Plant rhizosphere specificity and variability in the insect and plant adhesins, *Mad1* and *Mad2*, within the genus *Metarhizium* suggest plant adaptation as an evolutionary force

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Abstract

Metarhizium is a soil-inhabiting fungus currently used as a biological control agent against various insect species, and research efforts are typically focused on its ability to kill insects. In section 1, we tested the hypothesis that species of *Metarhizium* are not randomly distributed in soils but show plant rhizosphere-specific associations. Results indicated an association of three *Metarhizium* species (*Metarhizium robertsii*, *M. brunneum* and *M. guizhouense*) with the rhizosphere of certain types of plant species. *M. robertsii* was the only species that was found associated with grass roots, suggesting a possible exclusion of *M. brunneum* and *M. guizhouense*, which was supported by *in vitro* experiments with grass root exudate. *M. guizhouense* and *M. brunneum* only associated with wildflower rhizosphere when co-occurring with *M. robertsii*. With the exception of these co-occurrences, *M. guizhouense* was found to associate exclusively with the rhizosphere of tree species, while *M. brunneum* was found to associate exclusively with the rhizosphere of shrubs and trees. These associations demonstrate that different species of *Metarhizium* associate with specific plant types.

In section 2, we explored the variation in the insect adhesin, *Mad1*, and the plant adhesin, *Mad2*, in fourteen isolates of *Metarhizium* representing seven different species. Analysis of the transcriptional elements within the *Mad2* promoter region revealed variable STRE, PDS, degenerative TATA box, and TATA box-like regions. Phylogenetic analysis of 5' EF-1α, which is used for species identification, as well as *Mad1* and *Mad2* sequences demonstrated that the *Mad2* phylogeny is more congruent with 5' EF-1α than *Mad1*. This suggests *Mad2* has diverged among *Metarhizium* lineages, contributing to clade- and species-specific variation. While other abiotic and biotic factors cannot be excluded in contributing to divergence, it appears that plant associations have been the driving factor causing divergence among *Metarhizium* species.

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1.1. Introduction

Metarhizium is an Ascomycete fungus, within the order Hypocreales. It is of the family Clavicipitaceae, within the anamorphic genera of fungi that have no known sexual cycle (Spatafora et al., 2007; Biscoff et al., 2009). The teleomorph of *Metarhizium* species appear to be members of the *Metacordyceps* genus (Sung et al., 2007). *Metarhizium* is an entomopathogenic fungi, with a broad host range. Insect infection takes place through infection structures known as an appressorium and penetration peg, which allow penetration of the insect cuticle and entry into the insect hemolymph (St. Leger, 1993). Inside the insect hemolymph, toxins and nutrient depletion contribute to host death. When nutrients become depleted, the fungus emerges and conidiates on the surface of the insect cadaver.

Traditionally, *Metarhizium* is viewed as an insect pathogen, and has been used as a biological control agent against various insect pests (Lomer *et al.*, 1997; 2001; Milner & Pereire, 2000; Hunter *et al.*, 2001; Maniania *et al.*, 2003; Shah & Pell, 2003). Commercial formulations of *Metarhizium* also exist, such as Green Muscle. *Metarhizium anisopliae*, the type species of the genus, has also been shown to be effective against mosquitoes, with estimations that applications could reduce the intensity of malaria transmission by 75% (Scholte et al., 2005; Kanzok and Jacobs-Lorena, 2006).

While chemical pesticides are fast and effective ways of controlling pests on agriculturally important crops, there are environmental and health issues associated with their use (Hoppin et al. 2002; Huang et al. 2003). Biological control agents offer an environmentally friendly and safe means of crop protection. However, most current biocontrol agents are slow acting in comparison to pesticides, costly to maintain, and have a short shelf life (Bailey et al.,

2010). Also, while *Metarhizium* has been used as a biocontrol agent, results have been inconsistent and the efficacy unclear (St. Leger and Screen, 2001). Clearly, further research is required in order to exploit *Metarhizium* successfully and effectively in an agricultural setting.

1.2. Rhizosphere Competence

One of the first studies involving *Metarhizium* and plants tested the effects of epicuticular waxes and leaf extracts of the family Cruciferae (now Brassicaceae) on the germination and virulence of *Metarhizium anisopliae* conidia (Inyang et al., 1999). Surface leachates and soluble extracts of leaves demonstrated the ability to stimulate germination more than yeast or starch extract. Formulation of conidia in leachates or leaf extracts was also shown to increase the virulence of *M. anisopliae* to the mustard beetle, *Phaedon cochleariae*. Interestingly, germination also increased on insect cuticle that were pretreated with leaf extracts.

Using a recombinant isolate of *M. anisopliae*, field studies revealed that it is rhizosphere competent (Hu and St. Leger, 2002). In order to be identified as rhizosphere competent, a microorganism must have the ability to colonize and persist in the rhizosphere, which is the zone of soil within a few millimetres of the plant root (Schmidt, 1979). In nonrhizosphere soil, *M. anisopliae* propagules decreased from 10^5 propagules/g to 10^3 propagules/g after several months. However, in rhizosphere soil propagules persisted at 10^5 propagules/g, identifying rhizospheric soils as a potential reservoir. Similarly, it has been found that *M. anisopliae* colonizes the rhizosphere of *Picea abies* in a soilless potting media, with the fungal population in the rhizosphere significantly greater than the bulk media surroundings (Bruck, 2005). This study also found that inoculation of plant roots with *M. anisopliae* was able to protect plants from a target insect. *P. abies* roots inoculated with *M. anisopliae* infected 76% of 2nd to 3rd instar black vine weevil (Bruck, 2005).

In addition to being rhizosphere competent, *Metarhizium robertsii* has also recently been found to be an endophyte that stimulates plant root development (Sasan and Bidochka, 2012). While initial exposure to *Metarhizium* increased root length, root hair density, and the number of lateral roots emerged, longer term associations (60 days) showed that *Metarhizium* had endophytically colonized cortical cells of haricot bean root. Other insect biocontrol agents within the Hypocreales order, such as *Trichoderma*, *Beauveria*, and *Lecanicillium* have also been shown to be endophytes (Ownley *et al.*, 2010).

Differential gene expression by *M. anisopliae* growing in bean root exudate, insect cuticle, and insect hemolymph demonstrated physiological adaptation to these different environments throughout the lifecycle of *M. anisopliae* (Wang et al., 2005). Expression patterns from insect cuticle and hemolymph clustered separately from those in root exudate, demonstrating differences in transcriptional control during the insect pathogenic and soil dwelling phases. This analysis also identified two very high frequency contigs, one in insect hemolymph and one in root exudate, of unknown function.

Characterization of these genes revealed that they encode insect cuticle and plant specific adhesins (Wang and St. Leger, 2007). *Metarhizium* adhesin-like protein 1 (*Mad1*) and *Metarhizium* adhesin-like protein 2 (*Mad2*) were shown to enable attachment to insect cuticle and plants, respectively. Adherence assays demonstrated that disruption of *Mad1* eliminated the ability to adhere to locust cuticle. Also, yeast cells expressing *Mad1*, but not *Mad2*, were able to adhere to locust cuticle. Likewise, a *Mad2* disrupted mutant showed the inability to adhere to onion epidermis, while yeast cells expressing only *Mad2* were able to adhere. These adhesins give *Metarhizium* the ability to anchor to insect and plant surfaces, enabling it to effectively colonize and persist in these different phases of its life cycle. Interestingly, the *Mad2* gene has

recently been shown to be upregulated by nutrient starvation (Barelli et al., 2011). Also, a *Mad2* disrupted mutant has demonstrated delayed root colonization and endophytic growth, suggesting that while it is an important factor, it is not solely responsible (Sasan and Bidochka, 2012).

Much is still unknown about the mechanisms by which Metarhizium is rhizosphere competent, however, the *Metarhizium* raffinose transporter (*Mrt*) gene has been shown to be necessary (Fang and St. Leger, 2010). Disruption mutants were shown to grow poorly in root exudate and the rhizosphere competence was greatly reduced. Notably, disruptions in *Mrt* did not have an effect on virulence to insects, demonstrating that this gene is exclusively used in *Metarhizium*'s interactions with plants. While this demonstrates that *Mrt* is necessary for rhizosphere competence, there are very likely other genes that are necessary and contribute to rhizosphere competence as well.

1.3. *Metarhizium* Adhesin-like Proteins

The open reading frames of *Mad1* and *Mad2* encode a 717 and 306 amino acid protein, respectively, with no introns (Wang and St. Leger, 2007). These adhesin-like proteins have a three-domain structure, similar to that of the *Candida albicans* cell wall agglutinin-like sequence (ALS) proteins (Hoyer, 2001; Sheppard et al., 2004). ALS proteins rapidly form extremely stable H-bond-dependent associations with host proteins and peptides (Gaur and Klotz, 2004). MAD1 and MAD2 also possess a hydrophobic signal peptide and a predicted glycosylphosphatidylinositol cell wall anchor site at their N- and C-terminal ends (Wang and St. Leger, 2007), respectively, which is consistent with the fact they are cell wall proteins.

The N-terminal regions (domain A) downstream of the signal peptides are predicted to be highly hydrophobic, with Thr-rich tandem repeats in the middle regions (domain B) (Wang and St. Leger, 2007), also similar to that of ALS proteins (Hoyer, 2001; Sundstrom, 2002). MAD1

and MAD2 show some sequence similarities within this middle region. In MAD1, this region contains six tandem repeats of 12 residues, GKETTPAQQTTP. In MAD2, it contains three repeats of TVPATMPG. This smaller number of repeats in MAD2 suggests that the distance between the cell surface and the N-terminal ligand binding region is shorter in MAD2 than in MAD1 (Wang and St. Leger, 2007).

1.4. Specific Plant-Fungi Associations

Numerous specific rhizosphere associations have been identified in fungi. Ectomycorrhizal fungi have a great range of host specificity (Molina et al., 1992; Roy et al., 2008). For example, the truffles *Tuber borchii* and *Tuber melanosporum* have specific ectomycorrhizal associations with oaks and hazels (Martin & Nehls, 2009). The *Suilloideae* are almost exclusively associated with Pinaceae, including the very host specific *Suillus pungens*, which colonizes only a few related pine species (Kretzer et al., 1996; Bruns et al., 2002). The ectomycorrhizal *Lactarius quietus*, commonly known as the oak milkcap, associates exclusively with oak species (Courty *et al.*, 2008; Martin & Nehls, 2009). Interestingly, *Lactarius deterrimus*, previously thought to be specific to the roots of *Picea abies* (Norway spruce) has also demonstrated an ability to colonize the roots of the extremely nonspecific phytobiont *Arctostaphylos uva-ursi* (Muhlmann and Gobl, 2006). Conversely, some ectomycorrhizal fungi, such as *Laccaria amethystina*, demonstrate a multihost ability (Roy et al., 2008). Oppositely, arbuscular mycorrhizal fungi have demonstrated that they are largely not host specific (Klironomos, 2000). In most instances, these associations have shown to be beneficial to the host plant, and are critical for nutrient cycling in sustainable ecosystems (Estrada-Luna *et al.*, 2000; Rai *et al.*, 2001; Sirrenberg *et al.*, 2007; Felten *et al.*, 2009; Martin & Nehls, 2009; Baldi *et al.*, 2010).

Chemical compounds found within plant root exudate may contribute to specificity through the exclusion of certain fungi by inhibiting their growth. Phenolic compounds have demonstrated an ability to inhibit colonization by arbuscular mycorrhizal fungi, however, they did not affect colonization by other root colonizing fungi (Piotrowski *et al.*, 2008). In *Sisymbrium loeselii* (tall hedge mustard), isothiocyanates have been implicated in inhibiting arbuscular mycorrhizal colonization (Bainard *et al.*, 2009). With *Alliaria petiolata* (garlic mustard), benzyl isothiocyanate has also been shown to inhibit growth of ectomycorrhizal fungi (Wolfe *et al.*, 2008).

In 2001, Bidochka *et al.* identified two cryptic species within *M. anisopliae* that displayed a habitat association. Ontario group 1 (OG1), which was deemed the heat active group, demonstrated an ability to grow at high temperatures (37°C), and was found to be resilient to UV exposure. Ontario group 2 (OG2), which was deemed cold active, showed an ability to grow at low temperatures (8°C). No association with insect host was identified, with variable bioassay results, but no consistent pattern of virulence for each group (Bidochka et al., 2001). This represented a significant paradigm shift, in that it demonstrated that habitat selection, not host insect selection, influenced the population structure of *Metarhizium*.

Metarhizium has also shown some specificity with respect to rhizosphere colonization. Two of three studied isolates were found to be rhizosphere competent on the roots of *P. abies*, with their populations increasing almost 10 fold over a 14 week period (Bruck, 2010). All three tested isolates were found to be rhizosphere competent on the roots of *Picea glauca*, with populations again increasing nearly 10 fold over a 14 week period. Oppositely, none of the three isolates responded favourably to the rhizosphere of *Taxus baccata* over a 14 week period. This demonstrated a distinctive response to the rhizosphere of different plant roots among various

Metarhizium isolates. However, this study was limited in that these Metarhizium isolates were broadly identified as *Metarhizium anisopliae*, and not identified based on the new multilocus phylogeny by Bischoff *et al.* (2009).

1.5. Genetic Variability in *Metarhizium*

A common paradigm in insect pathology suggests that the host insect is the predominant influence on the population genetics and distribution of insect-pathogenic fungi. As such, the population genetics of *Metarhizium* was assumed to be influenced primarily by host insect taxa (Riba *et al.*, 1986; St. Leger *et al.*, 1992; Bridge *et al.*, 1993; 1997; Fegan *et al.*, 1993; Leal *et al.*, 1994; 1997; Tigano-Milani *et al.*, 1995). Some of these studies have attempted to characterize strains of *Metarhizium* based on genes involved in insect infection. For example, Leal *et al.* (1994;1997), described a method for identifying strains of *Metarhizium* based on the restriction enzyme digest of the subtilisin-like protease 1 (*Pr1*). Using this technique, they identified 15 possible RFLP profiles that clustered into 4 groups. It was also found that the introns were more variable (6.28%) than the coding regions (2.95%), which is expected, as introns are typically under lower evolutionary constraint.

Genetic variability has also been explored in the putative virulence factor, neutral trehalase (*Ntl*) (Small et al., 2004). Kinetic properties of the neutral trehalases revealed no differences between *M. anisopliae* strains. Virulence was similar among these strains, with one exception that showed lower virulence, suggesting a factor other than neutral trehalase was responsible. Again, introns were found to be more variable (6.2%) than the coding regions (3.1%). Comparisons of translated nucleotide codons showed high levels of synonymous substitutions between strains, with another fraction of the mutations resulting in neutral substitutions of amino acids with similar functions. Overall, it was determined that while

nucleotide variation did exist between strains, it was not involved in pathogenicity, and was mostly neutral in nature. This suggests a strong stabilizing selection to maintain enzyme function.

1.6. Phylogeny of the *Metarhizium* Genus

The genus Metarhizium, originally described by Sorokin (1883), has undergone a number of taxonomic revisions. The first major taxonomic revision rejected most of the published names and reduced the genus to two species; *M. anisopliae* and *M. flavoviride* (Tulloch, 1976). This included the type, *M. anisopliae* var. *anisopliae*, and another variety, *M. anisopliae* var. *majus*.

Another taxonomic revision was undertaken using ITS and 28S rDNA D3 sequences and RAPD patterns (Driver et al., 2000). The species described by Tulloch (1976) were accepted, as well as the addition of *Metarhizium album*, *M. anisopliae* var. *lepidiotum* and *M. anisopliae* var. *acridum* were also recognized as additional varieties. *M. flavoviride* was recognized as four varieties, namely *M. flavoviride* var. *flavoviride*, *M. flavoviride* var. *minus*, *M. flavoviride* var. *novazealandicum*, and *M. flavoviride* var. *pemphigum*. These new lineages were described only as varieties, and not species, due to the limited resolution and support provided by the ITS sequence analysis.

While this was the most comprehensive morphological and molecular phylogenetic effort at the time, it was limited in that some taxa described from China, including *M. pingshaense* and *M. guizhouense*, were not available for study (Bischoff et al., 2009). These taxa, including *M. guizhouense*, *M. pingshaense* and *M. taii,* were later synonymized with *M. anisopliae* (Huang et al., 2005a, b). However, these studies used only ITS sequences, which perform poorly in resolving *Metarhizium* lineages.

Most recently, Bischoff et al. (2009) performed a multilocus phylogeny on the *Metarhizium anisopliae* lineage. This study employed a multigene approach, which used sequences from the nuclear encoded genes elongation factor 1-alpha (EF-1α), RNA polymerase II largest subunit (RPB1), RNA polymerase second largest subunit (RPB2) and ß-tubulin (Bt). Based on the phylogenetic evidence, nine terminal taxa were proposed in the *M. anisopliae* complex to be recognized as species. The 5' EF-1α region was also identified as the most informative region for routine species identification within the genus. Using this methodology, the authors identified one isolate from each of the two presumptive cryptic species by Bidochka *et al.* (2001, 2005), Ontario groups 1 and 2, as *M. robertsii* and *M. brunneum*, respectively.

1.7. Overview

Recent literature suggests that while *Metarhizium* is typically looked at as an insect pathogen, plants may also play an important part in its lifecycle. In addition to the rhizosphere competence, differential gene expression in plant root exudate, and plant specific genes such as *Mad2*, phylogenetic and genomic analysis also suggest *Metarhizium* has plant related origins. In a phylogenetic analysis of the Clavicipitaceae family, Metarhizium was shown to be closely related to grass endosymbionts *Claviceps* and *Epichloë* (Spatafora *et al.*, 2007). Genomic analyses indicated that *Metarhizium* spp. are more closely related to plant endophytes and plant pathogens than to animal pathogens, which suggests that *Metarhizium* evolved from fungi adapted to grow on plants (Gao et al., 2011).

The traditional approach to the fungal biological control of plants from insects has focused on a fungi's ability to kill a target insect and has not shown great success. A shift from this approach, to one more focused on the ecology of the fungi and its persistence in a target environment has shown promise and may lead to greater success in the future. Therefore,

research focusing on the ecology of *Metarhizium* and its persistence in the rhizosphere of different plants is crucial in order for it to be effectively used as a biological control agent.

Studies on the use of *Pochonia chlamydosporia* as a control agent against nematodes have demonstrated that nematode control is greatest on roots that support the highest level of rhizosphere colonization, with some isolates displaying differing levels of rhizosphere colonization on various plant cultivars (De Leij and Kerry, 1991; Bourne *et al.*, 1996; Kerry, 2000). The use of a rhizosphere competent isolate of *Metarhizium* has also been shown to be extremely effective, providing nearly 80% control of the insect target (Bruck, 2005). These studies underscore the importance of rhizosphere competence with respect to the effective use of a fungus as a biological control agent. In a recent review article, Vega *et al.* (2009) also emphasized this, suggesting that future research on entomopathogenic fungi should concentrate on understanding the ecology of the fungi, focusing on roles such as rhizosphere colonization, in order to use them more effectively as biological control agents.

Expanding on the habitat association previously observed by Bidochka *et al.* (2001), the first section seeks to determine if there are specific plant relationships with *Metarhizium* within the habitat. That is, the objective is to determine if different species of *Metarhizium* show specificity towards the rhizosphere of various plant types. The second section explores the variation in the insect adhesin, *Mad1*, and the plant adhesin, *Mad2*, in species of *Metarhizium*. The objective of this is to infer evolutionary relationships within the *Metarhizium* genus, by exploring the evolution of an insect and plant specific gene.

2. Section 1 - Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity

2.1. Abstract

Here we tested the hypothesis that species of the soil-inhabiting insect pathogenic fungus *Metarhizium* are not randomly distributed in soils but show plant rhizosphere-specific associations. We isolated *Metarhizium* from plant roots at two sites in Ontario, Canada, sequenced the 5' EF-1α gene to discern *Metarhizium* species, and developed an RFLP for rapid species identification. Results indicated a non-random association of three *Metarhizium* species (*Metarhizium robertsii*, *M. brunneum* and *M. guizhouense*) with the rhizosphere of certain types of plant species (identified to species and categorized as grasses, wildflowers, shrubs and trees). *M. robertsii* was the only species that was found associated with grass roots, suggesting a possible exclusion of *M. brunneum* and *M. guizhouense*. Supporting this, in vitro experiments showed that *M. robertsii* conidia germinated significantly better in *Panicum virgatum* (switchgrass) root exudate, than *M. brunneum* or *M. guizhouense*. *M. guizhouense* and *M. brunneum* only associated with wildflower rhizosphere when co-occurring with *M. robertsii*. With the exception of these co-occurrences, *M. guizhouense* was found to associate exclusively with the rhizosphere of tree species, predominately *Acer saccharum* (sugar maple), while *M. brunneum* was found to associate exclusively with the rhizosphere of shrubs and trees. These associations demonstrate that different species of *Metarhizium* associate with specific plant types.

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2.2. INTRODUCTION

Metarhizium is an insect pathogenic fungus currently used as a biological control agent against various insect species (Lomer *et al.*, 1997; 2001; Milner & Pereire, 2000; Hunter *et al.*, 2001; Maniania *et al.*, 2003; Shah & Pell, 2003). Recent studies suggest that another ecological role of this fungus is as a plant rhizosphere associate. A green fluorescent protein (GFP)-expressing *Metarhizium* applied in an agricultural setting was not randomly distributed in the soil but showed preferential association with the plant rhizosphere (Hu & St. Leger, 2002). The rhizosphere has been identified as a potential reservoir for *Metarhizium*, with propagules persisting in the inner rhizosphere, and decreasing over time in bulk soil (Hu & St. Leger, 2002). Furthermore, populations of *Metarhizium* have been shown to increase significantly over time within the rhizosphere (Bruck, 2005). Also, a plant adhesin (MAD2) has been shown to enable attachment to plants (Wang & St. Leger, 2007).

Notably, *Metarhizium* is phylogenetically related to the fungal grass endosymbionts *Claviceps* and *Epichloë* (Spatafora *et al.*, 2007), suggesting a possible origin of plant association in *Metarhizium*. In a recent review article, Bruck (2010) suggested that rhizosphere competence in insect pathogenic fungi was dependent upon host plant. Additionally, Vega *et al.* (2009) suggest that future research on entomopathogenic fungi should concentrate on understanding the ecology of the fungi, focusing on roles such as rhizosphere colonization, in order to use them more effectively. The use of *Metarhizium* as a biocontrol agent has mostly fixated on its ability to kill insects, with little consideration of plant associations or rhizosphere ecology thus limiting our ability to fully exploit this fungus for biocontrol efforts.

A common paradigm in insect pathology suggests that the host insect is the predominant influence on the population genetics of insect-pathogenic fungi. As such, the population genetics

of *Metarhizium* was assumed to be influenced primarily by host insect taxa (Riba *et al.*, 1986; St. Leger *et al.*, 1992; Bridge *et al.*, 1993; 1997; Fegan *et al.*, 1993; Leal *et al.*, 1994; 1997; Tigano-Milani *et al.*, 1995). However, this paradigm is being challenged. For example, in Ontario, the fungus previously known as *M. anisopliae* was comprised of two cryptic species, namely Ontario group 1 (OG1) and Ontario group 2 (OG2) (Bidochka *et al.*, 2001; 2005). These genetically distinctive, non-recombining groups were found to be strongly associated with habitat type, with isolates from agricultural/open field habitats belonging predominately to OG1, and OG2 isolates found in forested soils. Interestingly, no differences in insect host specificity or virulence was found between these groups (Bidochka *et al.*, 2001; 2005).

Here we further explored the "habitat hypothesis" (i.e. agricultural/open fields or forests) in defining the distribution of *Metarhizium* species (Bidochka *et al.*, 2001) and suggest that *Metarhizium* species show plant specific rhizosphere associations within a habitat. Our objectives were to randomly collect plant root samples along a linear transect from an agricultural/open field habitat through to a forested habitat, at two independent sites, and to isolate and identify the *Metarhizium* species associated with these plant roots. *Metarhizium* was isolated from plant roots using a selectable agar and the 5' EF-1α gene was sequenced in a selection of isolates and compared to previously deposited nucleotide sequences for confirmation of *Metarhizium* species (Bischoff *et al.*, 2009). The genus *Metarhizium* has recently been subdivided into different species according to the sequences of several genes (Bischoff *et al.*, 2009). The sequence for the 5' region of EF-1α was deemed to be a diagnostic marker for *Metarhizium* species identification (Bischoff *et al.*, 2009). We assessed the sequence data and designed an RFLP analysis of 5' EF-1α in order to rapidly identify, at the *Metarhizium* species

level, a large number of isolates. We also utilized EF-1α to identify *Metarhizium* species for representative isolates that we had previously referred to as OG1 and OG2.

2.3. METHODS

2.3.1. Root sample collection.

A site near Brock University (St. Catharines, ON), as well as a site in Guelph, ON were selected using GIS (Google Earth®). Locations were chosen that had a large, established forest area adjacent to a large open field with a strong delineation between habitats. The Brock site (43°06'50.95"N 79°14'53.21"W) forest was mature and contained mostly maple, ash, and beech, with the open field dominated by goldenrod species. The mature forest at the Guelph site (43°29'34.34"N 80°13'33.57"W) contained mostly cedar, ash, and maple, while the open field consisted of aster, goldenrod, and wild carrot. Digital photographs of the sites are available upon request.

One hundred plant root samples were randomly collected approximately every meter along a linear transect that ran from the field to the forest, with the first 50 samples primarily from field plants, including grasses and wildflowers, and the last 50 predominately forest plants, trees and shrubs. Plant roots were dug out with a spade, and excess soil was tapped from the roots before storage in separate, pre-labeled plastic reclosable bags. For mature trees and larger shrubs, fibrous roots found between the larger woody roots were collected. Additionally, representative foliage from each plant was photographed and collected in a pre-labeled envelope and was used to identify plant species using botanical keys and appropriate field guides.

2.3.2. *Metarhizium* isolation.

Roots were washed with sterile distilled water to remove excess soil. Soil that adhered closely to the root was kept as representative of the rhizosphere. The roots were cut into 0.5 cm pieces, placed in 5 ml distilled water, and homogenized using a rotary homogenizer (Greiner Scientific). One hundred microlitres of homogenate was spread, in duplicate, onto selective media, containing 39 g/L potato-dextrose agar (PDA) (Difco), 0.5g/L cycloheximide, 0.2g/L chloramphenicol, 0.5g/L 65% dodine, and 0.01g/L crystal violet. The plates were incubated at 27°C for 20 days. *Metarhizium* isolates were identified by colony morphology, namely white mycelia with green conidia, as well as microscopic identification of conidial morphology. Morphologically differing colonies were individually isolated from the selective plate of each plant root sample and grown on PDA plates at 27°C for 10 days. Morphologically similar colonies were also isolated multiple times from the same plant root sample.

Isolates HKB1-1b and 43a-2i (Bidochka *et al.*, 2001) were used as representative isolates of OG1 and OG2, respectively. 43a-2i has been previously identified as M. brunneum and accessioned as ARSEF 8680 (Bischoff *et al.*, 2009). ARSEF 2575 is the ex-type for *M. robertsii* (Bischoff *et al.*, 2009).

The construction of green fluorescent protein (GFP) expressing plasmids, transformation, and creation of transgenic fungal lines were performed as previously described (Fang *et al.*, 2006) in order to produce a GFP-expressing transformant of ARSEF 2575 (*M. robertsii*). The transformant did not show any differences in growth, insect virulence, or colony morphology in comparison to the wild-type.

2.3.3. DNA extraction.

Morphologically different isolates were inoculated into 50 mL 0.2% (w/v) yeast extract, 1% peptone, 2% dextrose (YPD) broth in flasks. The flasks were shaken at 200 rpm at 27°C for 3 to 4 days. The mycelia were removed by vacuum filtration onto Fisherbrand® P8 filter paper and washed with distilled water. The samples were then crushed in liquid nitrogen using a mortar and pestle, and DNA was extracted using either a DNeasy Plant Mini Kit (QIAGEN) or Plant/Fungi DNA Isolation Kit (Norgen).

2.3.4. PCR amplification and RFLP analysis.

The subtilisin-like protease 1 (*Prl*) and neutral trehalase (*Ntl*) genes were amplified for all isolates according to previously described conditions and digested with RsaI as previously described (Leal *et al.*, 1997; Bidochka *et al.*, 2001; Small *et al.*, 2004). Each isolate was identified as belonging to either OG1 or OG2 based on RFLP banding pattern (Bidochka *et al.*, 2001; Small *et al.*, 2004).

The 5' region of the translation elongation factor 1-alpha (EF-1α) gene was also amplified according to previously described conditions (Rehner & Buckley, 2005; Bischoff *et al.*, 2006; 2009). 5' EF-1α products were sequenced for five isolates of OG1 and two isolates from each of the possible genetic groups present in OG2, including representative isolates HKB1-1b (OG1), 43a-2i (OG2), and ARSEF 2575 (OG1). Putatively unique nucleotide polymorphisms were identified for the 5' EF-1α product of each species and restriction site analysis of the 5' EF-1α sequences revealed Ontario isolates could be differentiated based on RFLP banding patterns of the 5' EF-1α amplification product double digested with MseI and XhoI. This was performed in a total volume of 20 μL, which included 5μL 5' EF-1α PCR

product, 2 μL 10X NEBuffer 4 (NEB), 4 μL 10X BSA, 10 units of MseI (NEB), and 20 units of XhoI (NEB). Reactions were incubated at 37°C for 16 hours.

All amplified DNA sequences and RFLP products were visualized by electrophoresis on a 1% agarose gel, run at 70V for 45 minutes in 0.5X TBE buffer.

2.3.5. Sequencing.

Prior to sequencing, DNA samples were purified using a QIAquick PCR Purification Kit (QIAGEN). The 5' EF-1α PCR products were sent to the core molecular biology lab at York University for sequencing.

2.3.6. Nucleotide sequence accession numbers.

5' EF-1α sequences for *Metarhizium* isolates that were sequenced have been deposited in the GenBank database: HKB1-1b (Accession HM748301), B18-ai (HM748302), M31-ai (HM748303), G90-bi (HM748304), G55-ai (HM748305), B77-ai (HM748307), B34-aiii (HM748308), and B35-avi (HM748309).

2.3.7. Phylogenetic identification of *Metarhizium* isolates.

Molecular phylogenetic analysis of the 5' EF-1α sequences was conducted in order to identify the *Metarhizium* species of sequenced isolates. Alignments were made with Clustal X 2.1 (Larkin et al, 2007) using the default settings. A maximum parsimony (MP) phylogenetic tree was constructed using PHYLIP 3.69 (Felsenstein, 2009). Nonparametric bootstrapping was conducted using 1000 pseudoreplicates, with 10 random addition replicates per parsimony run, and subtree pruning and regrafting (SPR) branch swapping.

2.3.8. Seed sterilization, conidial inoculation, and microscopy.

Phaseolus vulgaris (haricot bean) and *Panicum virgatum* (switchgrass) seeds were surface sterilized using a modification of the method of Miche and Balandreau (2001). First,

seeds were immersed in sterile distilled water for 30 minutes. The seeds were then immersed in 4% sodium hypochlorite solution for 2.5 hours. The fluid was decanted and the seeds were washed with sterile distilled water. The seeds were then immersed in 15% hydrogen peroxide for 30 minutes and then washed 3 times with sterile distilled water. Sterilized seeds were kept at 4°C overnight to allow for synchronization of growth. Switchgrass seeds were immersed in 10^7 spores/mL inoculum of GFP-expressing *Metarhizium* (ARSEF 2575) for one hour, plated in sterile soil, and kept at 25°C for a photoperiod of 12 hours for 14 days. For haricot bean, sterilized seeds were allowed to develop into seedlings, and then inoculated. The treated bean roots were kept at 25°C for a photoperiod of 12 hours for 14 days. Sterile water was added regularly to avoid drying of the sample.

Roots were rinsed with sterile distilled water without a fixing reagent. A Leica DM RBE laser scanning confocal microscope, equipped with an argon/krypton laser was used for the observation of GFP-*Metarhizium* on roots.

2.3.9. Root exudate collection.

Surface sterilized switchgrass seeds were soaked in water in Petri dishes, kept on an orbital shaker, and provided with light. Once 90% of the seeds had germinated, samples were kept on the shaker for 4 additional days, after which root exudate was collected by vacuum filtration.

2.3.10. Conidial germination assay.

Conidial suspensions (10^7 conidia/mL) of ARSEF 2575 (*M. robertsii*), 43a-2i (*M. brunneum*), and B77-ai (*M. guizhouense*) were inoculated (1%vol/vol) into switchgrass root exudate and incubated at 15°C or 27°C. Positive controls for conidial germination were also

done in YPD. Conidial germination was assessed microscopically (Leitz DIAPLAN light microscope) at 12, 24 and 48 hour intervals.

2.4. RESULTS

2.4.1. *Metarhizium* species in the sampling sites

After plant root samples were collected, the *Metarhizium* colonies were isolated and DNA was extracted, RFLP analysis of the *Ntl* was initially used to assign each isolate to OG1 or OG2 (Fig. 1A). RFLP analysis of the *Prl* gene showed that isolates assigned to OG2, based on RFLP analysis of *Ntl*, displayed 2 possible banding patterns for *Prl*, which suggested the presence of 2 distinctive genetic groups within OG2 (Fig. 1B).

In order to resolve the exact species composition of each of these distinctive genetic groups, the 5' region of EF-1α was amplified and sequenced for four isolates of OG1 and two isolates from each of the possible genetic groups present in OG2, including representative isolates HKB1-1b (OG1), 43a-2i (OG2), and ARSEF 2575 (OG1). Using molecular phylogenetic identification, isolates initially assigned to OG1 were identified as *M. robertsii* (Fig. 2). OG2 was determined to be comprised of two species, namely *M. brunneum* and *M. guizhouense* (Fig. 2). Restriction enzymes MseI and XhoI were used to cleave amplified portions of the 5' EF-1α gene which resulted in RFLP patterns that differentiated Ontario isolates of *Metarhizium* (Fig. 3). All species displayed a distinct RFLP pattern, with the exception of *M. brunneum* isolates, which had two distinctive RFLP patterns identified while screening isolates. Sequence analysis of the 5' EF-1α gene for another *M. brunneum* RFLP pattern showed 6 single nucleotide polymorphisms, including one within an MseI site, resulting in a polymorphic RFLP pattern. This alternate *M.*

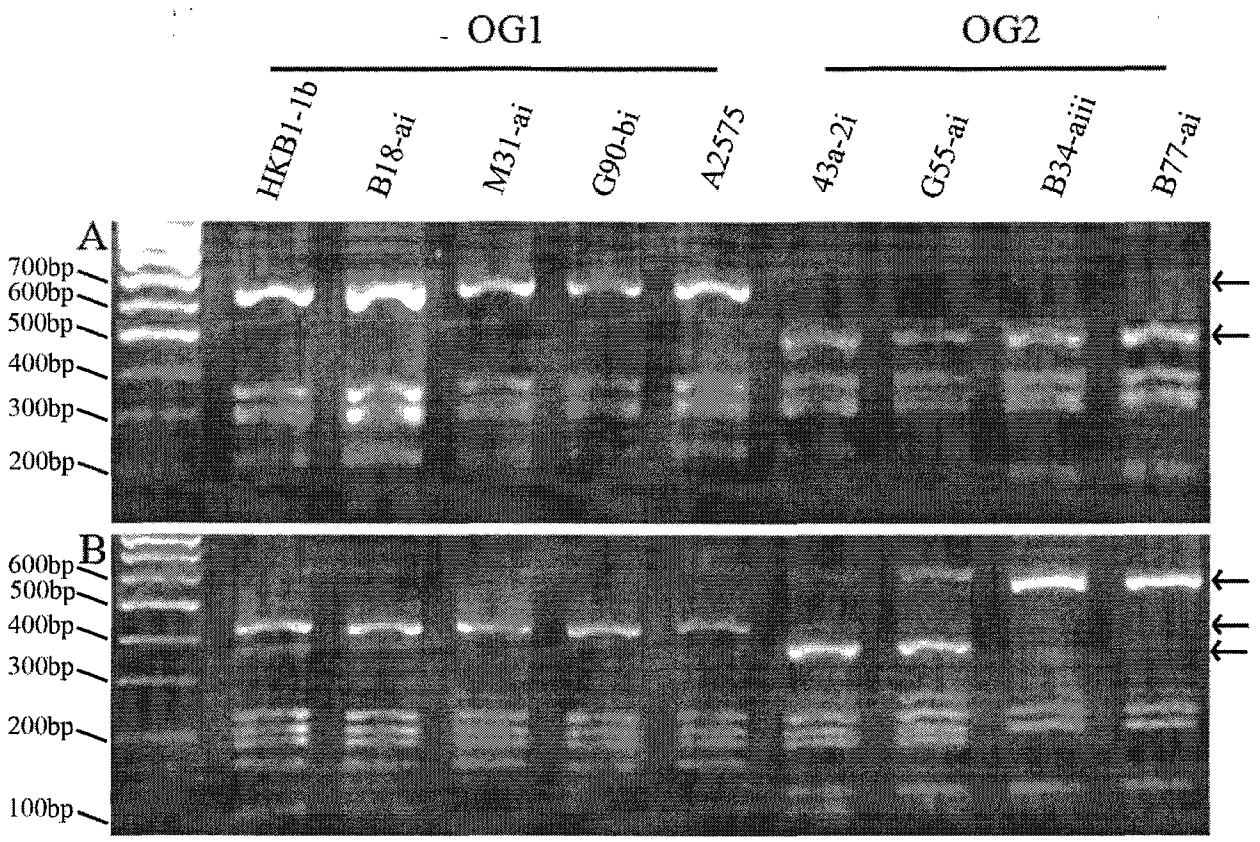

Figure 1. RFLP analysis of *Ntl* (A) and *Prl* (B) using RsaI for isolates of *Metarhizium.* Representative isolates HKB1-1b (OG1), 43a-2i (OG2), and ARSEF 2575 (OG1). Arrows indicate distinctive bands used for identification. *Ntl* and *Prl* RFLP products were separated on a 1.5% gel at 60V for 80 minutes and 2% gel at 100V for 45 minutes, respectively.

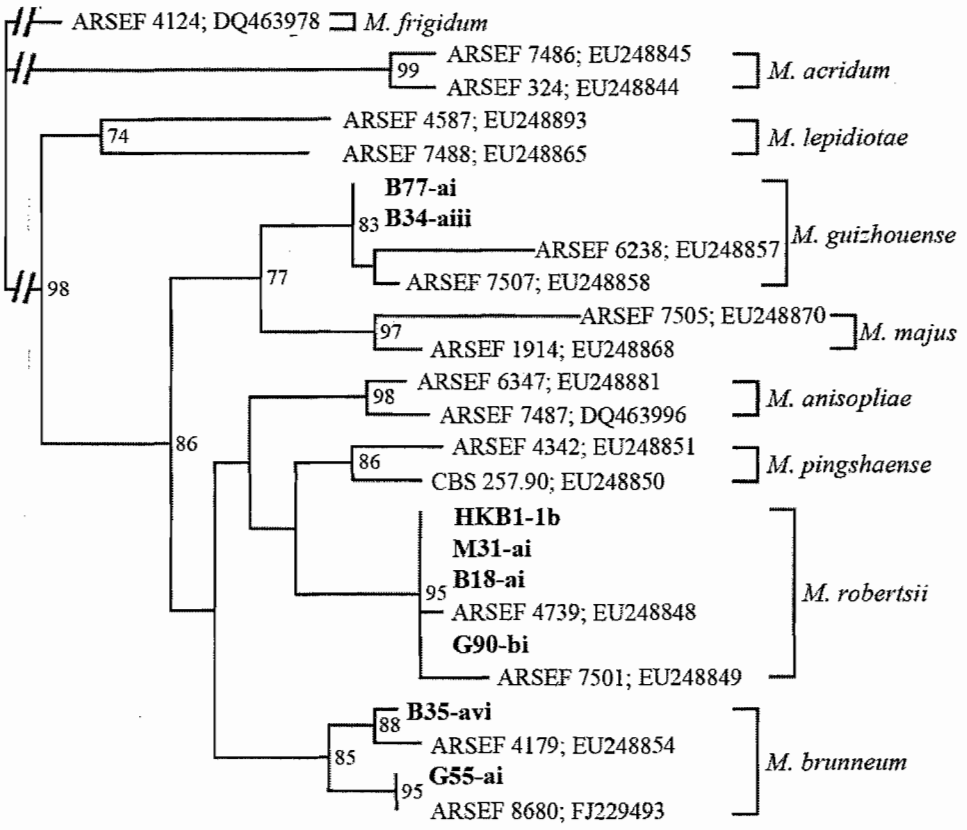

Figure 2. Maximum parsimony (MP) phylogenetic tree of 5' EF-1α sequences of *Metarhizium* isolates. Bootstrap values are based on 1000 pseudoreplicates; values > 70% are shown.

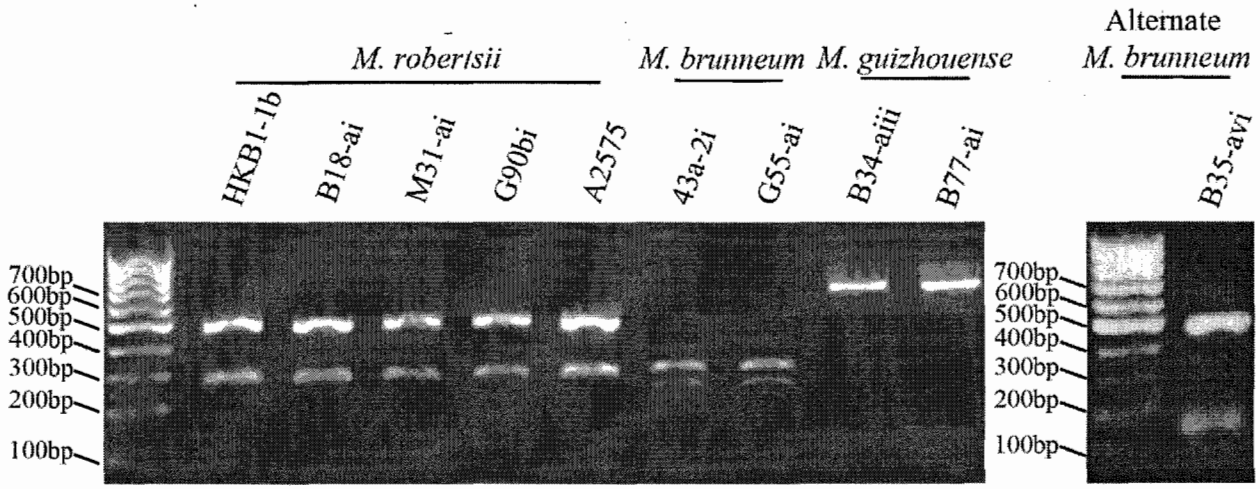

Figure 3. RFLP analysis of 5' EF-1α using MseI and XhoI for *Metarhizium* isolates.

brunneum 5' EF-1α gene shared 99.2% identity with the other *M. brunneum* 5' EF-1α sequence (705bp/711bp).

Using this RFLP analysis, screening of all isolates collected demonstrated that *M. robertsii* is the most prevalent species of *Metarhizium* in Ontario (Fig. 4), with 49 and 32 isolates collected from the Guelph and Brock sites, respectively. *M. brunneum* was also present at both sites, but in lower frequency, with 13 and 2 isolates collected from the Guelph and Brock sites, respectively. Interestingly, *M. guizhouense* was only isolated from the Brock site, with 11 isolates collected, but not at the Guelph site. Morphologically similar isolates from the same plant root sample were always identified as the same species, and thus were only counted as a single isolate.

2.4.2. Plant-specific associations.

Of all 200 plant root samples collected, 102 (51%) resulted in isolations of *Metarhizium*. *Metarhizium* was found to associate with 63%, 62%, 55%, and 35% of grass, wildflower, shrub, and tree root samples, respectively. Generally, *Metarhizium* was found most abundantly from wildflower species.

Three species of *Metarhizium* were isolated from the rhizosphere of plant root samples collected during this study, namely *M. robertsii*, *M. brunneum*, and *M. guizhouense*. After identifying the species of *Metarhizium* isolates collected, an association between *Metarhizium* species and habitat was observed (Fig. 4), confirming previous results (Bidochka *et al.*, 2001). That is, *M. robertsii* was found predominantly in the agricultural or open field habitats, while *M. brunneum* and *M. guizhouense* (at the Brock Site) were found predominantly in the forested habitats.

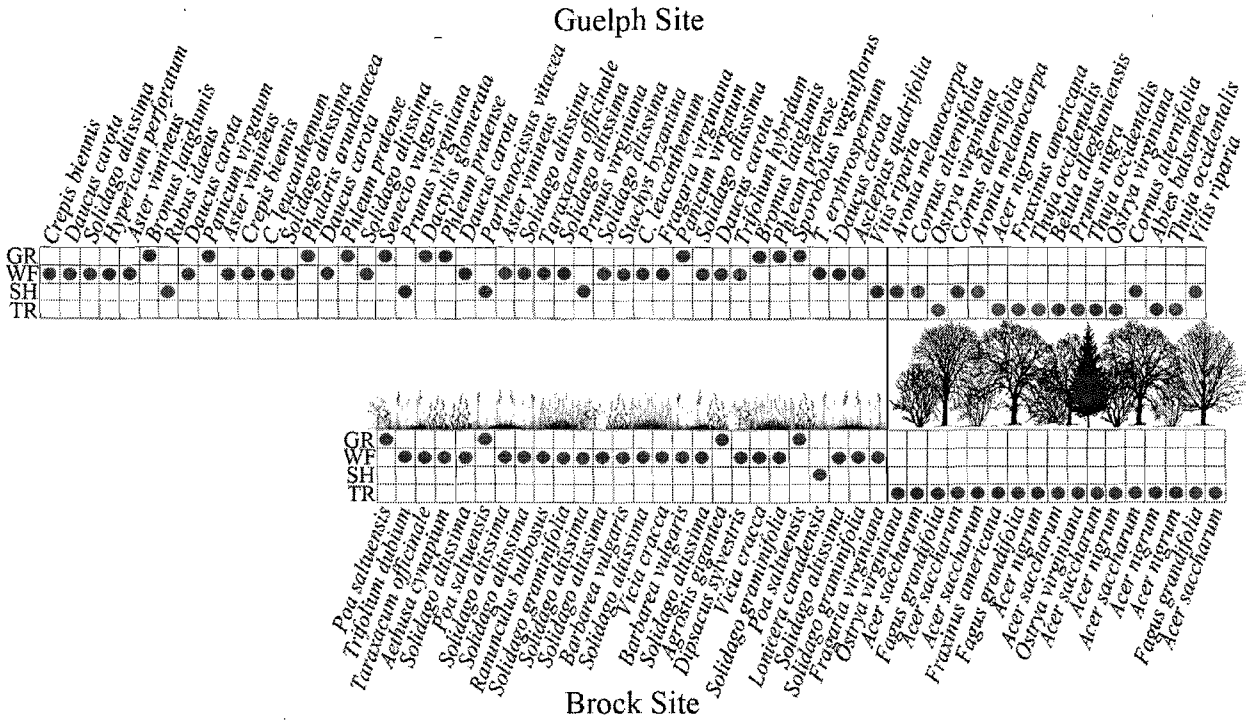

Figure 4. Plant species, plant type (GR = grass, WF = wildflower, SH = shrub, TR = tree), and *Metarhizium* species (Red = *M. robertsii*, Blue = *M. brunneum*, Green = *M. guizhouense*) isolated from plant root samples collected at the Guelph and Brock sites. Samples on the left represent transect samples in the agricultural forage field, and samples on the right represent the transect samples in a forest.

(Research assisted by Cristina Huber)

Plant root samples collected resulting in no *Metarhizium* isolations are as follows: **Guelph**; GR; *Phalaris arundinacea*, *Agrostis perennans*, WF; *Cirsium arvense*, *Convolvulus arvensis*, *Solidago altissima* (2), *Aster vimineus, Daucus carota*, *Thaspium trifoliatum*, SH; *Rubus idaeus*, *Salix bebbiana*, *Aronia melanocarpa* (2), *Parthenocissus vitacea*, *Frangula alnus*, TR; *Thuja occidentalis* (8), *Abies balsamea* (5), *Fraxinus americana* (2), *Acer saccharinum*, *Tsuga canadensis* (2), *Betula alleghaniensis* (3), *Acer nigrum* (2), *Fraxinus nigra*, *Prunus nigra*, *Ostrya virginiana*. **Brock**; GR; *Poa saltuensis* (4), *Schizanchne purpurascens*, *Glyceria canadensis*, *Agrostis gigantean*, WF; *Solidago altissima* (3), *Dipsacus sylvestris* (4), *Daucus carota* (3), *Barbarea vulgaris* (3), *Chrysanthemum leucanthemum*, *Hieracium pratense*, *Lepidium campestre*, *Solidago graminifolia* (2), *Petasites frigidus*, *Taraxacum officinale*, *Dalibarda repens*, *Plantago major*, SH; *Prunus virginiana* (2), *Cornus stolonifera*, *Sambucus racemosa ssp. Pubens*, TR; *Acer saccharum* (7), *Fraxinus americana* (8), *Fagus grandifolia* (6), *Acer nigrum* (2), *Ostrya virginiana.*

Additionally, it was found that within these habitats, the three species of *Metarhizium* associated with specific plant types (Fig. 4). There were significant associations of *Metarhizium* species with plant type at the Brock and Guelph sites (Yates' $\chi^2 = 19.808$, $p < 0.005$ and $\chi^2 = 21.192$, $p < 0.001$, respectively). Notably, *M. robertsii* was the only species isolated from grasses. It was also commonly isolated from wildflowers. Also notable, *M. brunneum* and *M. guizhouense* were only isolated from wildflowers when *M. robertsii* was also isolated from that same plant root sample.

With the exception of one co-occurrence with *M. robertsii* on the wildflower *Solidago altissima* (late goldenrod), *M. guizhouense* was isolated exclusively from trees, suggesting a specific association with this plant type. Additionally, it was observed that *M. guizhouense* was predominantly isolated from species of the *Acer* (maple) genus, primarily *Acer saccharum* (sugar maple) (Fig. 4). Of the eleven different occurrences of *M. guizhouense* at the Brock site, seven were isolated from trees of the genus *Acer*. Six of these were from *A. saccharum* and one was from *A. nigrum* (black maple). At the Guelph site, three *A. nigrum* and one *A. saccharinum* (silver maple) roots were sampled from which a single *M. brunneum* isolate was obtained from *A. nigrum* (Fig. 4). Additionally, *M. guizhouense* was the only species to be isolated from root samples of *Fraxinus americana* (white ash), with one of nine samples at the Brock site resulting in isolations of this species. Neither of two ash root samples at the Guelph site had *M. guizhouense* (Fig. 4).

M. brunneum was uncommon at the Brock site and was isolated on its own from one tree, *Fagus grandifolia* (American beech). It was also found to co-occur with *M. robertsii* on the wildflower *Barbarea vulgaris* (wintercress). At the Guelph site, *M. brunneum* was isolated exclusively from shrub and tree species, with the exception of one co-occurrence with *M.*

robertsii on the wildflower *Trifolium hybridum* (alsike clover) (Fig. 4). *M. brunneum* was also found to co-occur with *M. robertsii* on the shrub *Cornus alternifolia* (alternate-leaved dogwood) and the tree *Betula alleghaniensis* (yellow birch). Additionally, *M. brunneum* was the only species to be isolated from root samples of *Thuja occidentalis* (eastern white cedar), with four of eleven samples at the Guelph site resulting in isolations of this species.

2.4.3. *In vitro* microscopy.

In vitro experiments demonstrated the close association of a GFP-expressing *Metarhizium* isolate (ARSEF 2575) with *Phaseolus vulgaris* (haricot bean) root (Fig. 5A) and *Panicum virgatum* (switchgrass) root (Fig. 5B). Hyphae can be seen growing on the root surfaces, as well as around the root hairs in switchgrass. Plants appeared healthy even after long term (up to 20 days) root colonization by *Metarhizium.*

2.4.4. Conidial germination assays.

The level of conidial germination in switchgrass root exudate was monitored over 48 hours for an isolate of each species; ARSEF 2575 (*M. robertsii*), 43a-2i (*M. brunneum*), and B77-ai (*M. guizhouense*) (Fig. 6). Differences in germination and hyphal growth were observed in *Metarhizium* species grown in switchgrass root exudate, relative to growth in YPD as a positive control. *M. robertsii* demonstrated a high level of germination in switchgrass root exudate. A high level of hyphal growth was also seen after 24 hours. Additionally, the germination rate of *M. robertsii* conidia in switchgrass root exudate was higher than the rate in YPD after 48 hours at 27°C and 15°C. Conversely, *M. brunneum* and *M. guizhouense* showed minimal germination in switchgrass root exudate, relative to YPD. Similar results were obtained at 27°C and 15°C. The differences in relative rates of germination between *M. robertsii,* and *M. brunneum* or *M. guizhouense* was statistically significant at all time intervals (12, 24 and 48hr)

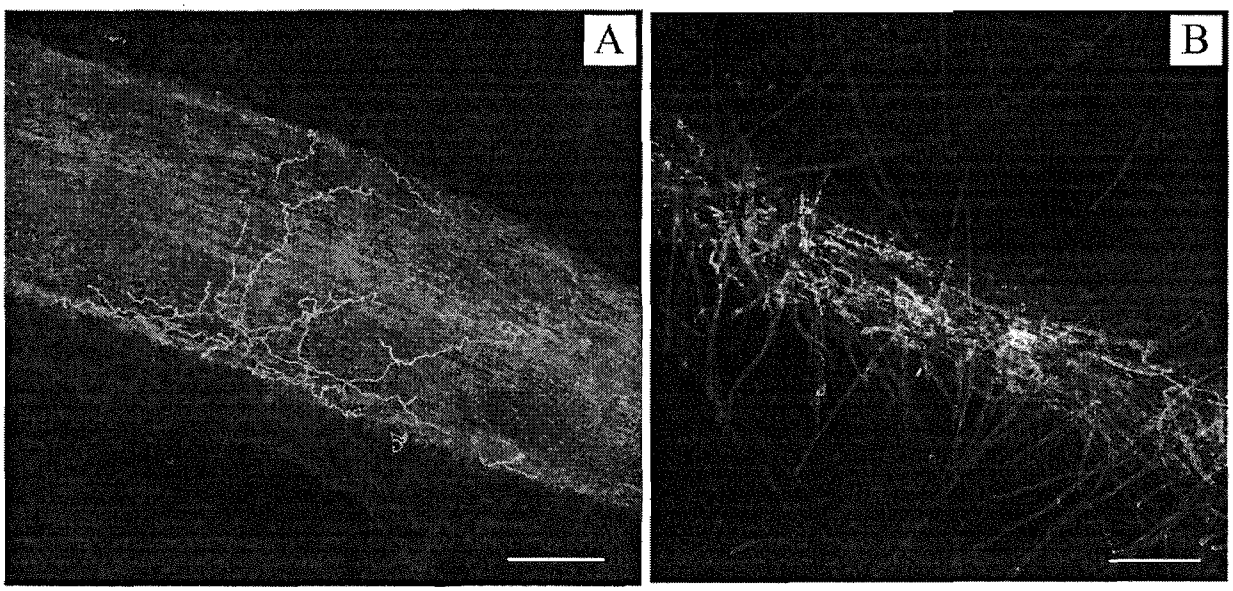

Figure 5. Confocal images of a GFP-expressing *Metarhizium* (ARSEF 2575) associating with *Phaseolus vulgaris* (haricot bean) root (A), and *Panicum virgatum* (switchgrass) root (B). The scale bar represents 200μm.

(Research performed by Ramanpreet Kaur Sasan)

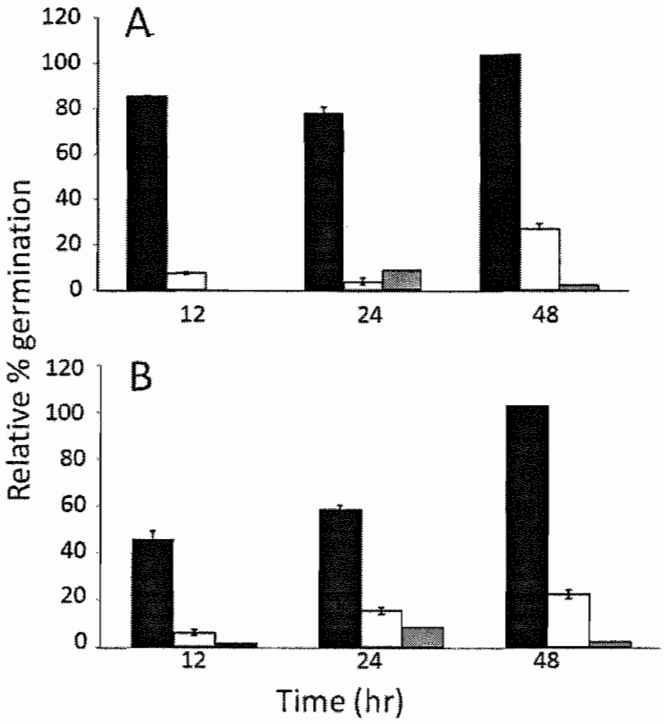

Figure 6. Relative germination rates of *M. robertsii* (black bars), *M. brunneum* (white bars), *M. guizhouense* (gray bars) conidia inoculated into *Panicum virgatum* (switchgrass) root exudate at 27°C (A) and 15°C (B). Germination rates were relative to YPD as the positive control. Conidia failed to germinate in water for all three species. Germination was scored from 100 conidia, standard deviations are shown, and n=3. The experiment was repeated twice with similar results. (Research performed by Ramanpreet Kaur Sasan)

and both temperatures (27°C and 15°C) (*t* tests $p \leq 0.0005$). Conidia failed to germinate in water for all three species.

2.5. DISCUSSION

Here we identified three sympatrically occurring species of *Metarhizium* in Ontario that associated with the rhizosphere of specific plant types. We suggest that plant rhizosphere specific associations within a habitat determine the distribution of *Metarhizium* species. *M. robertsii* preferentially associated with grasses and wildflowers. *M. brunneum* preferentially associated with shrubs and trees and *M. guizhouense* was isolated almost exclusively from trees, predominately *A. saccharum*.

Field data demonstrated that *M. robertsii* is the most prevalent *Metarhizium* species in Ontario (Fig. 4), a finding supported by bulk soil sampling (Bidochka *et al.*, 2001). There may be some selective advantage to this species, which may not be due to superior virulence or ability to infect a host (Bidochka *et al.,* 2001), but rather an ecological ability to survive or inhabit a certain plant rhizosphere. Moreover, this ecological ability may be mechanistically unrelated to pathogenicity, as Ontario species have demonstrated no significant differences in host specificity or virulence. A similar hypothesis has been proposed for the plant pathogen, *Fusarium graminearum*, in which one lineage was found to be more prevalent than others, while all lineages had similar pathogenicity (Lee *et al.*, 2009).

In addition to being the most prevalent species found in Ontario soils, *M. robertsii* also demonstrated an ability to associate with a wider range of plant types and species (Fig. 4). In particular, *M. robertsii* was prevalent among wildflower species, and was the only species of *Metarhizium* to associate with wildflowers on its own. On the other hand, *M. brunneum* and *M.*

guizhouense were only found to associate with wildflowers when in conjunction with *M. robertsii*. The exact nature and mechanisms of these associations are unknown, and there is a paucity of research conducted on plant associations of similar rhizosphere competent fungi. However, examples of specific mycorrhizal associations are known. Synergistic colonization has been suggested in arbuscular mycorrhizal fungi, in which *Gigaspora rosea* and *Scutellospora castanea* colonization was significantly increased in the presence of *Glomus* species, particularly *G. mosseae* (Van Tuinen *et al.*, 1998). In the case of *S. castanea*, when inoculated on its own, root colonization frequency was zero after 8 weeks, but increased to approximately 35% in the presence of *Glomus* species.

While *M. robertsii* was found to co-occur in the rhizosphere with other *Metarhizium* species, *M. brunneum* and *M. guizhouense* were never isolated from the rhizosphere of the same plant. There may be competition between *M. brunneum* and *M. guizhouense*, wherein one species outcompetes the other with respect to root colonization. In the ectomycorrhizal fungus *Rhizopogon*, the timing of colonization, as well as the proportion of the root system colonized are key factors in competitive success (Kennedy *et al.*, 2009). In arbuscular mycorrhizal fungi, colonization of a plant by one fungus has demonstrated altered root exudation that may inhibit development of subsequent mycorrhizal interactions (Pinior *et al.*, 1999; Vierheilig *et al.*, 2003).

Host plant specificity has been suggested to be responsible for differences in ectomycorrhizal community composition and diversity (Molina *et al.*, 1992; Kernaghan *et al.*, 2003). This finding may have implications in *Metarhizium* colonization of the plant rhizosphere where we found that this is not a loose association but rather that *Metarhizium* is attracted to and associates intimately on root surfaces (Fig. 5). In the field, *M. robertsii* was the only species found to associate with grass roots. Additionally, the in vitro conidial germination experiments

demonstrated that switchgrass root exudate was a favourable medium for germination of *M. robertsii* conidia compared with *M. brunneum* or *M. guizhouense* (Fig. 6).

The *in vitro* experiments complement the field data and demonstrate that this exclusion is a plant associated effect, rather than an artifact of the habitat association or due to a specific insect host. While the mechanism for this exclusion is unknown, it may be due to compounds in the grass root exudate. Phenolics, and other compounds such as isothiocyanates have been implicated in the inhibition of root colonization by some fungi (Piotrowski *et al.*, 2008; Wolfe *et al.*, 2008; Bainard *et al.*, 2009). Grass species may exude a compound that is toxic or inhibitory to the growth of *M. brunneum* and *M. guizhouense*. Alternately, *M. brunneum* (Guelph site) was isolated from *T. occidentalis*, and *M. guizhouense*, was isolated from *F. americana*, at the exclusion of other *Metarhizium* species. The associations observed in this study are unlikely to be due to chance alone, and indicate specific rhizosphere associations.

Specific rhizosphere associations have been noted in ectomycorrhizal fungi *Tuber borchii*, *Tuber melanosporum*, and *Lactarius quietus* (Courty *et al.*, 2008; Martin & Nehls, 2009). Conversely, arbuscular mycorrhizal fungi are largely not host specific (Klironomos, 2000). In most instances, these associations have shown to be beneficial to the host plant, and are critical for nutrient cycling in sustainable ecosystems (Estrada-Luna *et al.*, 2000; Rai *et al.*, 2001; Sirrenberg *et al.*, 2007; Felten *et al.*, 2009; Martin & Nehls, 2009; Baldi *et al.*, 2010). Specific rhizosphere associations, with respect to rhizosphere competence, have also been demonstrated in *Metarhizium*, wherein certain isolates are only rhizosphere competent with certain plants (Bruck, 2010).

Our findings of plant rhizosphere specific associations in *Metarhizium* have significant implications for the use of *Metarhizium* as a biological control agent. The use of a rhizosphere

competent isolate of *Metarhizium* provided nearly 80% control against a target insect (Bruck, 2005). Plant rhizosphere specificity must be taken into account in order to optimize delivery against a target insect and ensure sustainability in the plant rhizosphere.

ACKNOWLEDGEMENTS. This research was conducted with the assistance of an NSERC Discovery Grant to MJB.

3. Section 2 - Variability in the insect and plant adhesins, *Mad1* and *Mad2*, within the genus *Metarhizium* suggest plant adaptation as an evolutionary force

3.1. Abstract

Several species of the insect pathogenic fungus *Metarhizium* are associated with certain plant types and genome analyses suggested a bifunctional lifestyle; as an insect pathogen and as a plant symbiont. Here we wanted to explore whether there was more variation in genes devoted to plant association (*Mad2*) or to insect association (*Mad1*) overall in the genus *Metarhizium*. Greater divergence within the genus *Metarhizium* in one of these genes may provide evidence for whether host insect or plant is a driving force in adaptation and evolution in the genus *Metarhizium*. We compared differences in variation in the insect adhesin gene, *Mad1*, to the plant adhesin gene, *Mad2*, as well as to EF-1α in 14 isolates representing 7 different species in the genus *Metarhizium*. Overall variation for the *Mad1* promoter region (7.1%), *Mad1* open reading frame (6.7%), and *Mad2* open reading frame (7.4%) were similar, while it was higher in the *Mad2* promoter region (9.9%). Analysis of the transcriptional elements within the *Mad2* promoter region revealed variable STRE, PDS, degenerative TATA box, and TATA box-like regions, while this level of variation was not found for *Mad1*. Phylogenetic analysis of 5' EF-1α, which is used for species identification, as well as the *Mad1* and *Mad2* sequences, demonstrated that the *Mad2* phylogeny is more congruent with 5' EF-1α than *Mad1*. This would suggest that *Mad2* has diverged among *Metarhizium* lineages, contributing to clade- and species-specific variation, while it appears that *Mad1* has been largely conserved. While other abiotic and biotic factors cannot be excluded in contributing to divergence, it suggests that plant relationships, rather than insect host, have been a major driving factor in the divergence of the genus *Metarhizium*.

3.2. INTRODUCTION

Species within the genus *Metarhizium* are insect pathogenic fungi with a broad range of insect hosts. The genus was recently divided into several separate species based on a multilocus phylogeny (Bischoff *et al.*, 2009). The EF-1α sequence was found to be diagnostic for species identification. The population biology (and now species association) of *Metarhizium* had been assumed to be influenced primarily by host insect taxa (Riba *et al.*, 1986; Bridge *et al.*, 1993; 1997; Fegan *et al.*, 1993; Leal *et al.*, 1994; 1997; Tigano-Milani *et al.*, 1995). That is, different species of *Metarhizium* were associated with different insect species. However, an association between *Metarhizium* species and habitat and/or plant types has been observed (Bidochka *et al.*, 2001; Wyrebek *et al.*, 2011). This represents a significant paradigm shift, in that it demonstrated that habitat/plant selection, not host insect selection, influenced the population structure of *Metarhizium*. In addition, *M. robertsii* has been shown to be rhizosphere competent (Hu & St. Leger, 2002; Bruck 2005; 2010), further supported by research demonstrating *M. robertsii* is a plant endophyte (Sasan and Bidochka, 2012).

Metarhizium is phylogenetically related to the fungal grass endosymbionts *Claviceps* and *Epichloë* (Spatafora *et al.*, 2007). Genomic analyses also indicated that *Metarhizium* spp. are more closely related to plant endophytes and plant pathogens than to animal pathogens, suggesting that *Metarhizium* evolved from fungi that are plant associates (Gao *et al.*, 2011).

Two adhesin genes have been identified that are specifically involved with insect pathogenesis and plant association, *Metarhizium* adhesin-like protein 1 (*Mad1*) and *Metarhizium* adhesin-like protein 2 (*Mad2*), respectively (Wang and St. Leger, 2007). The MAD1 adhesin allows *Metarhizium* to adhere to insect cuticle, while the MAD2 adhesin enables attachment to

plants, and were expressed differentially on their respective hosts (Wang and St. Leger, 2007). Both proteins contain a middle region (domain B) that contains Thr-rich tandem repeats.

We propose three possible models of evolution within the *Metarhizium* genus: (1) insect host has caused divergence among species; (2) plant host has caused divergence among species; (3) another abiotic or biotic factor has caused the divergence and evolution among *Metarhizium* species. In this study, we explored the genetic differences in 14 *Metarhizium* isolates, representing 7 different species, through the amplification and cloning of the *Mad1* insect adhesin and *Mad2* plant adhesin genes. The open reading frames and promoter regions were sequenced, in order to analyze genetic differences. Sequences were also compared to the EF-1α gene, which allows for species identification (Bischoff *et al.*, 2009), in order to infer evolutionary relationships.

3.3. METHODS

3.3.1. Metarhizium isolates.

Fourteen isolates of *Metarhizium* were used in this study. Eight isolates were obtained from soil and plant root samples from various locations in Ontario, Canada (Bidochka *et al.*, 2001; Wyrebek *et al.*, 2011); *M. robertsii* isolates HKB1-1b, B18-ai, M31-ai, and G90-ai; *M. brunneum* isolates 43a-2i, and G55-ai; and *M. guizhouense* isolates B34-aiii, and B77-ai. *Metarhizium* isolates representing six species were obtained from the USDA-ARSEF (Ithaca, New York); ARSEF 6238 (*M. guizhouense*), ARSEF 439 (*M. pingshaense*) and ex-type isolates ARSEF 7488 (*M. lepidiotae*), ARSEF 7486 (*M. acridum*), ARSEF 2575 (*M. robertsii*), and ARSEF 1914 (*M. majus*) (Bischoff *et al.*, 2009). Isolates were grown on potato dextrose agar (Difco) plates at 27°C for 10 days in order to obtain conidia.

3.3.2. DNA extraction.

Conidia were inoculated into 50 mL 0.2% (w/v) yeast extract, 1% peptone, 2% dextrose (YPD) broth in flasks. The flasks were incubated at 27°C and shaken at 200 rpm for 3 to 4 days, until sufficient mycelia had accumulated. The mycelia were removed by vacuum filtration onto Fisherbrand® P8 filter paper, washed with distilled water, and crushed in liquid nitrogen using a mortar and pestle. DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN). Extracted DNA was quantified using a NanoVue spectrophotometer (GE).

3.3.3. PCR, primer walking, gene cloning, and sequencing.

Primers were designed by using the sequences of the *Metarhizium* adhesin-like protein 1 (*Mad1*), and *Metarhizium* adhesin-like protein 2 (*Mad2*) derived from ARSEF 2575 (Wang and St. Leger, 2007), and deposited in GenBank (accession No. DQ338437 and DQ338439, respectively). The upstream sequence for *Mad1* was obtained using Y-shaped adaptor dependent extension (YADE) (Fang *et al.,* 2005).

PCR amplifications were performed in a total volume of 50 μL, which included 5μL 10X Standard PCR Buffer (NEB), 1 μL dNTPs (10mM each dATP, dCTP, dGTP, dTTP) (QIAGEN), 10 pmol each of the opposing amplification primers (Sigma), 0.5 μL *Taq* polymerase (NEB), and 500ng genomic DNA. The following PCR conditions were used for *Mad1* amplification: initial denaturation, 1 minute at 94°C, then 30 cycles of denaturation, 1 minute at 94°C; annealing, 1 minute at 60°C; extension, 4.5 minutes at 72°C; and final extension, 10 minutes at 72°C. The same PCR conditions were used for *Mad2* amplification, with an annealing temperature of 56°C and an extension time of 3 minutes. Table 1 lists the primers used to amplify all *Mad1* and *Mad2* sequences.

Table 1 – Primers used to amplify all *Mad1* and *Mad2* sequences.

Gene: Isolates amplified	**Sequence (5' – 3')**
Mad1: All Isolates	(F) GCT TGT GCC CTG TGT TCC (R) AAG ATT ACA GAA TGC CAG CCC T
Mad2: A2575, HKB1-1b, B18-ai, M31-ai, G90-bi, 43a-2i, G55-ai, A7488, A7486	(F) GCG GCT AAT TTT TGA CTA C (R) TCA TAG CAC AAA TGA GTT GTA T
Mad2: A439	(F) GGA TAT TCA GTC GTG GCT (R) TCA TAG CAC AAA TGA GTT GTA
Mad2: A6238, A1914	(F) GCT TGC TCG TTA GAC ACA (R) TTA GTG TCG GAG GAA TAG AT
Mad2: B34-aiii, B77-ai	(F) AGT GAC TTG GTG GGA TAA G (R) TTA GTG TCG GAG GAA TAG AT

The 5' region of the translation elongation factor 1-alpha (EF-1α) gene was amplified according to previously described conditions (Rehner & Buckley, 2005; Bischoff *et al.*, 2006; 2009) for isolates ARSEF 2575 (*M. robertsii*) and ARSEF 439 (*M. pingshaense*).

Full DNA sequences for *Mad1* and *Mad2* were obtained for all isolates by primer walking (DNA Walking Speedup kit; Seegene). Amplified PCR products were separated by gel electrophoresis, excised, and purified with a QIAquick gel extraction kit (QIAGEN). Purified PCR products were cloned using pGEM-T Easy, as per manufacturer's instructions (Promega). Plasmid DNA was extracted using a GenElute Plasmid Miniprep Kit (Sigma), and inserts were sequenced using vector sequencing primers (SP6 and T7) by the Core Molecular Biology Facility at York University (Toronto, Canada).

5' EF-1α sequences were obtained from GenBank for isolates HKB1-1b (HM748301), B18-ai (HM748302), M31-ai (HM748303), G90-bi (HM748304), 43a-2i (FJ229493), G55-ai (HM748305), B77-ai (HM748307), B34-aiii (HM748308), ARSEF 7488 (EU248865), ARSEF 7486 (EU248845), ARSEF 6238 (EU248857), and ARSEF 1914 (EU248868).

3.3.4. Sequence and phylogenetic analysis.

DNA sequences were aligned using Clustal_X 2.1 (Larkin *et al*, 2007) using the default settings. Translated protein sequences were identified through a multiple sequence alignment with protein sequences derived from ARSEF 2575 (Wang and St. Leger, 2007), for MAD1 and MAD2 (accession No. ABC65821 and ABC65823, respectively). Pairwise comparisons were carried out using EMBOSS Needle (Rice *et al.*, 2000). The nonsynonymous/synonymous rate ratio (dN/dS) was estimated using the ETH Codon Suite, which uses an empirical codon substitution matrix (Schneider *et al.*, 2005), and estimates dN and dS according to Nei and Gojobori (1986).

Molecular phylogenetic analysis of the 5' EF-1α, *Mad1* and *Mad2* sequences was conducted in order to evaluate the phylogenetic relationship of the genes. Maximum parsimony (MP) phylogenetic trees were constructed using PHYLIP 3.69 (Felsenstein, 2009), as previously described (Wyrebek *et al.*, 2011). Nonparametric bootstrapping was conducted using 1000 pseudoreplicates, with 10 random addition replicates per parsimony run, and subtree pruning and regrafting (SPR) branch swapping. The congruency index (I_{cong}) and maximum agreement subtree (MAST) were calculated using the I_{cong} online tool (De Vienne *et al.*, 2007), which calculates the MAST values following Berry and Nicolas (2006).

3.4. RESULTS

3.4.1. *Mad1* variability

Inter-isolate, interspecies, and intraspecies variation were calculated for the open reading frame and promoter regions through pairwise nucleotide comparisons. The greatest inter-isolate divergence within the open reading frame of *Mad1* was 14.2% found between isolates ARSEF 7486 (*M. acridum*) and ARSEF 6238 (*M. guizhouense*). However, when considering the average inter-isolate variation between species, the greatest interspecies divergence was 12.3% between *M. acridum* and *M. majus*, with the least divergence between *M. robertsii* and *M. brunneum* (2.9%). The overall average interspecies variation for the *Mad1* open reading frame for all *Metarhizium* species examined was 6.7%. The average interspecies variation for the promoter region was 7.1%. For the open reading frame, the intraspecies variation was low in *M. robertsii* (0.2%) and *M. brunneum* (0.3%), while it was relatively higher for *M. guizhouense* (3.9%). Similarly, in the promoter region, intraspecies variation was low in *M. robertsii* (0.1%) and *M.*

brunneum (0.1%), and higher for *M. guizhouense* (3.9%). The average estimated nonsynonymous/synonymous substitution rate ratio (dN/dS) for *Mad1* was calculated at 0.20.

Initial analysis of the MAD1 proteins showed that *M. robertsii* isolates had a conserved protein length at 717 amino acids. The MAD1 protein for ARSEF 6238 (*M. guizhouense*) was also 717 a.a., while the Ontario isolates of *M. guizhouense* had proteins that contained 706 a.a.. The MAD1 protein for *M. brunneum* isolates was 711 a.a., while the *M. pingshaense* MAD1 was 704 a.a. Overall, *M. acridum* has the longest MAD1 protein at 723 a.a., including an insertion of 11 amino acids within domain B, which contained Thr-rich tandem repeats. These 11 extra amino acids provided *M. acridum* with eight tandem repeats, while all other species contained six.

3.4.2. *Mad2* variability

The greatest inter-isolate divergence within the open reading frame for *Mad2* was 15.9% found between isolates ARSEF 7486 (*M. acridum*) and HKB1-1b (*M. robertsii*). Similarly, the greatest interspecies divergence was 15.7% between *M. acridum* and *M. robertsii*, with the least divergence between *M. guizhouense* and *M. majus* (2.5%). The overall average interspecies variation for the *Mad2* open reading frame and promoter region for all *Metarhizium* species examined was 7.4% and 9.9%, respectively. The intraspecies variation within the open reading frame was low in *M. robertsii* (0.2%) and *M. brunneum* (0.0%), and moderately higher in *M. guizhouense* (2.2%). In the promoter region, intraspecies variation was also low in *M. robertsii* (0.04%) and *M. brunneum* (0.1%), and higher in *M. guizhouense* (1.5%). The average estimated dN/dS ratio for *Mad2* was 0.31.

The length of the MAD2 proteins was conserved for isolates within the PARB clade, which included *M. pingshaense*, *M. robertsii*, and *M. brunneum*, as well as Ontario isolates of *M.*

guizhouense, with a length of 306 amino acids. ARSEF 6238 (*M. guizhouense*) has the longest MAD2 protein at 310 a.a. *M. majus* and *M. lepidiotae* have a MAD2 protein length of 305 and 307 a.a., respectively. Overall, *M. acridum* has the shortest MAD2 protein at 295 a.a., including a 12 amino acid deletion directly after the Thr-rich tandem repeats present in domain B.

Analysis of the *Mad2* promoter regions revealed differences in putative transcriptional elements. A stress responsive element (STRE) (AGGGG) was present twice within all species, except for *M. robertsii* and *M. acridum* isolates. *M robertsii* and *M. acridum* possessed a post-diauxic shift (PDS) element (AAGGGA) in place of the second STRE copy (upstream location -109 in *M. robertsii*). Interestingly, a degenerative TATA box (TATG) was present in the promoter of *M. robertsii* as a repeat sequence, containing five repeats (upstream location -604). *M. pingshaense* contained three repeats in this region, while *M. majus* and *M. guizhouense* contained one. *M. brunneum*, *M. acridum*, and *M. lepidiotae* all lacked a TATG repeat in this region. This degenerative TATA box was also present prior to a TATA box-like sequence (TACATA) in isolates of the PARB clade, which included *M. pingshaense*, *M. robertsii*, and *M. brunneum* (upstream location -266 in *M. robertsii*). The TATA-box-like sequence was also present within the promoter region of the MGT isolates, which included *M. majus* and *M. guizhouense*, although they lacked the degenerative TATA box. *M. lepidiotae* had two TATG repeats in this region, while *M. acridum* had one TATG sequence present. *M. acridum* and *M. lepidiotae* lacked the TATA-box-like sequence.

3.4.3. Phylogenetic analysis of 5' EF-1α, *Mad1*, and *Mad2*

The 5' EF-1α phylogenetic tree for all fourteen isolates segregated according to species, including the division of the PARB clade, which included isolates of *M. pingshaense*, *M.*

robertsii, and *M. brunneum*, and the MGT clade, which included isolates of *M. majus*, and *M. guizhouense* (Fig. 7).

The phylogenetic trees for the *Mad1* and *Mad2* full gene sequences also formed divisions that were consistent with the PARB and MGT clades (Fig. 8 and 9). The PARB clade isolates all formed species-specific nodes consistent with their 5' EF-1α identification, however, in the *Mad1* tree *M. pingshaense* and *M. brunneum* were grouped together (Fig. 8), while *M. pingshaense* and *M. robertsii* grouped together in the 5' EF-1α and *Mad2* trees (Fig. 7 and 9). In the *Mad1* and *Mad2* phylogenetic trees, ARSEF 6238 (*M. guizhouense*) and ARSEF 1914 (*M. majus*) grouped together to form a separate node from the Ontario isolates of *M. guizhouense* within the MGT clade. Overall, the *Mad2* tree had the best resolution, with the highest bootstrap values for each node.

The congruency indices (I_{cong}) calculated for both trees derived from the promoter regions of *Mad1* and *Mad2* in comparison to 5' EF-1α were each 2.03 ($p = 1.66 \times 10^{-6}$) (Table 2). That is, the phylogenetic trees of the *Mad1* and *Mad2* promoter regions were equally congruent to the phylogenetic tree for 5' EF-1α. The maximum agreement subtree (MAST), for 5' EF-1α and the phylogenetic trees of the promoter regions each contained 11 terminal nodes in order for perfect congruence to occur (Table 2). The phylogenetic trees derived from the full gene DNA sequence of *Mad1*, as well as trees derived from the open reading frame DNA sequence and protein sequence were equally congruent to the 5' EF-1α tree. In each case, the congruency index was 1.66 ($p = 2.31 \times 10^{-4}$). In each pairwise tree comparison the maximum agreement subtree (MAST) for 5' EF-1α and each of the *Mad1* trees (full gene DNA sequence, open reading frame DNA sequence, and protein sequence) all contained 9 terminal nodes. For the 5' EF-1α tree and *Mad2* trees derived from the full gene DNA sequence, open reading frame DNA sequence, and

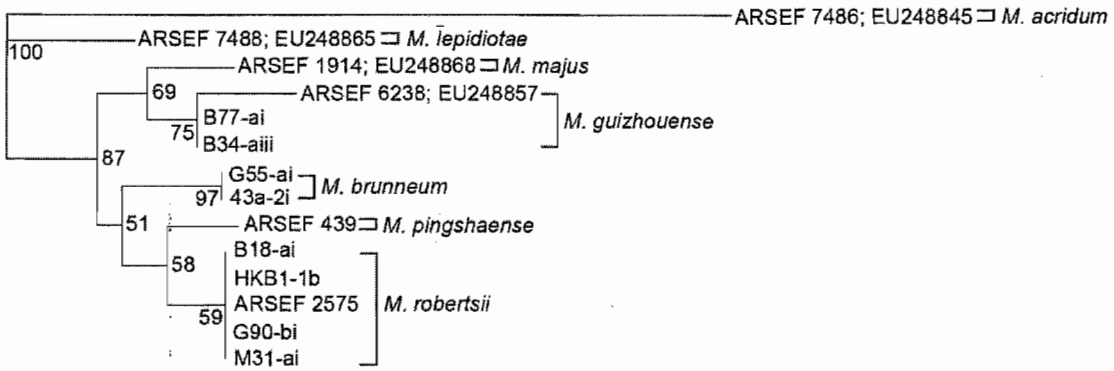

Figure 7. Maximum parsimony (MP) phylogenetic tree of 5' EF-1α sequences of *Metarhizium* isolates. Bootstrap values are based on 1000 pseudoreplicates.

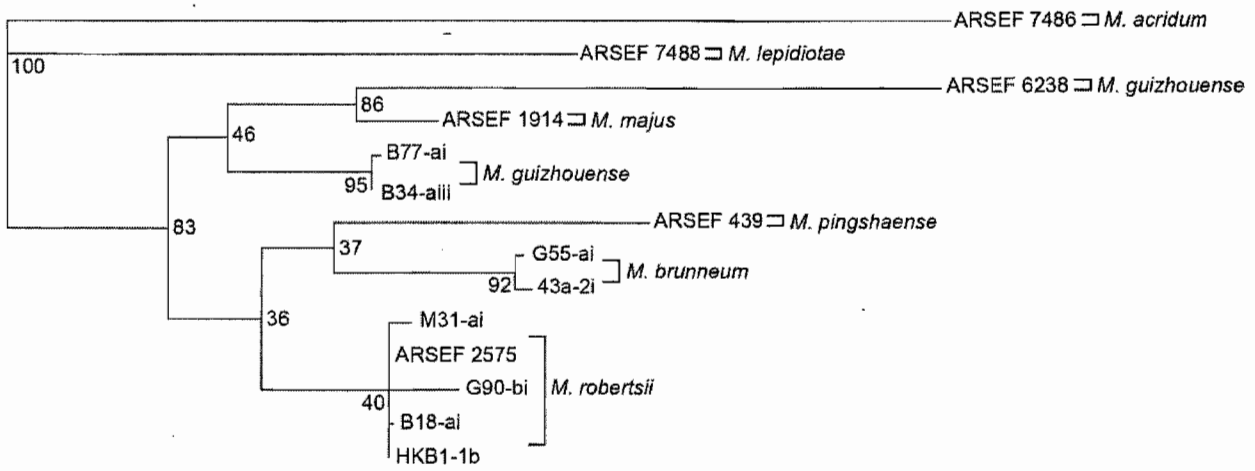

Figure 8. Maximum parsimony (MP) phylogenetic tree of *Mad1* full gene sequences of *Metarhizium* isolates. Bootstrap values are based on 1000 pseudoreplicates.

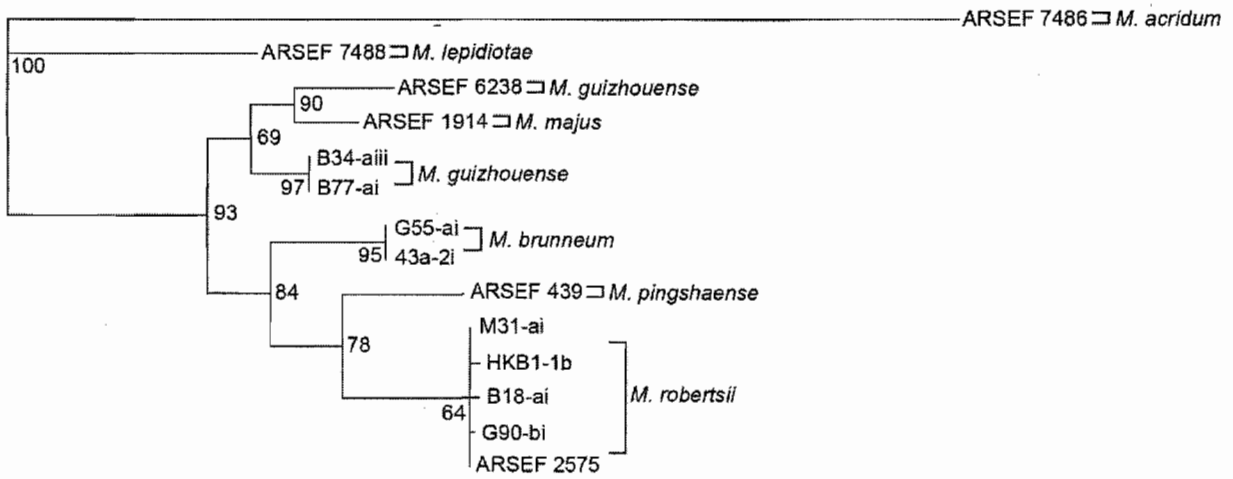

Figure 9. Maximum parsimony (MP) phylogenetic tree of *Mad2* full gene sequences of *Metarhizium* isolates. Bootstrap values are based on 1000 pseudoreplicates.

Table 2 – Congruency values for pairwise comparisons of *Mad1* and *Mad2* phylogenetic trees to the 5' EF-1α phylogenetic tree.

		Promoter region* (DNA)	ORF (DNA)	ORF (Protein)	Full gene** (DNA)
Mad1	I-cong	2.03	1.66	1.66	1.66
	p-value	1.66×10^{-6}	2.31×10^{-4}	2.31×10^{-4}	2.31×10^{-4}
	MAST	11	9	9	9
Mad2	I-cong	2.03	1.84	1.84	1.84
	p-value	1.66×10^{-6}	1.96×10^{-5}	1.96×10^{-5}	1.96×10^{-5}
	MAST	11	10	10	10

*~800 base pair DNA sequence prior to open reading frame (ORF).
**Promoter and open reading frame DNA sequences combined.

protein sequence, I_{cong} for each pairwise comparison was calculated to be 1.84 ($p = 1.96x10^{-5}$). The MAST for 5' EF-1α and the *Mad2* trees all contained 10 terminal nodes. The topology of the phylogenetic trees, as well as a higher I_{cong} and MAST (Table 2), indicated that the *Mad2* phylogenetic trees were more congruent with the 5' EF-1α tree than the *Mad1* trees.

3.5. DISCUSSION

Here, we amplified and cloned the full *Mad1* and *Mad2* genes in fourteen isolates of seven different species of *Metarhizium* in order to assess the gene variability. *M. acridum*, the acridid-specific pathogen (Gao *et al.*, 2011; Wang *et al.*, 2011), was found to have relatively more insertions and deletions within the open reading frames of *Mad1* and *Mad2*, respectively, specifically within the Thr-rich tandem repeat region in domain B of both proteins. *Mad2* variability between species was identified within putative transcriptional elements, including STRE, PDS, the degenerative TATA box, and TATA box-like regions. Additionally, phylogenetic analysis of 5' EF-1α, *Mad1*, and *Mad2* revealed that the evolution of the *Mad2* gene was more congruent with the phylogeny of 5' EF-1α than *Mad1*, suggesting plant host, rather than insect host, was a probable influence in the divergence among *Metarhizium* species.

In general, it was found that *Mad1* and *Mad2* were largely conserved within a species. However, intraspecies variation for *M. guizhouense* was high in comparison to *M. robertsii* and *M. brunneum*. This was especially notable for the *Mad1* open reading frame, in which variation within *M. guizhouense* was greater than the variation between *M. robertsii* and *M. brunneum*. However, Ontario isolates of *M. guizhouense* had very low intraspecies variation, similar to that of *M. robertsii* and *M. brunneum*. Additionally, ARSEF 6238 (*M. guizhouense*) formed a group with ARSEF 1914 (*M. majus*), separate from the Ontario isolates of *M. guizhouense* in all *Mad1*

and *Mad2* phylogenetic trees. This influenced the incongruencies in the *Mad1* and *Mad2* phylogenies when compared to the 5' EF-1α tree. This may be due to geographic divergence within *M. guizhouense*, since ARSEF 1914 and ARSEF 6238 were isolated in the Philippines and China, respectively (Bischoff *et al.*, 2009). Interestingly, Bischoff *et al.* (2009) accepted *M. majus* and *M. guizhouense* at the species rank due to congruence between conidial size and the 5' EF-1α phylogeny, although these species did not meet the molecular genealogical concordance criteria. However, Japanese isolates demonstrated that the conidial sizes of *M. majus* and *M. guizhouense* were incongruent with the 5' EF-1α phylogeny (Nishi *et al.*, 2011). This incongruence within the MGT clade warrants further investigation in order to fully resolve species ranks which may be obfuscated by population genetic differences within a *Metarhizium* species.

M. acridum, which is a species that displays insect host specificity, particularly pathogenic to acridids (grasshoppers and locusts) (Gao *et al.*, 2011; Wang *et al.*, 2011), had the longest MAD1 protein. This includes an 11 amino acid insertion that gave the *M. acridum* MAD1 protein eight tandem repeats of GKETTPAQQTTP within domain B, as opposed to the six repeats in all other isolates. This is putatively a functional difference, as it is presumed that a higher number of repeats could increase the distance between the cell wall and the N-terminal ligand binding region (Wang and St. Leger, 2007). However, when the *Mad1* gene from *M. acridum* was inserted into *M. robertsii*, there was no difference in cuticle adhesion or virulence (St. Leger, pers. comm.). Conversely, *M. acridum* had the shortest MAD2 protein, including a 12 amino acid deletion directly after the Thr-rich repeats in domain B. This may also have a functional implication that may limit its ability to associate with plants. Phylogenetic analysis of

5' EF-1α, *Mad1*, and *Mad2* also shows this species is highly divergent from other *Metarhizium* species.

Overall, genetic variation was slightly greater in the *Mad2* open reading frame (7.4%) in comparison to *Mad1* (6.7%), but noticeably higher in the *Mad2* promoter region (9.9%) in comparison to the *Mad1* promoter (7.1%). Analysis of the *Mad1* promoter did not identify any variable transcriptional elements. Future research could focus on the expression of *Mad2* between species as the expression may have a varied response due to the variation present within the promoter region. Several putative transcriptional elements have been identified within the *Mad2* promoter (Barelli et al., 2011), however, the analyses presented here focused on the variable STRE, PDS, degenerative TATA box, and TATA box-like regions. The stress response element (STRE) activates genes under various stress conditions, including glucose starvation (Marchler *et al.*, 1993; Gorner *et al.*, 1998). Similarly, the post diauxic shift (PDS) element mediates transcriptional activation in response to nutritional limitation (Boorstein and Craig, 1990; Pedruzzi *et al.*, 2000). The presence of these transcriptional elements is consistent with the finding that *Mad2* is upregulated under nutrient deprivation (Barelli *et al.*, 2011).

Interestingly, it has been found that the expression of cell wall and stress response genes evolved at an accelerated rate following the transfer of *M. robertsii* from a semitropical to a temperate soil community (Wang *et al.*, 2011). It was also found that cell wall genes with significantly altered expression were enriched for TATA boxes. Conversely, virulence determinants were unaltered (Wang *et al.*, 2011). *M. robertsii*, which has demonstrated a more generalist ability to colonize plant rhizosphere when compared to *M. brunneum* and *M. guizhouense* (Wyrebek *et al.*, 2011), contained the most TATG repeats within the degenerative TATA box region. It also contains a TATG repeat prior to the TATA box-like sequence, which

the other species lack. Whether this contributes to the generalist nature of the plant association is unknown. Also, the length of the MAD2 protein is conserved within the PARB clade, including the Ontario isolates of *M. guizhouense*. This is notable, since Ontario isolates of *M. robertsii*, *M. brunneum*, and *M. guizhouense* have shown plant rhizosphere associations (Wyrebek *et al.*, 2011).

Overall, variation within the DNA and protein sequences of the *Mad1* and *Mad2* genes, were largely species-specific. This is expected, as these genes would have diverged during speciation. However, the higher amount of variation, especially in the promoter region, suggests *Mad2* had diverged more than *Mad1*, and phylogenetic analysis indicated that *Mad2* is more congruent with the 5' EF-1α phylogeny, which is used for species identification (Bischoff *et al.*, 2009). Also, variation within the TATA box-like region of the *Mad2* promoter was conserved within a clade. This would suggest that in evolutionary terms, *Mad2*, the plant adhesin, has diverged among *Metarhizium* lineages, contributing to clade- and species-specific variation. Conversely, it appears that *Mad1* has been largely conserved. This is reflected in the average estimated dN/dS ratio, which is higher in *Mad2* (0.31) than in *Mad1* (0.20), suggesting that there is more stabilizing selection for *Mad1*, as there is a higher relative abundance of nonsynonymous mutations.

One explanation for the results observed is that the stabilizing selection for *Mad1* has reduced variation and caused incongruency with 5' EF-1α. The promoter regions are both equally congruent to 5' EF-1α. While EF-1α is highly conserved (Nakazato *et al.*, 2006), the 5' region used in these analyses contains a large portion of intronic nucleotides (>60% when aligned with GenBank Accession AAR16425). As such, the promoter regions of the *Mad* genes and the intronic regions of 5' EF-1α would both accumulate random substitutions during

evolution. *Mad2*, which has demonstrated a degree of stabilizing selection, would have fewer accumulated random mutations. Lastly, *Mad1*, which has shown more stabilizing selection and less variation than *Mad2*, would have accumulated even fewer random mutations, resulting in more incongruency with the 5' EF-1α phylogenetic tree. Previous studies on insect infection related genes (i.e. *Pr*1 and *Ntl*) have also demonstrated a high degree of stabilizing selection (Leal *et al.*, 1997; Small *et al.*, 2004).

There is evidence that plant host association may play an important role in the evolutionary divergence within the genus *Metarhizium* with the exception of the acridid-specific *M. acridum* and possibly *M. majus*, which has demonstrated specificity for Coleopteran insects, particularly scarabs (Lin and Roberts, 1986; Wang *et al.*, 2011; Wang *et al.*, 2012). Ontario species of *Metarhizium* have shown plant rhizosphere specificity (Wyrebek *et al.*, 2011) and *M. robertsii* is a plant endophyte (Sasan and Bidochka, 2012). Whole genome analysis has also suggested that the genus *Metarhizium* evolved from plant endophytes or pathogens (Gao *et al.*, 2011). While the *Mad2* plant adhesin gene showed a higher amount of variability than *Mad1*, and was more congruent with 5' EF-1α, it is difficult to ascertain whether this is due to plant relationships alone. In reality, it may be that a number of factors have contributed differentially to the evolution of various *Metarhizium* species. While other abiotic and biotic factors cannot be excluded in contributing to divergence, it appears that plant relationships has been a driving factor causing divergence among most *Metarhizium* species.

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4. Concluding Remarks

In section 1, we have demonstrated that *Metarhizium* displays plant rhizosphere specific associations. In section 2, phylogenetic analysis of the *Mad* genes has also revealed that the evolution of the *Mad2* gene is more congruent with 5' EF-1α phylogeny than *Mad1*. These main findings demonstrate that the plant life cycle of *Metarhizium* must be taken into account for effective use as a biological control agent, and also suggests that the plant environment has more of an impact on the evolution, and subsequently the population genetics, of *Metarhizium* than insect hosts.

For the purposes of biocontrol, emphasis is typically put on virulence assays, with an isolate that has high virulence against a target insect usually selected as a candidate for the biological control of that insect. However, more emphasis is required on the ecological side, examining where each isolate came from, and which one would be most appropriate to use on a specific plant type or species. For example, if one wished to protect an agriculturally important field crop, such as switchgrass, the efficacy would likely be low if an isolate other than *M. robertsii* were used. This is due to the fact that *M. robertsii* is the only species that has demonstrated an ability to colonize and associate with grass rhizosphere. A more effective means of isolating a candidate for use as a biocontrol agent would begin by randomly sampling the population of *Metarhizium* that naturally associates with the plant you wish to protect. From this sample, which will contain isolates able to persist well in that specific environment, virulence assays can then be done, in order to select the most effective isolate.

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6. Appendix A - Mad1/Mad2 percent identity raw data

Table 3 - Inter-isolate percent identity in pairwise comparisons for *Mad1* open reading frame in all fourteen studied isolates

%ident	A2575	HKB1-1b	B18-ai	M31-ai	G90-bi	A439	43a-2i	G55-ai	B34-aiii	B77-ai	A6238	A1914	A7488	A7486
A2575	---	100	99.9	99.9	99.7	96.1	97.1	97.2	96.4	96.4	93.7	96.2	94.7	88.4
HKB1-1b		---	99.9	99.9	99.7	96.1	97.1	97.2	96.4	96.4	93.7	96.2	94.7	88.4
B18-ai			---	99.9	99.6	96.1	97.0	97.1	96.4	96.4	93.7	96.2	94.7	88.3
M31-ai				---	99.6	96.1	97.0	97.1	96.4	96.4	93.6	96.1	94.7	88.3
G90-bi					---	95.9	96.8	96.9	96.2	96.2	93.4	95.9	94.4	88.1
A439						---	96.9	97.0	97.3	97.4	91.7	94.2	94.4	88.5
43a-2i							---	99.7	96.8	96.8	92.6	95.3	93.7	89.1
G55-ai								---	96.9	96.9	92.7	95.3	93.8	89.1
B34-aiii									---	99.9	94.2	96.3	95.2	89.2
B77-ai										---	94.3	96.4	95.3	89.3
A6238											---	94.7	91.7	85.8
A1914												---	93.6	87.7
A7488													---	88.7
A7486														---

Table 4 - Interspecies percent identity for *Mad1* open reading frame, calculated by averaging inter-isolate percent identities across each species. Intraspecies percent identity in parentheses.

	M. rob	*M. pin*	*M. bru*	*M. gui*	*M. maj*	*M. lep*	*M. acr*
M. robertsii	(99.81)	96.06	97.05	95.45	96.12	94.64	88.3
M. pingshaense		---	96.95	95.47	94.2	94.4	88.5
M. brunneum			(99.7)	95.45	95.3	93.75	89.1
M. guizhouense				(96.13)	95.8	94.07	88.1
M. majus					---	93.6	87.7
M. lepidiotae						---	88.7
M. acridum							---

Interspecies average percent identity (excluding intraspecies percent identity) = 93.27% (Variation = 6.73%)

Table 5 - Inter-isolate percent identity in pairwise comparisons for *Mad1* promoter region in all fourteen studied isolates

%ident	A2575	HKB1-1b	B18-ai	M31-ai	G90-bi	A439	43a-2i	G55-ai	B34-aiii	B77-ai	A6238	A1914	A7488	A7486
A2575	---	99.9	99.9	99.8	99.8	97.5	95.4	95.5	97.0	96.8	92.8	96.8	90.6	90.1
HKB1-1b		---	100	99.9	99.9	97.6	95.5	95.7	97.1	96.9	92.9	96.9	90.8	90.2
B18-ai			---	99.9	99.9	97.6	95.5	95.7	97.1	96.9	92.9	96.9	90.8	90.2
M31-ai				---	99.8	97.5	95.4	95.5	97.0	96.8	92.8	96.8	90.6	90.1
G90-bi					---	97.5	95.4	95.5	97.0	96.8	92.8	96.8	90.6	90.1
A439						---	94.7	94.8	96.3	96.0	92.0	96.0	89.7	89.4
43a-2i							---	99.9	96.8	96.5	92.4	96.4	90.4	89.9
G55-ai								---	96.9	96.6	92.5	96.5	90.5	90.0
B34-aiii									---	99.7	94.5	98.7	91.7	91.3
B77-ai										---	94.2	98.5	91.5	91.0
A6238											---	95.1	90.9	87.2
A1914												---	92.0	91.0
A7488													---	86.9
A7486														---

Table 6 - Interspecies percent identity for *Mad1* promoter region, calculated by averaging inter-isolate percent identities across each species. Intraspecies percent identity in parentheses.

	M. rob	*M. pin*	*M. bru*	*M. gui*	*M. maj*	*M. lep*	*M. acr*
M. robertsii	(99.88)	97.54	95.51	95.57	96.84	90.68	90.14
M. pingshaense		---	94.75	94.77	96.0	89.7	89.4
M. brunneum			(99.9)	95.28	96.45	90.45	89.95
M. guizhouense				(96.13)	97.43	91.37	89.83
M. majus					---	92.0	91.0
M. lepidiotae						---	86.9
M. acridum							---

Interspecies average percent identity (excluding intraspecies percent identity) = 92.93% (Variation = 7.07%)

Table 7 - Inter-isolate percent identity in pairwise comparisons for *Mad2* open reading frame in all fourteen studied isolates

%ident	A2575	HKB1-1b	B18-ai	M31-ai	G90-bi	A439	43a-2i	G55-ai	B34-aiii	B77-ai	A6238	A1914	A7488	A7486
A2575	---	99.8	99.9	100	99.9	96.4	97.2	97.2	97.2	97.2	95.8	97.1	93.7	84.3
HKB1-1b		---	99.7	99.8	99.7	96.2	97.0	97.0	97.0	97.0	95.6	96.9	93.5	84.1
B18-ai			---	99.9	99.8	96.3	97.1	97.1	97.1	97.1	95.7	97.0	93.6	84.3
M31-ai				---	99.9	96.4	97.2	97.2	97.2	97.2	95.8	97.1	93.7	84.3
G90-bi					---	96.3	97.1	97.1	97.1	97.1	95.7	97.0	93.6	84.4
A439						---	97.0	97.0	97.0	97.0	95.6	96.4	93.8	84.4
43a-2i							---	100	97.3	97.3	95.5	96.5	93.9	85.0
G55-ai								---	97.3	97.3	95.5	96.5	93.9	85.0
B34-aiii									---	100	96.7	97.7	94.8	85.1
B77-ai										---	96.7	97.7	94.8	85.1
A6238											---	97.2	93.5	84.3
A1914												---	94.0	84.6
A7488													---	84.8
A7486														---

Table 8 - Interspecies percent identity for *Mad2* open reading frame, calculated by averaging inter-isolate percent identities across each species. Intraspecies percent identity in parentheses.

	M. rob	*M. pin*	*M. bru*	*M. gui*	*M. maj*	*M. lep*	*M. acr*
M. robertsii	(99.84)	96.32	97.12	96.65	97.02	93.62	84.28
M. pingshaense		---	97.0	96.53	96.4	93.8	84.4
M. brunneum			(100)	96.7	96.5	93.9	85.0
M. guizhouense				(97.8)	97.53	94.37	84.83
M. majus					---	94.0	84.6
M. lepidiotae						---	84.8
M. acridum							---

Interspecies average percent identity (excluding intraspecies percent identity) = 92.64% (Variation = 7.36%)

Table 9 - Inter-isolate percent identity in pairwise comparisons for *Mad2* promoter region in all fourteen studied isolates

%ident	A2575	HKB1-1b	B18-ai	M31-ai	G90-bi	A439	43a-2i	G55-ai	B34-aiii	B77-ai	A6238	A1914	A7488	A7486
A2575	---	100	99.9	100	100	96.8	94.5	94.5	94.1	94.1	93.5	93.3	88.9	81.0
HKB1-1b		---	99.9	100	100	96.8	94.5	94.5	94.1	94.1	93.5	93.3	88.9	81.0
B18-ai			---	99.9	99.9	96.6	94.5	94.5	94.1	94.1	93.4	93.3	88.9	81.0
M31-ai				---	100	96.8	94.5	94.5	94.1	94.1	93.5	93.3	88.9	81.0
G90-bi					---	96.8	94.5	94.5	94.1	94.1	93.5	93.3	88.9	81.0
A439						---	94.7	94.7	94.3	94.3	93.7	93.4	89.3	80.7
43a-2i							---	100	95.7	95.7	95.4	95.0	90.9	81.0
G55-ai								---	95.7	95.7	95.4	95.0	90.9	81.0
B34-aiii									---	100	97.8	97.7	91.6	82.5
B77-ai										---	97.8	97.7	91.6	82.5
A6238											---	98.3	91.3	82.6
A1914												---	92.2	82.7
A7488													---	82.7
A7486														---

Table 10 - Interspecies percent identity for *Mad2* promoter region, calculated by averaging inter-isolate percent identities across each species. Intraspecies percent identity in parentheses.

	M. rob	*M. pin*	*M. bru*	*M. gui*	*M. maj*	*M. lep*	*M. acr*
M. robertsii	(99.96)	96.76	94.5	93.89	93.3	88.9	81.0
M. pingshaense		---	94.7	94.1	93.4	89.3	80.7
M. brunneum			(100)	95.6	95.0	90.9	81.0
M. guizhouense				(98.53)	97.9	91.5	82.53
M. majus					---	92.2	82.7
M. lepidiotae						---	82.7
M. acridum							---

Interspecies average percent identity (excluding intraspecies percent identity) = 90.12% (Variation = 9.88%)

7. Appendix B – dN/dS raw data

Table 11 – Inter-isolate estimated Nei-Gojobori number of non- synonymous (dN) substitutions per site for *Mad1* open reading frame in all fourteen studied isolates

	A2575	HKB1-1b	B18-ai	M31-ai	G90-bi	A439	43a-2i	G55-ai	B34-aiii	B77-ai	A6238	A1914	A7488	A7486
A2575	---	0	0.000623	0.000623	0.003121	0.009576	0.010748	0.009477	0.006369	0.00573	0.012169	0.009468	0.015926	0.050274
HKB1-1b		---	0.000623	0.000623	0.003121	0.009576	0.010748	0.009477	0.006369	0.00573	0.012169	0.009468	0.015926	0.050274
B18-ai			---	0.001247	0.003747	0.010219	0.011385	0.010113	0.007009	0.00637	0.011523	0.008833	0.01657	0.05095
M31-ai				---	0.003747	0.01022	0.011387	0.010114	0.007009	0.00637	0.012816	0.010105	0.016571	0.050956
G90-bi					---	0.012799	0.013303	0.012027	0.008934	0.008293	0.014759	0.012654	0.019156	0.052993
A439						---	0.014081	0.012792	0.011522	0.010879	0.017663	0.012871	0.02194	0.051745
43a-2i							---	0.003775	0.013432	0.012788	0.01944	0.01531	0.020585	0.053975
G55-ai								---	0.012144	0.011501	0.018789	0.014024	0.019285	0.052627
B34-aiii									---	0.000633	0.009743	0.005101	0.015397	0.047245
B77-ai										---	0.00909	0.004462	0.014751	0.046573
A6238											---	0.011492	0.023485	0.052562
A1914												---	0.017297	0.048487
A7488													---	0.050558
A7486														---

Table 12 – Interspecies Nei-Gojobori dN for *Mad1*, calculated by averaging inter-isolate dN across each species. Intraspecies dN in parentheses.

	M. rob	*M. pin*	*M. bru*	*M. gui*	*M. maj*	*M. lep*	*M. acr*
M. robertsii	(0.001748)	0.010478	0.010878	0.008775	0.010106	0.016830	0.051089
M. pingshaense		---	0.013437	0.013355	0.012871	0.021940	0.051745
M. brunneum			(0.003775)	0.014682	0.014667	0.019935	0.053301
M. guizhouense				(0.006489)	0.007018	0.017878	0.048793
M. majus					---	0.017297	0.048487
M. lepidiotae						---	0.050558
M. acridum							---

Average dN for *Mad1* (excluding intraspecies dN) = 0.024482

Table 13 – Inter-isolate estimated Nei-Gojobori number of synonymous (dS) substitutions per site for *Mad1* open reading frame in all fourteen studied isolates

	A2575	HKB1-1b	B18-ai	M31-ai	G90-bi	A439	43a-2i	G55-ai	B34-aiii	B77-ai	A6238	A1914	A7488	A7486
A2575	---	0	0	0	0.003674	0.056198	0.053733	0.053709	0.044257	0.044243	0.070512	0.063533	0.12087	0.237425
HKB1-1b		---	0	0	0.003674	0.056198	0.053733	0.053709	0.044257	0.044243	0.070512	0.063533	0.12087	0.237425
B18-ai			---	0	0.003674	0.056198	0.053733	0.053709	0.044257	0.044243	0.070512	0.063533	0.12087	0.237425
M31-ai				---	0.003673	0.05618	0.053716	0.053692	0.044243	0.044228	0.070489	0.063513	0.12083	0.237339
G90-bi					---	0.058173	0.055687	0.055662	0.048199	0.048183	0.076645	0.067516	0.125165	0.242333
A439						---	0.044277	0.044257	0.054275	0.054257	0.075917	0.072877	0.119632	0.241057
43a-2i							---	0	0.040354	0.040341	0.0711	0.060034	0.124166	0.239038
G55-ai								---	0.040336	0.040323	0.071046	0.060007	0.124107	0.238916
B34-aiii									---	0.001863	0.036993	0.028559	0.102117	0.204415
B77-ai										---	0.034989	0.026612	0.099942	0.2019
A6238											---	0.042159	0.12546	0.216519
A1914												---	0.119058	0.235681
A7488													---	0.2165
A7486														---

Table 14 – Interspecies Nei-Gojobori dS for *Mad1*, calculated by averaging inter-isolate dS across each species. Intraspecies dS in parentheses.

	M. rob	*M. pin*	*M. bru*	*M. gui*	*M. maj*	*M. lep*	*M. acr*
M. robertsii	(0.001470)	0.056589	0.054108	0.053935	0.064326	0.121721	0.238389
M. pingshaense		---	0.044267	0.061483	0.072877	0.119632	0.241057
M. brunneum			(0)	0.050583	0.060021	0.124137	0.238977
M. guizhouense				(0.018461)	0.032443	0.109173	0.207611
M. majus					---	0.119058	0.235681
M. lepidiotae						---	0.216500
M. acridum							---

Average dS for *Mad1* (excluding intraspecies dS) = 0.120122

Table 15 – Inter-isolate estimated Nei-Gojobori number of non- synonymous (dN) substitutions per site for *Mad2* open reading frame in all fourteen studied isolates

	A2575	HKB1-1b	B18-ai	M31-ai	G90-bi	A439	43a-2i	G55-ai	B34-aiii	B77-ai	A6238	A1914	A7488	A7486
A2575	---	0.001499	0	0	0	0.024339	0.026625	0.026625	0.025869	0.025869	0.022787	0.024411	0.046248	0.077275
HKB1-1b		---	0.001499	0.001499	0.001499	0.025886	0.028176	0.028176	0.027419	0.027419	0.024331	0.025964	0.04784	0.079015
B18-ai			---	0	0	0.024339	0.026625	0.026625	0.025869	0.025869	0.022787	0.024411	0.046248	0.077275
M31-ai				---	0	0.024339	0.026625	0.026625	0.025869	0.025869	0.022787	0.024411	0.046248	0.077275
G90-bi					---	0.024339	0.026625	0.026625	0.025869	0.025869	0.022787	0.024411	0.046248	0.077275
A439						---	0.019684	0.019684	0.015106	0.015106	0.015111	0.01977	0.035976	0.068335
43a-2i							---	0	0.022744	0.022744	0.025838	0.027477	0.046175	0.07457
G55-ai								---	0.022744	0.022744	0.025838	0.027477	0.046175	0.07457
B34-aiii									---	0	0.012057	0.013622	0.028933	0.065723
B77-ai										---	0.012057	0.013622	0.028933	0.065723
A6238											---	0.00754	0.028943	0.060627
A1914												---	0.033728	0.071144
A7488													---	0.06807
A7486														---

Table 16 – Interspecies Nei-Gojobori dN for *Mad2*, calculated by averaging inter-isolate dN across each species. Intraspecies dN in parentheses.

	M. rob	*M. pin*	*M. bru*	*M. gui*	*M. maj*	*M. lep*	*M. acr*
M. robertsii	(0.000600)	0.024648	0.026935	0.025151	0.024722	0.046566	0.077623
M. pingshaense		---	0.019684	0.015108	0.019770	0.035976	0.068335
M. brunneum			(0)	0.023775	0.027477	0.046175	0.074570
M. guizhouense				(0.008038)	0.011595	0.028936	0.064024
M. majus					---	0.033728	0.071144
M. lepidiotae						---	0.068070
M. acridum							---

Average dN for *Mad2* (excluding intraspecies dN) = 0.039715

Table 17 – Inter-isolate estimated Nei-Gojobori number of synonymous (dS) substitutions per site for *Mad2* open reading frame in all fourteen studied isolates

	A2575	HKB1-1b	B18-ai	M31-ai	G90-bi	A439	43a-2i	G55-ai	B34-aiii	B77-ai	A6238	A1914	A7488	A7486
A2575	---	0.004011	0.004011	0	0.004011	0.071317	0.034939	0.034939	0.036976	0.036976	0.049671	0.032858	0.107735	0.264962
HKB1-1b		---	0.008043	0.004011	0.008043	0.07573	0.039159	0.039159	0.041199	0.041199	0.053963	0.037068	0.112384	0.270964
B18-ai			---	0.004011	0.008043	0.07573	0.039159	0.039159	0.041199	0.041199	0.053963	0.037068	0.112384	0.264962
M31-ai				---	0.004011	0.071317	0.034939	0.034939	0.036976	0.036976	0.049671	0.032858	0.107735	0.264962
G90-bi					---	0.07573	0.039159	0.039159	0.041199	0.041199	0.053963	0.037068	0.112384	0.259009
A439						---	0.062833	0.062833	0.07591	0.07591	0.080284	0.071668	0.13374	0.290477
43a-2i							---	0	0.041373	0.041373	0.054192	0.045754	0.098957	0.261234
G55-ai								---	0.041373	0.041373	0.054192	0.045754	0.098957	0.261234
B34-aiii									---	0	0.045509	0.037154	0.112658	0.263635
B77-ai										---	0.045509	0.037154	0.112658	0.263635
A6238											---	0.024541	0.12669	0.245704
A1914												---	0.117658	0.252613
A7488													---	0.25345
A7486														---

Table 18 – Interspecies Nei-Gojobori dS for *Mad2*, calculated by averaging inter-isolate dS across each species. Intraspecies dS in parentheses.

	M. rob	*M. pin*	*M. bru*	*M. gui*	*M. maj*	*M. lep*	*M. acr*
M. robertsii	(0.004820)	0.073965	0.037471	0.043755	0.035384	0.110524	0.264972
M. pingshaense		---	0.062833	0.077368	0.071668	0.133740	0.290477
M. brunneum			(0)	0.045646	0.045754	0.098957	0.261234
M. guizhouense				(0.030339)	0.032950	0.117335	0.257658
M. majus					---	0.117658	0.252613
M. lepidiotae						---	0.253450
M. acridum							---

Average dS for *Mad2* (excluding intraspecies dS) = 0.127877