 mL of sterile distilled water and placed in a rotary shaker at 0.05 *g* and 20–23 °C for 4 d. After this period, the material was passed through a filter membrane (Puradisc FP 30 Cellulose Acetate Syringe Filter 0.2 μm, GE Healthcare Life Sciences) and freeze-dried. The resulting dried root exudate was diluted in sterile distilled water to produce a 1% v/v solution and then stocked at −4 °C. The sterility of the solution was assessed by plating five samples (200 μL) in Sabouraud Dextrose Agar and incubating at 26 °C for 7 d.

Insect cuticle was obtained by cutting off the wings, hind legs and pronotum of freeze-killed locusts (*Locusta migratoria*). The cuticle sections were grinded under liquid nitrogen, mixed with sterile distilled water to produce a 1% w/v suspension and stocked at −4 °C.

2.4.2. Sample preparation and RNA extraction

Isolates were grown in Petri dishes containing one-quarter strength Sabouraud dextrose agar plus yeast extract (SDAY/4: 0.25% peptone, 0.25% yeast extract, 1% dextrose, 2% agar) for 10 d (26 °C, 12 h photophase), after which inoculum of each isolate was prepared (2 mL; $1x10^8$ conidia mL^{-1}) and applied in Erlenmeyer flasks containing 200 mL of YPD broth. Culture flasks were placed on a rotary shaker for 4 d at 0.45 *g* and 20–23 °C. The resulting fungal material was vacuum filtered and divided into portions of 1 g wet weight, which were inoculated in flasks containing 50 mL of the substrates (root exudate solution or insect cuticle suspension), and then placed on a rotary shaker at 0.2 *g* and 20–23 °C. There was one flask per treatment (isolate plus substrate). After 24 h, cultures were vacuum filtered. The fungal material was collected for RNA extraction, performed using RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions with the following changes: (i) after crushing the material, 450 μL of buffer RLT and 5 μL of 2-mercaptoethanol were added and samples were shaken; (ii) 500 μL of 25:24:1 phenol/chloroform/isoamyl alcohol were added to the samples, which were shaken and centrifuged at 18,395 *g* and 4 °C for 5 min; (iii) after transferring 450 μL of the supernatants to new tubes, 450 μL of 24:1 chloroform/isoamyl alcohol were added to the samples, and they were centrifuged again at 18,395 *g* and 4 °C for 5 min; (iv) 400 μL of the supernatants were transferred to the lilac spin columns of the kit, and the procedure continued according to the manufacturer's instructions. Quantification of RNA was performed on mySPEC. To conduct reverse transcriptions, tubes of each isolate containing 200 ng μL^{-1} of RNA were prepared (total volume: 10 μL), and then 0.8 μL of 100 mM Oligo-dT solution (5′-TTT TTT TTT TTT TTT TTT-3′) and 0.8 μL of 100 mM random hexamers (5′-NNN NNN-Wobble-3′) were added. The samples were incubated at 70 °C for 5 min and put on ice right after. Next, a master mix (total volume = 4.2 μL) composed of 3 μL of M-MLV RT-Buffer 5X (Promega), 0.8 μL of 10 mM dNTPs and 0.4 μL of M-MLV Revertase (Promega) were added to each tube. Reactions were vortexed and incubated at 42 °C for 1 h and then at 72 °C for 15 min. All procedures were repeated three times as independent replicates.

2.4.3. Relative quantification of gene expression

For each combination of fungal isolate + gene + substrate, nine qPCR reactions (three for each flask of each independent replicate) were performed. Table 2 contains the list of target and reference genes evaluated, primers used and annealing temperatures. Target genes were selected for their known relevance for either insect or plant association, based on Pava-Ripoll et al. (2011) and Barelli et al. (2011). Genes *18S rRNA*, *gpd* and *try* were selected as reference genes, based on Fang and Bidochka (2006). Primers for *18S rRNA* and EST AJ274118 were obtained from Barelli et al. (2011), primers for *gpd* and *try* were obtained from Fang and Bidochka (2006), and the remaining primers were designed using CLC Main Workbench 8.1 or the Primer-BLAST Tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/); in this case, before designing the primers, sequences corresponding to *Pr1A*, *hyd1* and *rib* were downloaded from GenBank (accession codes presented in Supplemental Table S3) and aligned, using the default settings of CLC Main Workbench 8.1, to obtain consensus sequences for each gene. Reactions were performed in volumes of 25 μL, consisting of 9.5 μL of nuclease-free water, 12.50 μL of SYBR Green, 0.5 μL of each primer and 2 μL of cDNA. qPCR conditions were: 95 °C for 15 min; 40 cycles of 95 °C for 30 s, annealing temperature (depending on the target gene) for 30 s and 72 °C for 1 min. An additional cycle of 95 °C for 1 min, 50 °C for 30 s, and 95 °C for 30 s was performed to generate dissociation curves to address the specificity of the primers.

Gene expression was analyzed using relative quantification, which quantifies the expression of a target gene in a particular condition relative to its expression in another condition. Expression results are thus shown as fold changes between conditions for a given gene. Before the analysis, linear regression tests [(pcr_test('lm'); α = 5%) from the "pcr" package (Ahmed and Kim, 2018)] were performed between isolates of the same species (due to large genomic and expressional differences, *M. robertsii* isolates were separated into two groups ESALQ, from Brazil, and ARSEF, from the USA), and data were combined after we did not find intraspecific differences between isolates (or intragroup differences, in the case of *M. robertsii*). Data for the target genes were normalized against the geometric mean of the values obtained for the three reference genes. Analyses were performed applying the ΔΔCt method and the linear regression test (pcr_test('lm'); α = 5%) (Ahmed and Kim, 2018) in R (R Core Team, 2020). Comparisons between species were relative to the species group with the lowest expression level for a given target gene.

2.5. Phylogenies of Pr1A, hyd1 and rib

Consensus sequences for *Pr1A*, *hyd1* and *rib* were obtained in the same manner described in section 2.4.3. Then, BLAST searches were performed against the orthogroups defined by OrthoFinder (section 2.2.4.), and matching sequences with >90% of similarity were retrieved. Multiple alignments for each gene were performed using the algorithm ClustalW with default options in MEGA X (Kumar et al., 2018), and phylogenetic trees were constructed implementing the amino acid substitutions models WAG (*Pr1A*), Dayhoff (*hyd1*) and LG (*rib*), based on AIC and BIC, with 1,000 bootstrap replicates.

2.6. Genetic diversity

Estimates of genetic diversity were calculated for *Mad1*, *Mad2*, *Pr1A*, *hyd1* and *rib*. For each gene, the amino acid sequences of the 14 isolates were aligned using ClustalW with default options, and the overall mean genetic distances were calculated using the "Distance" module of MEGA X (Kumar et al., 2018), implementing the Poisson correction model and treating gaps as complete deletions, with 1,000 bootstrap replicates.

To identify if differences in the sequences would result in changes in functional domains or active sites, the amino acid sequences of the 14

Table 2
Target and reference genes used to evaluate differential expression among the selected isolates of *Metarhizium* spp., with their respective forward and reverse primers, annealing temperatures (Ta; °C) and amplicon sizes (bp) for qPCR reactions.

Target genes			
Gene or EST	Primers (5' – 3')	Ta (°C)	Amplicon size (bp)
Metarhizium adhesin-like protein 1 (*Mad1*; insect adhesin)	F: GGGTCATCTACCCCAAGCAG R: GAGGTGCTAGGCAGTGTGAG	60	142
Metarhizium adhesin-like protein 2 (*Mad2*; plant adhesin)	F: GTCAAGCTTCCCCTTGGCAT R: CAGTCGCAAGGGTGGACATA	56	160
Subtilisin-like protease (*Pr1A)*	F: CCATTGGTAGCAAAAGCTACGG R: CGGGTCTTGGAGTCACTGG	60	136
Ribosomal protein (*rib*)	F: GCCTACCTGCGAGTTTCCTT R: CTTGGAGACACCGAACTGCT	60	183
Hydrophobin-like protein (*hyd1*)	F: GCAACAAAGTGGCGCAAAAC R: GTCTGCGAGGTGCATTTGTC	60	121
[a] EST AJ274118 – unknown product (constitutive)	F: GGGGGTTTGATTATGTGGTTGGTATTAGCA R: TAACTTCAGTCGTGCGTGCCATTTCTAC	57	100
Reference genes			
[a] *18S rRNA*	F: AGGCCCGGGTAATCTTGT R: GACCTTGTTACGACTTTTACTTCCTCT	60	266
[b] Glyceraldehyde 3-phosphate dehydrogenase (*gpd*)	F: GACTGCCCGCATTGAGAAG R: AGATGGAGGAGTTGGTGTTG	60	149
[b] Tryptophan biosynthesis enzyme (*try*)	F: TTGCAATGCATGTTTGATGTC R: CAAAGAGTGGTATCGAGTTAC	60	147

[a] Primer sequences obtained from Barelli et al. (2011).
[b] Primer sequences obtained from Fang and Bidochka (2006).

isolates were scanned for matches against the InterPro protein database (available at: http://www.ebi.ac.uk/interpro/search/sequence/). Identified amino acid substitutions were assessed for their placement within or matching domains or active sites.

3. Results

3.1. WGR

3.1.1. General features and phylogenomic tree

Genome sizes varied among the species: 38.5 Mb for *M. anisopliae*, 37.0 Mb for *M. brunneum,* and 41.6 Mb for *M. robertsii*. According to BUSCO, genomes were estimated to be, on average, 98% complete. The BUSCO output for each isolate is shown in Supplemental Table S4.

A total of 153,128 genes were identified across the 14 isolates, with 153,011 (99.9%) being assigned to 11,999 orthogroups; of these, 9,648 contained genes from all 14 isolates, and 9,497 consisted entirely of single-copy genes. The number of predicted genes was 10,854–10,859 for *M. anisopliae*, 11,203–11,223 for *M. robertsii,* and 10,636–10,641 for *M. brunneum*. Supplemental Table S5 contains general statistics for each isolate obtained from OrthoFinder. A phylogenomic analysis confirmed that isolates of each species grouped in the same clade (Fig. 2).

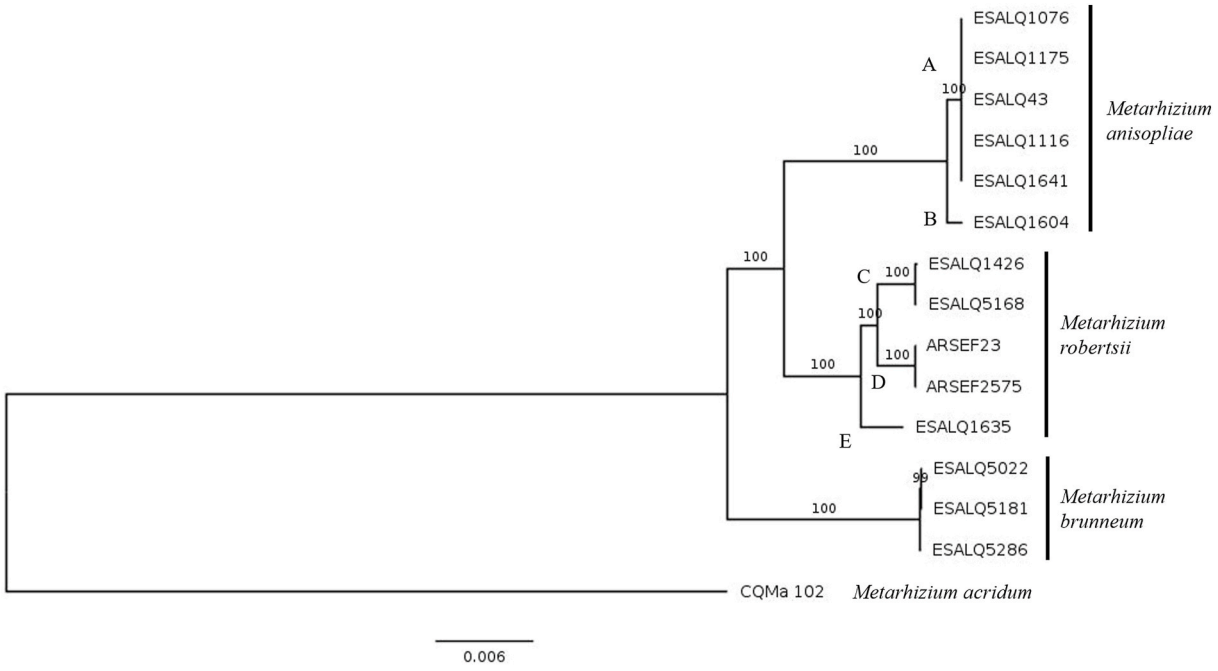

Fig. 2. Phylogenomic tree based on a concatenated multiple alignments of 7,848 amino acid sequences, showing the relationship between fourteen isolates of *Metarhizium anisopliae* s.str. Mani 2, *M. robertsii* and *M. brunneum* (Table 1). *Metarhizium acridum* CQMa 102 was used as an outgroup. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site. Two clades were identified for *M. anisopliae* (clades A and B), and three clades for *M. robertsii* (clades C, D and E).

Subgroups identified within *M. robertsii* and *M. anisopliae* were designated A-E for further comparative analyses.

3.1.2. Relevant protein families

The number of cytochrome P450s was higher in isolates of *M. robertsii* (128 or 129) compared to isolates of *M. anisopliae* (122) and *M. brunneum* (126) (Supplemental Table S6). Our analysis also showed that isolates of *M. anisopliae* had a higher number of oligosaccharide transporters (70) compared to *M. brunneum* (68) and *M. robertsii* (67–69) (Supplemental Table S6).

Proteins involved in fungal pathogenicity to insects include hydrophobins, subtilisins and trypsins. Out of these three protein families, isolates of *M. robertsii* had the highest numbers of subtilisins (52) and trypsins (30–33). Few hydrophobins were identified (3–4), with isolates of *M. anisopliae* having one protein more than isolates of *M. brunneum* and *M. robertsii* (Supplemental Table S6).

The numbers of domains of NRPS and PKS were conserved within species, while variability was found between species (Supplemental Table S7). This was also the case of the domains of the NRPS destruxin synthetase dtxs1, involved in the biosynthesis of destruxins (Supplemental Table S8).

3.1.3. Whole genome similarity comparison

Dot-plots comparing pairs of isolates of the same species showed the same percentages of similarity (for *M. anisopliae*: 99.76% of contigs with similarity >75%; for *M. brunneum*: 99.93% of contigs with similarity >75%; for *M. robertsii*: 93.74% of contigs with similarity >75%), thus only one isolate of each species is shown in the interspecific comparisons (Fig. 3). Alignments indicated higher similarity between *M. anisopliae* and *M. robertsii* (62.43% of contigs with similarity >75%, 27.13% contigs with similarity between 50 and 75%, 9.94% with no match), compared to the similarity between *M. anisopliae* and *M. brunneum* (21.44% contigs with similarity >75%, 66.14% contigs with similarity between 50 and 75%, and 11.56% contigs with no match) or between *M. brunneum* and *M. robertsii* (43.23% contigs with similarity >75%, 34.06% contigs with similarity between 50 and 75%, 22.36% contigs with no match). Some degree of rearrangement of chromosomes can be visualized between species as shifted non-linear sections of dot-plots (Fig. 3D–F), while the intraspecific comparisons represent almost complete diagonal lines (Fig. 3A–C).

3.2. Coding sequences of Mad1 and Mad2

The six isolates of *M. anisopliae* had the shortest CDS of *Mad1*, 2,097 bp (698 a.a.), and they all shared the same sequence, except for a single bp difference in ESALQ 1604. The length of CDS of *Mad1* for the three isolates of *M. brunneum* was 2,136 bp (711 a.a.), and no bp differences were found. For *M. robertsii*, the length of CDS of *Mad1* was 2,154 bp (717 a.a.) for ESALQ 1635, ARSEF 23 and ARSEF 2575, and 2,169 bp (722 a.a.) for ESALQ 1426 and ESALQ 5168. The sequence of ESALQ 1635 was more similar to the sequences of the two ARSEF isolates (8 bp differences to ARSEF isolates vs 11 bp differences + 15 deletions to ESALQ 1426/5168). The maximum-likelihood phylogenetic tree (Fig. 4) shows that all 14 isolates used in the present study grouped with their respective species, although in separate branches (in the case of *M. brunneum* and *M. robertsii*). A BLAST search against the NCBI database confirmed that the translated sequences matched the protein MAD1 (queries cover = 99%, percentages of identity >99.31%, E-values = 0.0).

The lengths of *Mad2* were comparable among species: 921 bp (306 a.a.) for *M. anisopliae* and *M. robertsii*, and 915 bp (304 a.a.) for *M. brunneum*. Isolates of *M. anisopliae* and *M. brunneum* from Brazil had the same intraspecific sequences, but the two *M. brunneum* isolates studied by Wyrebek and Bidochka (2013) clustered in a separate clade far from their Brazilian conspecifics. Regarding *M. robertsii*, a single bp difference was found between the Brazilian isolates ESALQ 1635 and ESALQ

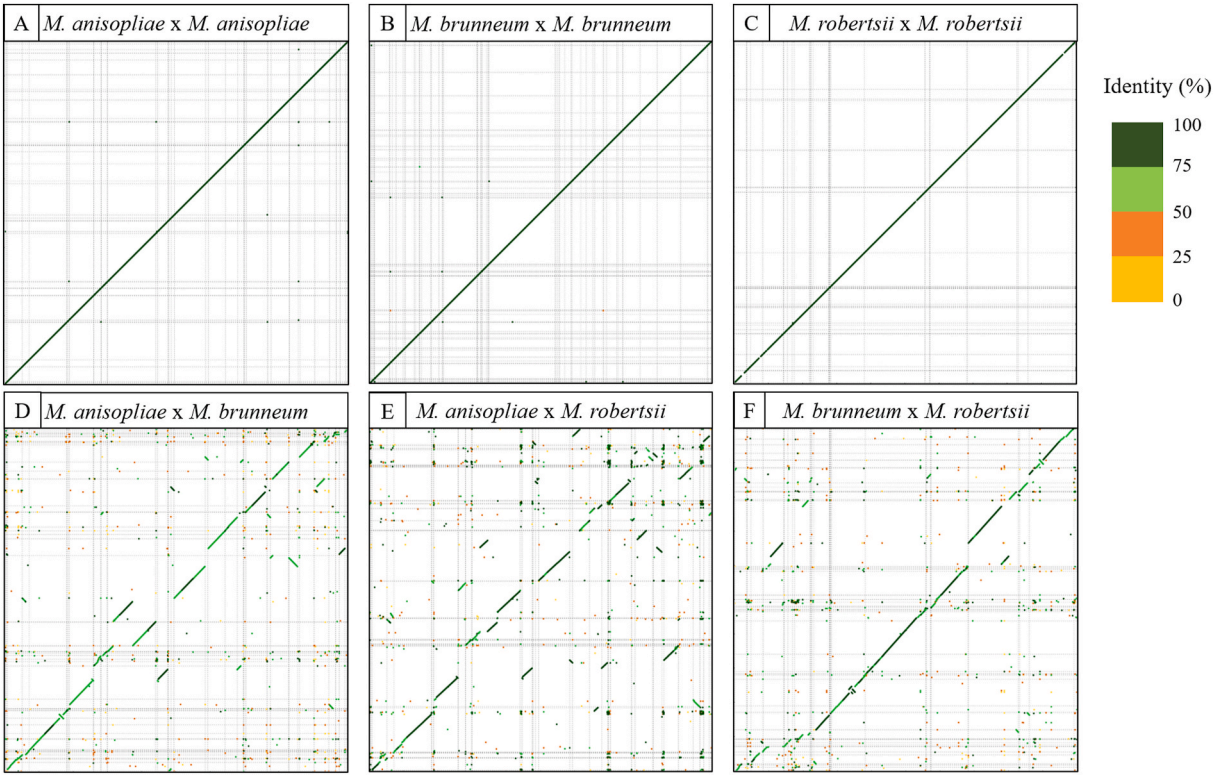

Fig. 3. Whole genome pairwise comparison between isolates of *Metarhizium anisopliae* s.str. Mani 2 (A), *M. brunneum* (B), *M. robertsii* (C), *M. anisopliae* Mani 2 x *M. brunneum* (D), *M. anisopliae* Mani 2 x *M. robertsii* (E), and *M. brunneum* x *M. robertsii* (F). Diagonal lines represent matches (identical sequences), gaps or inversions. The colors on the plots correspond to the percentage of similarity between regions.

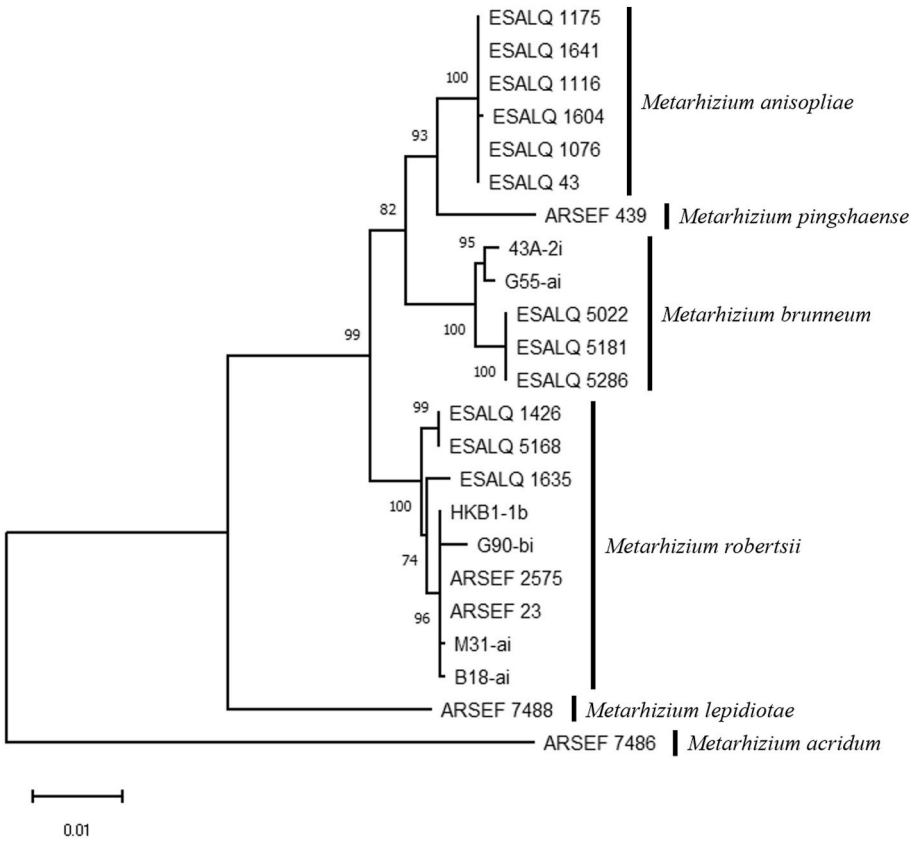

Fig. 4. Maximum-likelihood phylogenetic tree of coding sequences of *Mad1* for *Metarhizium* species. It includes 14 isolates from this study (ESALQ and ARSEF; GenBank accession numbers in Supplemental Table S1) and 9 obtained from GenBank [from Wyrebek and Bidochka (2013); accession numbers in Supplemental Table S2]. Alignment had a length of 2, 211 bp. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site.

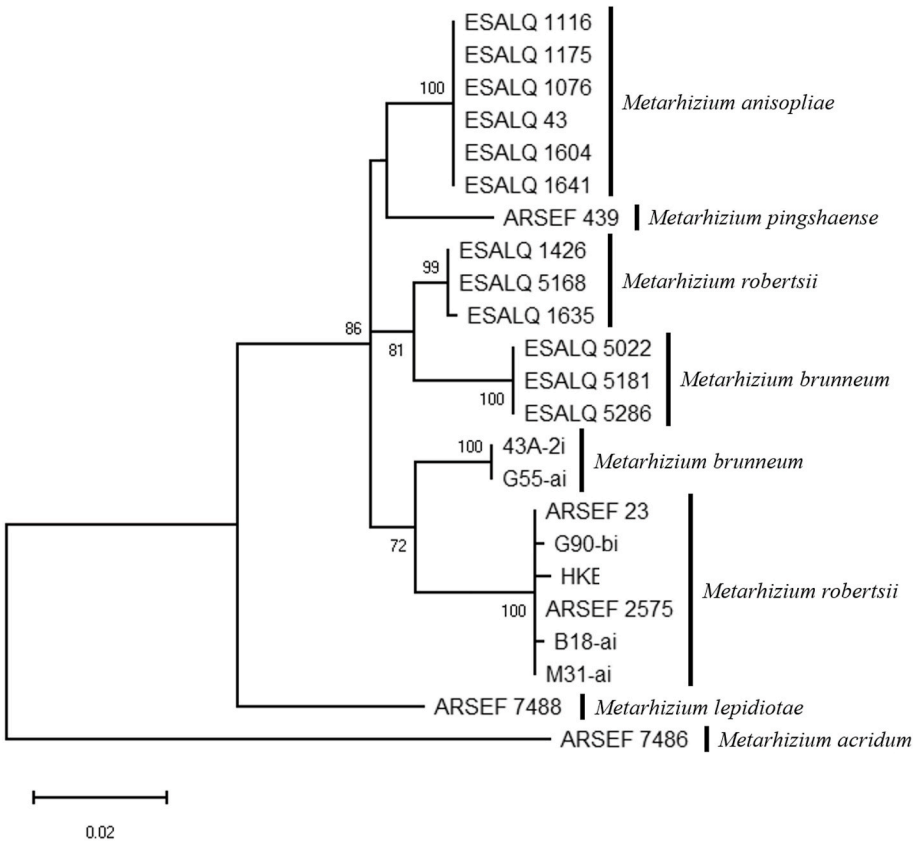

Fig. 5. Maximum likelihood phylogenetic tree of coding sequences of *Mad2* for *Metarhizium* species. It includes 14 isolates from this study (ESALQ and ARSEF; GenBank accession numbers in Supplemental Table S1) and 9 obtained from GenBank [from Wyrebek and Bidochka (2013); accession codes in Supplemental Table S2]. Alignment had a length of 930 bp. Bootstrap values, based on 1000 replicates, are shown in the nodes. Branch lengths represent the number of substitutions per site.

Table 3
Estimated genetic distances (number of amino acid substitutions per site) for five target genes among 14 isolates of *Metarhizium* representing three species (*Metarhizium anisopliae* s.str. Mani 2, *M. brunneum*, and *M. robertsii*). Analyses were conducted using the Poisson correction model. Intraspecific genetic distances between isolates of *M. brunneum* (n = 3) were estimated as zero for all genes, and all isolates were therefore combined. *Metarhizium anisopliae* isolates were divided in two clades based on the phylogenomic tree in Fig. 2 (clade A, n = 5; clade B, n = 1), and similarly *M. robertsii* isolates were separated in three clades (clade C, n = 2; clade D, n = 2; clade E, n = 1). Standard error estimates (S.E.) were obtained by 1,000 bootstrap replicates.

Groups	Genes				
	Mad1	Mad2	Pr1A	hyd1	rib
Overall distance	0.012 (0.003)	0.030 (0.006)	0.031 (0.007)	0.348 (0.051)	0.006 (0.003)
M. anisopliae clade A x *M. anisopliae* clade B	0.001 (0.001)	0.000	0.000	0.000	0.000
M. anisopliae clade A x *M. robertsii* clade C	0.013 (0.005)	0.030 (0.010)	0.053 (0.012)	0.500 (0.082)	0.000
M. anisopliae clade A x *M. robertsii* clade D	0.013 (0.005)	0.050 (0.013)	0.053 (0.012)	0.500 (0.082)	0.000
M. anisopliae clade A x *M. robertsii* clade E	0.014 (0.005)	0.030 (0.010)	0.053 (0.012)	0.500 (0.082)	0.000
M. anisopliae clade A x *M. brunneum*	0.019 (0.005)	0.040 (0.012)	0.031 (0.009)	0.599 (0.097)	0.016 (0.009)
M. anisopliae clade B x *M. robertsii* clade C	0.014 (0.005)	0.030 (0.010)	0.053 (0.012)	0.500 (0.082)	0.000
M. anisopliae clade B x *M. robertsii* clade D	0.014 (0.005)	0.050 (0.013)	0.053 (0.012)	0.500 (0.082)	0.000
M. anisopliae clade B x *M. robertsii* clade E	0.016 (0.005)	0.030 (0.010)	0.053 (0.012)	0.500 (0.082)	0.000
M. anisopliae clade B x *M. brunneum*	0.021 (0.005)	0.040 (0.012)	0.031 (0.009)	0.599 (0.097)	0.016 (0.009)
M. robertsii clade C x *M. robertsii* clade D	0.000	0.043 (0.012)	0.000	0.000	0.000
M. robertsii clade C x *M. robertsii* clade E	0.001 (0.001)	0.000	0.000	0.000	0.000
M. robertsii clade C x *M. brunneum*	0.021 (0.006)	0.023 (0.009)	0.045 (0.011)	0.389 (0.074)	0.016 (0.009)
M. robertsii clade D x *M. robertsii* clade E	0.001 (0.001)	0.043 (0.012)	0.000	0.000	0.000
M. robertsii clade D x *M. brunneum*	0.021 (0.006)	0.068 (0.015)	0.045 (0.011)	0.389 (0.074)	0.016 (0.009)
M. robertsii clade E x *M. brunneum*	0.023 (0.006)	0.023 (0.009)	0.045 (0.011)	0.389 (0.074)	0.016 (0.009)

1426/ESALQ 5168, while ARSEF 23 and ARSEF 2575 shared the same sequence and clustered in a separate distant clade with other North American isolates studied by Wyrebek and Bidochka (2013) (Fig. 5). Translated sequences were confirmed to match the protein MAD2 after conducting a BLAST search against the NCBI database (queries cover = 99%, percentages of identity >98%, E-values = 0.0).

3.3. Phylogenies of Pr1A, hyd1 and rib

The sequences of the three genes *Pr1A*, *hyd1* and *rib* were conserved intraspecifically, while interspecific variation was detected. Phylogenies of *Pr1A* and *hyd1* showed that each species was represented on their own branch, with *M. brunneum* being more closely related to *M. anisopliae* for *Pr1A*, and to *M. robertsii* for *hyd1* (Supplemental Figs. S1 and S2, respectively). In the case of *rib*, isolates of *M. anisopliae* clustered with isolates of *M. robertsii*, while isolates of *M. brunneum* were in a separate branch (Supplemental Fig. S3).

3.4. Genetic distance analysis

Comparing the two adhesin genes, the genetic distance for *Mad1* was lower than for *Mad2*, indicating that the degree of divergence is smaller in the insect adhesin (Table 3). Overall, the lowest genetic distance estimated was of the gene *rib*, which encodes a ribosomal protein, and the degree of divergence was a factor of 10 higher for the gene *hyd1*. Table 3 shows the mean genetic distances calculated for the five target genes based on amino acid substitutions per site. It is worth noting that for genes *Mad1* and *Mad2*, we observed genetic distances between groups of conspecific isolates (clade A and B from *M. anisopliae*, and clades C, D and E of *M. robertsii*, determined by the phylogenomic tree in Fig. 2), whereas for genes *Pr1A*, *hyd1* and *rib*, no genetic distances between conspecifics were observed.

Scanning the amino acid sequences against the InterPro protein database, we found regions of MAD1 and PR1A matching domains and active sites of the database. Among the amino acid differences between the sequences of the 14 isolates, very few changes were detected in domain regions and no changes were observed within active sites (Supplemental Table S9).

3.5. In vitro gene expression in root exudate and cuticle infused media

No differences between the species were found in the relative expression of *Mad1* in root exudate solution ($P > 0.05$ for all pairwise comparisons; Fig. 6A). Analysis in cuticle condition showed that the group of two *M. robertsii* ARSEF isolates had significantly higher relative expression compared to the group of six *M. anisopliae* isolates (2.49-fold, $P = 0.011$), while there were no significant differences regarding the other comparisons ($P > 0.05$ for all pairwise comparisons; Supplemental Table S10).

Expression of *Mad2* was similar for the groups of *M. anisopliae* and *M. robertsii* ESALQ isolates in both exudate (Fig. 6B; $P = 0.306$) and cuticle conditions (Supplemental Table S11; $P = 0.433$), while being significantly higher than the expression in the groups of *M. brunneum* and *M. robertsii* ARSEF isolates (>200-fold, $P < 0.001$ for pairwise comparisons between these species and *M. anisopliae/M. robertsii* ESALQ in both conditions); between groups of *M. brunneum* and *M. robertsii* ARSEF isolates, the former showed higher expression than the latter (>40-fold, $P < 0.001$ in both conditions).

The group of six isolates of *M. anisopliae* had significantly higher expression of *Pr1A* compared to the other three groups of *Metarhizium*

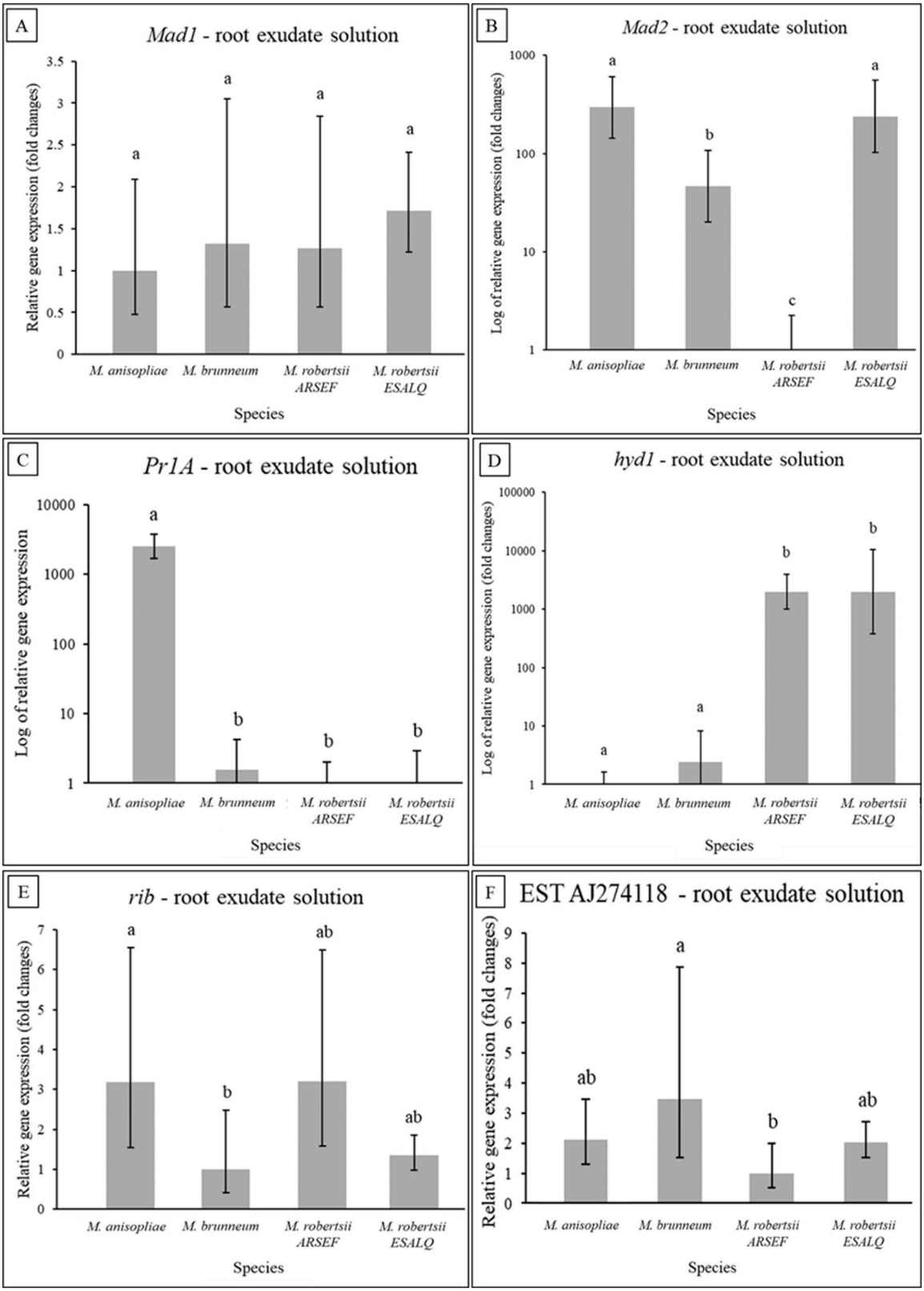

Fig. 6. Relative gene expression (in fold changes) of six target genes for isolates of *Metarhizium anisopliae* s.str. Mani 2 (n = 6), *M. brunneum* (n = 3) and *M. robertsii* (ESALQ, n = 3, and ARSEF, n = 2) in 1% v/v root exudate solution using the double delta Ct method. (A) Gene *Mad1*, expressions are relative to that of *M. anisopliae*; (B) gene *Mad2*, expressions are relative to that of *M. robertsii* ARSEF; (C) gene *Pr1A*, expressions are relative to that of *M. robertsii* ESALQ; (D) gene *hyd1*, expressions are relative to that of *M. anisopliae*; (E) gene *rib*, expressions are relative to that of *M. brunneum*; (F) EST AJ274118, expressions are relative to that of *M. robertsii* ARSEF. For each gene, different letters indicate significant differences between groups [linear regression test (pcr_test('lm'), from the R-package "pcr"), $P < 0.05$]. Plot bars represent the standard deviation.

spp. in both conditions (>2,000-fold, $P < 0.001$ for all pairwise comparisons). Analysis of gene expression in root exudate solution showed no differences between groups of *M. brunneum*, *M. robertsii* ESALQ and *M. robertsii* ARSEF isolates ($P > 0.05$ for all pairwise comparisons; Fig. 6C). In contrast, in insect cuticle suspension, the group of *M. brunneum* isolates exhibited significantly higher expression than *M. robertsii* ESALQ ($P = 0.021$; Supplemental Table S12), while no difference was found between groups of *M. robertsii* ESALQ and ARSEF ($P = 0.113$) or between groups of *M. robertsii* ARSEF and *M. brunneum* ($P = 0.501$).

In both root exudate and insect cuticle conditions, the ARSEF and ESALQ groups of *M. robertsii* showed higher expression of *hyd1* compared to groups of *M. anisopliae* and *M. brunneum* (>1,500-fold, $P < 0.001$ for all pairwise comparisons; Fig. 6D for expression in root exudate, Supplemental Table S13 for insect cuticle). Between the two *M. robertsii* groups, gene expression was similar (root exudate: $P = 0.999$; insect cuticle: $P = 0.539$), as it was between the groups of *M. anisopliae* and *M. brunneum* (root exudate: $P = 0.275$; insect cuticle: $P = 0.786$).

Variation was limited for the gene *rib*, although the group of *M. anisopliae* isolates had significantly higher expression than the group of *M. brunneum* isolates in root exudate solution (3.18-fold, $P = 0.036$), while the other groups did not differ from each other ($P > 0.05$ for all pairwise comparisons; Fig. 6E). In cuticle condition, significant differences were found between *M. brunneum* and both groups of *M. robertsii* (vs *M. robertsii* ESALQ: $P = 0.023$; vs *M. robertsii* ARSEF: $P = 0.018$), whereas other pairwise comparisons did not reveal differences ($P > 0.05$ for all pairwise comparisons; Supplemental Table S14).

Expression of the EST AJ274118 in root exudate solution was higher for the group of *M. brunneum* isolates compared to the group of *M. robertsii* ARSEF isolates (3.45-fold, $P = 0.012$), while there were no differences between the other species ($P > 0.05$ for all pairwise comparisons; Fig. 6F). Analysis of expression in insect cuticle condition showed no differences between the groups ($P > 0.05$ for all pairwise comparisons; Supplemental Table S15).

4. Discussion

Genomic studies usually focus on just one isolate of a determined fungal species (Gao et al., 2011; Hu et al., 2014; Pattemore et al., 2014). Although the data generated by such an approach are relevant to improve knowledge of the genome composition of the particular species, it is not sufficient to account for potential intraspecific variability. Our results demonstrate the existence of intraspecific differences in the number of genes in *Metarhizium*, and it is worth noting that ESALQ isolates of *M. robertsii* had up to 10–20 more genes than ARSEF isolates of *M. robertsii* (Supplemental Table S5). Isolates of *M. robertsii* had the largest genomes (41.6 Mb), and isolates of *M. brunneum* the shortest (37 Mb), similar to previous findings (Hu et al., 2014), and this correlated with the number of genes, in which case isolates of *M. robertsii* also possessed more protein-coding genes than the other two species (Supplemental Table S5). However, we found fewer genes than previously reported (Hu et al., 2014), possibly due to the methods used for mapping and assembly of the genomes.

Hu et al. (2014) stated that, due to their lifestyle, generalist species of *Metarhizium* show an expansion of protein families related to pathogenicity and detoxification compared to specialists. The three species of our study are all considered generalists. We found that the number of the selected protein families (e.g., trypsins, cytochrome P450s) were similar to the ones reported for the same species by the authors mentioned above, supporting their findings of this expansion across multiple isolates.

The genome comparisons showed some chromosome rearrangement between species, and an overall high similarity between genomes. The fungi *Metarhizium anisopliae* and *M. robertsii* had more regions with similarity above 75%, opposed to pairwise comparisons with *M. brunneum*. This is also evident in the phylogenomic tree, in which *M. anisopliae* and *M. robertsii* formed sister groups. This result is in accordance with Hu et al. (2014), who reported *M. brunneum* as a sister species to the clade with the other two species.

The phylogenomic tree reflects a variability not previously reported, especially for *M. robertsii*. The isolates of this species formed a monophyletic group; however, isolate ESALQ 1635 clustered separately from the other isolates of *M. robertsii*, which formed two sister groups, one composed of ARSEF isolates and the other composed of ESALQ 1426 and ESALQ 5168. The phylogenies of adhesin genes *Mad1* and *Mad2* also revealed an important intraspecific variability: in the phylogenetic tree of *Mad1*, isolates of the same species clustered together in the same clade, but the isolates were placed on separate branches within each clade according to their continental origin. In the phylogeny of *Mad2*, Brazilian isolates of *M. brunneum* and *M. robertsii* clustered in individual clades, while North American isolates of the two species were place in other conspecific clades. No divergence was observed within the Brazilian *M. anisopliae* group. All of these results indicate some degree of geographic divergence, potentially reflecting adaptations benefitting survival in different habitats with contrasting environmental conditions.

The analysis of genetic distances showed that *Mad1* diverged less than *Mad2*, whereas analysis of relative expression revealed limited interspecific differences for *Mad1*, but high intra- and interspecific variability for *Mad2*. This might be related to the fact that *Mad1* is primarily conserved in the genus *Metarhizium* (Wyrebek and Bidochka, 2013). The higher divergence of *Mad2* may be related to the hypothesis that plant association has been an important factor in the evolution of *Metarhizium* (Wyrebek and Bidochka, 2013) and that adaptation to new environments is dependent on the habitat and interaction with plants rather than insects (Wang et al., 2011; St. Leger and Wang, 2020). At present, we cannot conclude on potential comparable geographical variation of *Mad2* within *M. anisopliae* based on the current isolate sampling.

We found interspecific variation in the relative expression of the insect pathogenicity-related genes *Pr1A* and *hyd1* in both root exudate solution and cuticle suspension. These two genes are expressed under nutrient-deprived conditions, e.g., formation of appressoria, and it has been previously demonstrated that *hyd1/ssgA* is expressed coordinately with *Pr1* (St. Leger et al., 1989; 1992a, b). Besides functioning during adhesion to (*hyd1*) or penetration of (*Pr1A*) the cuticle, these genes also play a role in other processes, e.g., sporulation (Small and Bidochka, 2005; Sevim et al., 2012). Although we expected that *M. anisopliae* would exhibit the highest relative expression of both genes, this was only observed for *Pr1A*. In contrast, relative expression of *hyd1* was much higher in both groups of *M. robertsii*. In the phylogenies of both genes, *M. anisopliae* and *M. robertsii* grouped in different clades, and the genetic distances show some degree of divergence, especially for *hyd1*. This may indicate, at least under *in vitro* conditions, that *M. anisopliae* and *M. robertsii* have developed differential strategies reflecting their lifestyles: most of the infections in insects above-ground are caused by isolates of *M. anisopliae* Mani 2 (Rezende et al., 2015; Iwanicki et al., 2019), so it seems reasonable that this species invests more in expressing *Pr1A* than the other species groups studied when exposed to an inducing substrate. On the other hand, studies by Fang and Bidochka (2006) and Sevim et al. (2012) reported that, although *hyd1* is expressed in most developmental stages, its expression levels vary. As such, *hyd1* is highly expressed in mycelia and mycelia with conidiophores (but weakly in swollen and germinated conidia), which would be consistent with the presumed niche of *M. robertsii* as a species better adapted to a below-ground lifestyle in the environment. Here, the fungus can form associations with plant roots and potentially transfer insect-derived nitrogen to the plant via mycelia (Behie et al., 2012, 2017; Rezende et al., 2015; Steinwender et al., 2015), which could explain why *M. robertsii* would invest in expressing more *hyd1* than *M. anisopliae*.

Contrary to our hypothesis that expression of constitutive genes would be similar in all groups, there was also interspecific variation in the relative expression of *rib* in both substrates, and of EST AJ274118 in

root exudate solution. However, it is worth noting that the differences were relatively minor, and this observation could be related to the nature of their encoded products (a ribosomal protein and a constitutive product), which may not show significant variability between species. The genetic distance analysis showed that *rib* is a highly conserved gene among the species, and the phylogeny demonstrated a very close relationship among the isolates.

Our study provides new information about the diversification of genomes representing three species of the entomopathogenic fungal genus *Metarhizium*. The detailed phylogenies of the adhesins *Mad1* and *Mad2* and documented variability in the relative expression of the six selected genes further demonstrate both genotypic and functional divergence within *M. robertsii*. The intraspecific variation between isolates from different geographical origins may be related to adaptations to differential environmental factors. Our data emphasize the importance of conducting gene expression coupled with genomic analyses on a diversity of fungal isolates to capture the natural variability within this group of entomopathogenic fungi.

Author contributions

Conceptualization: J.C.C., I.D.J., H.H.F.L. and N.V.M.; methodology: J.C.C., I.D.J., H.H.F.L. and N.V.M.; formal analysis: J.C.C.; investigation: J.C.C.; data curation: J.C.C.; writing—original draft preparation: J.C.C.; writing—review and editing: J.C.C., I.D.J., H.H.F.L. and N.V.M.; supervision: I.D.J., H.H.F.L. and N.V.M. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the São Paulo Research Foundation (FAPESP), grant numbers 2017/20491–0 and 2018/13282–8, and financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. H.H.D.F.L was supported by an Independent Research Fund Denmark Sapere Aude Grant (grant number 8049-00086B) and a Young Investigator Grant from Carlsberg Foundation (grant number CF20-0609).

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

J.C.C. would like to thank Didde Hedegaard Sørensen for technical support in the laboratory and Dinah Maran Parker for adjusting a protocol and extracting fungal DNA for the genomic analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2022.101190.

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