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Genetic variability of *Metarhizium* isolates from the Ticino Valley Natural Park (Northern Italy) as a possible microbiological resource for the management of *Popillia japonica*

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ABSTRACT

The natural occurrence of entomopathogenic fungi (EPF) was investigated along the Ticino River (Ticino River Natural Park, Novara Province, Piedmont, Italy), at the center of the area of the first settlement of the invasive alien pest *Popillia japonica*. Using Zimmermann's "*Galleria* bait method", EPF were successfully isolated from 83 out of 155 soil samples from different habitats (perennial, cultivated, or uncultivated meadows, woodlands, and riverbanks). Sequencing of the 5' end of the Translation Elongation Factor 1 alfa (5'-TEF) region allowed the assignment of 94% of the isolates to *Metarhizium* spp., while 8% and 7% were assigned to *Beauveria* spp. and *Paecilomyces* spp., respectively. Four *Metarhizium* species were identified: *Metarhizium robertsii* was the most common one (61.5% of the isolates), followed by *M. brunneum* (24.4%), *M. lepidiotae* (9%), and *M. guizhouense* (5.1%). Microsatellite marker analysis of the *Metarhizium* isolates revealed the presence of 27 different genotypes, i.e., 10 genotypes among *M. robertsii*, 8 among *M. brunneum*, 5 among *M. lepidiotae,* and 4 among *M. guizhouense*. *Metarhizium brunneum* appeared to be associated with woodlands and more acid soils, while the other species showed no clear association with a particular habitat. Laboratory virulence tests against *P. japonica* 3rd instar larvae allowed the identification of one *M. robertsii* isolate that showed efficacy as high as 80.3%. The importance of this kind of study in the frame of eco-friendly microbiological control is discussed.

1. Introduction

Popillia japonica Newman (Coleoptera: Scarabeidae) (Pj) is a pest native to Japan and far eastern Russia (EPPO Global Database, https://gd.eppo.int/taxon/POPIJA) with a wide host range (more than 300 plant species, Potter and Held, 2002). In 2014, it was detected in Italy in the Ticino Valley Natural Park, a natural environment located between the Lombardy and Piedmont regions (Pavesi, 2014). Since this first report, which represents the first interception in mainland Europe, the beetle quickly spread into the new territory, damaging important crops such as corn, grapevines, plum trees, apple trees, and soya (EPPO, 2014; Marianelli et al., 2017; Santoiemma et al., 2021), and crossing the border to Switzerland in 2017 (EPPO, 2017). To date, Pj in mainland Europe is confirmed only in Northern Italy (Piedmont, Lombardy, Aosta Valley, and Emilia-Romagna Regions) and in Southern Switzerland (Canton Ticino) (EPPO Global Database, https://gd.eppo.int/taxon/POPIJA).

Popillia japonica has a one-year life cycle that includes 3 larval instars: first-instar larvae can be found in the soil from the end of June, the second instar from mid-July onwards, and the third instar can be found starting from early August. Larval abundance seems to be related to less acidic soils, especially with sandy-skeletal particles (Simonetto et al., 2022). Pj larvae feed on plant roots and organic matter all summer long and overwinter as third instar. During the following March-April, Pj larvae start feeding again, pupate (starting from mid-May), and after 2–3 weeks adults start emerging and feeding on leaves, flowers, and fruits of their numerous host plants (Marianelli et al., 2017). In contrast to North America (Althoff and Rice, 2022), in Northern Italy, Pj adults show a longer oviposition period (from May to September), with the flight peaks in July (Marianelli et al., 2017). Females mate upon emergence and lay their eggs into the first layer of the soil (up to 7.5 cm below the soil surface; Potter and Held, 2002).

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In the last decades, concerns regarding the use of chemicals in agriculture reached the attention of public opinion. On one hand, public awareness of such a problem has fostered a more conscious consumption by the consumer (e.g., organic products), and on the other hand, promoted research and application of environmentally friendly alternatives for pest control.

Biological control approaches are crucial alternatives to pesticide use and provide important components of sustainable agriculture. These approaches are applied worldwide and are usually included in integrated pest management strategies (Barbosa, 1998; Baker et al., 2020).

To make microorganisms applicable as Biological Control Agents (BCAs) it is pivotal to know their natural occurrence and ecology in respect of the environment where they are supposed to be applied or exploited (Meyling and Eilenberg, 2007).

Several entomopathogenic fungi (EPF), in particular *Metarhizium* spp. Sorok. (Hypocreales: Clavicipitaceae) and *Beauveria* sp. Vuill. (Hypocreales: Cordycipitaceae) have been tested against larvae, pupae, and adults of Pj in laboratory, semi-field, and field trials worldwide but with contrasting results (Potter and Held, 2002; Ramoutar et al., 2010; Behle et al, 2015). The treatment with a commercial product of *Beauveria bassiana* in Italy was found to be ineffective, while the trials carried out with a commercial product based on *Metarhizium* sp. appeared to be more promising and thus deserve further research (e.g., Benvenuti et al., 2019; Mori et al., 2022).

Metarhizium spp. are ubiquitous, mostly soilborne entomopathogenic fungi (Roberts and St. Leger, 2004; Inglis et al., 2019), and together with *Beauveria* spp., are the most widely used EPF in biological control (Schneider et al., 2012). In 2007, a review by de Faria and Wraight (2007) showed that the list of *Metarhizium*-based mycoinsecticides is almost completely covered by the species *M. anisopliae* (Metch.) Sorok. According to Bischoff et al. (2009), *M. anisopliae* is a species complex including at least ten different species (Kepler et al., 2014, Rehner and Kepler, 2017, Lopes et al., 2018). Following this new classification, *M. brunneum* Petch appears to be the only *Metarhizium* species developed as BCA in Europe. Moreover, only two *M. brunneum* strains (BIPESCO 5/F52 and CB 15-III, the latter with an emergency authorization) have been registered as active substances and used in commercial phytosanitary products to date in Europe (EPPO database, EU Pesticides Database).

Given the necessity to develop and implement an eco-friendly approach to cope with the spread of *P. japonica* in the newly infested territory, the aims of this study were to 1) assess the presence of indigenous EPF in the Pj-infested area, and 2) molecularly characterize the recovered isolates. Moreover, 3) the selected isolates were tested for virulence to allow the selection of promising *Metarhizium* strains for *P. japonica* biological control. To the best of our knowledge, no data are available on EPF occurrence in this region. To achieve our goal, we have sampled soils in areas recently infested by *Popillia japonica* along the Ticino River (Ticino River Natural Park, Novara Province, Piedmont, Italy) and collected EPF isolates. Since *Beauveria* and *Paecilomyces* were isolated in low percentages (see the Results section), our study focused only on the *Metarhizium* genus.

2. Materials and methods

2.1. Soil sampling and fungal isolation

A sampling campaign was performed in April-May 2017 along the western bank of the Ticino River (Novara province, Piedmont region, Northern Italy), at the center of the area invaded by *Popillia japonica*. Sampling sites included habitats such as permanent meadows and uncultivated areas (with a variable density of Pj larvae in the samples), and woodlands (without Pj larvae in the samples) within the *Popillia japonica*-infested area, where no BCAs products had previously been applied. Each sampling site was described by recording geographical coordinates, agricultural/soil management, or vegetation cover (Supplementary S1).

Soil samples were collected from a total of 155 sampling sites distributed 30 km along the Ticino River (Novara Province, Piedmont, Italy). At each sampling site, a plot of 25 m^2 was specified, within which five randomly distributed soil cores (20x25 cm and 15 cm depth) were collected. Approximately 200 cm^3 of each soil core were combined into a single 1 Kg bulk soil sample per sampling site, taken to the laboratory in a cool box, and stored at room temperature until processed as in Torrini et al. (2020). The pH of each soil sample was analyzed in the laboratory following standard procedures (Violante, 2000).

Entomopathogenic fungi were collected from soil samples using the "*Galleria* bait method" (Zimmermann, 1986). Each soil sample was thoroughly mixed, and a sub-sample of 500 g was placed in a plastic box, covered with a perforated lid, and marked with the sampling site number. Five *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae obtained from Agripetgarden s.r.l. (Italy) and five *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae from the same Company were inserted in each box. Boxes were kept at room temperature for 10 days, checked daily for dead larvae, and the soil was sprayed with distilled water when needed to avoid drying out of the soil. After a sodium hypochlorite wash (1 min) and three rinses in sterile distilled water, dead larvae with symptoms of mycosis were individually transferred to sterile plastic Petri dishes that were lined with sterile wet filter paper and sealed with lab tape and incubated at room temperature until fungal sporulation. A sterile scalpel blade was used to isolate fungi from cadavers and plate them on Rose Bengal Chloramphenicol Agar (RBCA, VWR International PBI s.r.l.). Grown colonies were then transferred to quarter-strength Sabouraud Dextrose Agar (SDA, VWR International PBI s.r.l.) plus 0.25 % Yeast Extract (YE, Sigma-Aldrich Chemie GmbH) (SDAY1/4, Liu et al., 2003) and incubated at 24 °C in the dark for two weeks. Isolates were then morphologically identified, purified as single spore colonies, and preserved as part of the Entomopathogenic Fungi Collection maintained at CREA-DC, Florence, Italy.

2.2. DNA extraction, amplification, sequencing, and analysis

DNA extraction was performed from lyophilized mycelium. For this purpose, isolates were grown on sterile cellophane sheets laid on SDAY1/4 medium for 7–10 days at 24 °C in the dark. Mycelium was collected with a sterile spatula, placed in 1.5 ml plastic microcentrifuge tubes, and lyophilized overnight in a Modulyo (Edwards High Vacuum) freeze-dryer. A small portion of lyophilized mycelium (50–100 mg) was subsequently transferred to a 2 ml screw-capped plastic tube along with about 150 mg of glass beads (1:1 mix v/v of 0.5 and 2.0 mm in diameter) and homogenized with a Precellys 24 mechanical beater (Precellys). Genomic DNA was subsequently extracted from disrupted mycelium using the Qiagen Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. One hundred micro-liters of double-distilled sterile water were used for the final elution. Diluted DNA was then lyophilized and conserved for subsequent analyses.

Multilocus genotyping (MLG) with 15 microsatellite markers of all the collected isolates was performed based on the protocols described by Mayerhofer et al. (2015). Microsatellite allele sizes were determined on an ABI 3500 Series genetic analyzer using POP-7 polymer (Applied Biosystems, Foster City, CA, USA). GenScan ROX400 (Applied Biosystems) was used as an internal size standard. Data were analyzed using GenMarker V2.4.0 (SoftGenetics, State College, PA, USA) and allele sizes were corrected according to fragment sizes of reference strains *M. brunneum* ARSEF7524 and *M. robertsii* ARSEF7532.

MLGs were assigned to species by sequencing the 5′ end of the Translation Elongation Factor 1 alfa (5′-TEF) region of one isolate per genotype and subsequent sequence alignment with confirmed *Metarhizium* reference sequences. The 5′-TEF region was amplified using primers EF1T 5′-ATGGGTAAGGARGACAAGAC-3′ and EFjmetaR 5′-TGCTCACGRGTCTGGCCATCCTT-3′ and sequenced as described by Mayerhofer et al. (2019). Sequences were deposited at GeneBank

(accession numbers from OP688426 to OP688452, Supplementary S2). Obtained sequences were aligned with reference sequences of *Metarhizium* spp. for species allocation. Reference sequences were downloaded from GenBank, and alignments were performed using Clustal-W implemented in MEGA 11.0.9 (Kumar et al., 2018) followed by manual editing. A Maximum likelihood phylogenetic analysis was performed based on the Kimura 2-parameter model with default settings in MEGA 11.0.9. Bootstrap values were determined from 1000 bootstrap iterations.

2.3. Virulence tests

A single representative of each MLG (n = 27, see results section) was assayed against 3rd instar Pj larvae collected in autumn 2019 from an infested cornfield (Novara Province, Italy) and maintained at 4 °C in native soil at the CREA-DC laboratory in Florence (Italy) until their use. Before performing the virulence test, larvae were individually transferred in plastic cups containing about 30 g of sterile soil (autoclaved twice, 121 °C, 20 min) and ryegrass seeds and acclimated at 20 °C for 4

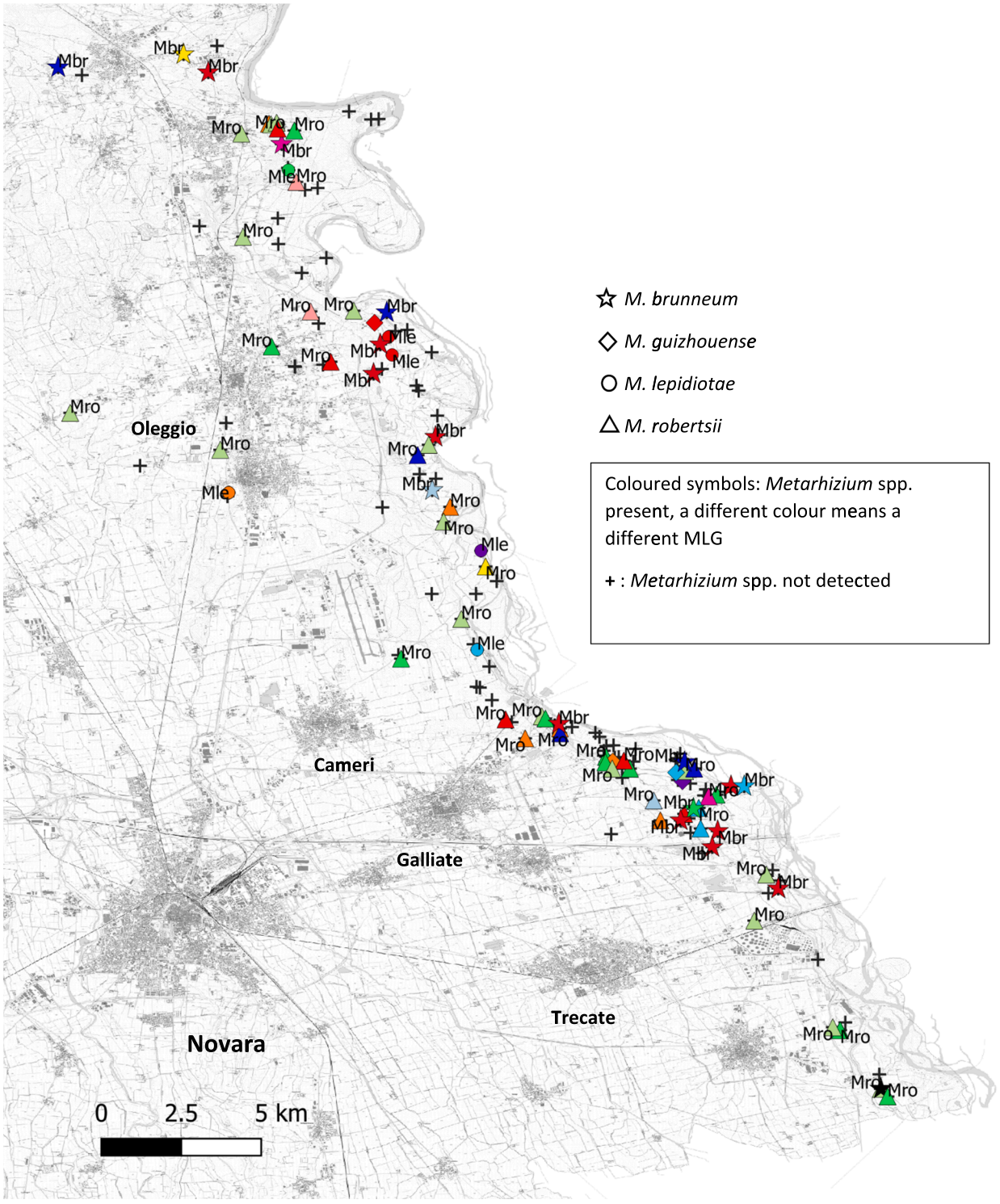

Fig. 1. *Metarhizium* species (and genotypes) isolated along the Ticino River (Piedmont region). Different colors indicate different genotypes within the four species identified (symbols in Supplementary Table S2). A cross indicates a sampling point where *Metarhizium* was not detected.

days for quarantine purposes (Torrini et al., 2020). Only healthy-looking larvae according to Koppenhöfer et al. (2012) were selected for the following tests. Sterile plastic plates (Cytoone, Starlab Int. GmbH), with 12 wells each (2.5 cm diameter, 2 cm depth) and lid, were used in laboratory trials to test the virulence of *Metarhizium* MLGs from Ticino against Pj larvae. A single larva was placed inside each well together with approximately 4 g of sterilized field soil inoculated with $1x10^8$ dry fungal conidia collected from colonies grown on SDAY1/4 overlayed with a sterile cellophane sheet. Some perennial ryegrass seeds were added to each well as food for the larva. Boxes were kept at room temperature (20–22 °C) and relative humidity (about 60 %), and larval mortality was assessed at 14 days, after which the experiment was closed. Dead larvae were individually incubated in plastic Petri dishes lined with sterile wet filter paper and sealed with lab tape and incubated at room temperature until fungal outgrowth to confirm the larvae were killed by the fungus. In 2 replicates, a total of 24 larvae were treated per *Metarhizium* MLG and the experiment was repeated twice.

2.4. Statistical analysis

A chi-square test was performed to assess the correlation between *Metarhizium* species and environment, while pH values were checked for normality and homoskedacity by the Shapiro-Wilk and Bartlett tests respectively, and then subjected to the ANOVA procedure followed by Tukey's HSD post-hoc test. Mortality data from the virulence test were corrected according to Schneider-Orelli's formula (Schneider-Orelli, 1947) to obtain efficacy values (i.e., treatment mortality corrected considering control mortality). After the arcsine transformation, percent mortality values were subjected to the ANOVA procedure followed by the Tukey HSD post-hoc test to evaluate the best-performing strain. All the analyses were performed with R statistical software version 4.1.1 (R Core Team, 2021).

3. Results

3.1. Soil sampling and fungal isolation

Seventy-eight isolates of *Metarhizium*, seven *Beauveria*, and six *Paecilomyces* were obtained from a total of 83 soil samples positive for EPF presence (Fig. 1). Three soil samples harbored 2 different *Metarhizium* species or genotypes, while in 4 cases *Metarhizium* and *Paecilomyces* and in 1 case *Metarhizium* and *Beauveria* were isolated from the same sample. *Beauveria* and *Paecilomyces* alone were isolated from 6 and 2 samples respectively. Due to their low abundance, *Beauveria* and *Paecilomyces* were excluded from further investigations in this study. *Metarhizium* spp. was present in soil samples of all habitat types sampled, the only exception was a single soil sample from cropland that yielded no EPF isolates (Table 1, Supplementary S1).

3.2. Microsatellite marker Genotyping and species affiliation

Microsatellite marker-based genotyping of the 78 *Metarhizium* isolates revealed the presence of 27 different MLGs (Supplementary S2). Seventeen MLGs were represented by single isolates, one MLG (no. 3) was shared by 18, and two (MLG2, MLG13) by 10 isolates each (Supplementary S3). The remaining MLGs were shared by 2 to 5 isolates (Supplementary S3). Genotype n° 13, which was one of the most common genotypes (10 isolates), as well as genotype n° 17 (3 isolates) were detected exclusively in soil samples from woodlands. Genotype n° 5 (2 isolates) was isolated from perennial meadow only and the remaining multiple-occurring genotypes had a variable origin (Supplementary S3).

One single isolate per MLG was selected for species affiliation. Molecular identification was performed by sequencing the 5′ end of the nuclear EF1-α gene and subsequent alignment of the sequences with sequences of 22 *Metarhizum* reference strains. This allowed the assignment of the 27 MLGs to 4 different species, namely *M. robertsii* J. F. Bisch., Rehner and Humber, *M. brunneum*, *M. lepidiotae* J. F. Bisch., Rehner and Humber, and *M. guizhouense* Q. T. Chen and H. l. Guo (Fig. 2).

Metarhizium robertsii was the most common species with 10 MLGs (37 %) and 48 (61.5 %) isolates followed by *M. brunneum*, *M. lepidiotae*, and *M. guizhouense* with 8 (30 %), 5 (19 %) and 4 (15 %) MLGs, and 19 (24.4 %), 7 (9 %), and 4 (5.1 %) isolates, respectively (Supplementary S3 and Table 1). The most common genotype (MLG 3, 18 isolates) was identical to that of the reference strain Ma500 (ARSEF7532, MLG 3). A single isolate had the same MLG as BIPESCO 5 strain (MLG 24), while all four *M. guizhouense* isolates had unique genotypes.

Metarhizium robertsii was also the most widespread species, having been isolated from each habitat, while *M. brunneum* appeared to be associated with woodlands ($\chi^2 = 14.651$, df = 1, p-value < 0.001) (Table 1, Supplementary S3). Furthermore, *M. robertsii* was isolated from soils with a wider pH range (3.7 – 6.19, mean 5.09), *M. guizhouense* was isolated from soils with a narrow pH range (4.56 – 5.78, mean 5.19), and *M. brunneum* was isolated from significantly more acid soils (3.48 – 5.24, mean 4.21; $F_{(3, 74)} = 9.4677$, p-value < 0.0001; Fig. 3, Supplementary S3).

3.3. Virulence tests

The isolates tested for virulence showed a highly variable efficacy against Pj larvae among and within species, with significantly different results ($F_{(27, 40)} = 3.75$, p-value < 0.0001) ranging from ineffective to 80 % efficacy (Table 2). *Metarhizium robertsii* (efficacy range 80.3 % − 1.2 %) appeared to be the most effective species with one isolate (MLG no. 2) reaching 80.3 %, one 78.1 %, and two more with over 60 % of efficacy (Table 2). Two *M. lepidiotae* isolates (range 70.4 % − 25.9 %) achieved 70 % efficacy, while one *M. brunneum* isolate reached almost 68 %, and two more exceeded 50 % (range 67.8 % - −8.7 %). *Metarhizium guizhouense* isolates (range 40.7 % - −3.8 %) generally exhibited low virulence (<41 %, Table 2).

Table 1
Metarhizium species and number of isolates obtained in each habitat (meadows, woodlands, riverbanks, croplands). *Metarhizium* species: Mro = *M. robertsii*, Mbr = *M. brunneum*, Mle = *M. lepidiotae*, Mgu = *M. guizhouense*.

	No. of sites	Mro	Mbr	Mle	Mgu	Total isolates
Meadows	72	28	1	3	3	**35**
Woodlands	79	18	18	4	1	**41**
Riverbanks	3	2	–	–	–	**2**
Croplands	1	–	–	–	–	**–**
Total	**155**	**48**	**19**	**7**	**4**	**78**

4. Discussion and conclusions

In our work, the *Metarhizium* genus was detected and isolated from approximately 50 percent of the soil samples collected along the Western Ticino riverbank. The genus was represented by four species: *M. robertsii*, *M. brunneum, M. lepidiotae,* and *M. guizouhense.* To the best of our knowledge, this is the first time that the combination of all these four species has been found in a single study area. According to Quesada-Moraga et al. (2007), *Metarhizium* species predominated in soils with pH lower than 7, as found in our study area, and the low abundance of the other EPF (e.g., *Beauveria*) could be linked to the acidic soils, but this topic deserves further investigations.

Several studies have indicated a heterogenous distribution of EPF in natural, semi-natural, or cultivated habitats (Bidochka et al., 1998; Meyling and Eilenberg, 2007; Quesada-Moraga et al., 2007; Garrido-Jurado et al., 2015, Fernández-Bravo et al., 2021). In particular, *Metarhizium* species have been reported to be associated with arable land, grassland, and forests (Bidochka et al., 2001; Meyling and Eilenberg, 2007; Schneider et al., 2012; Keyser et al., 2015; Fernández-Bravo et al.,

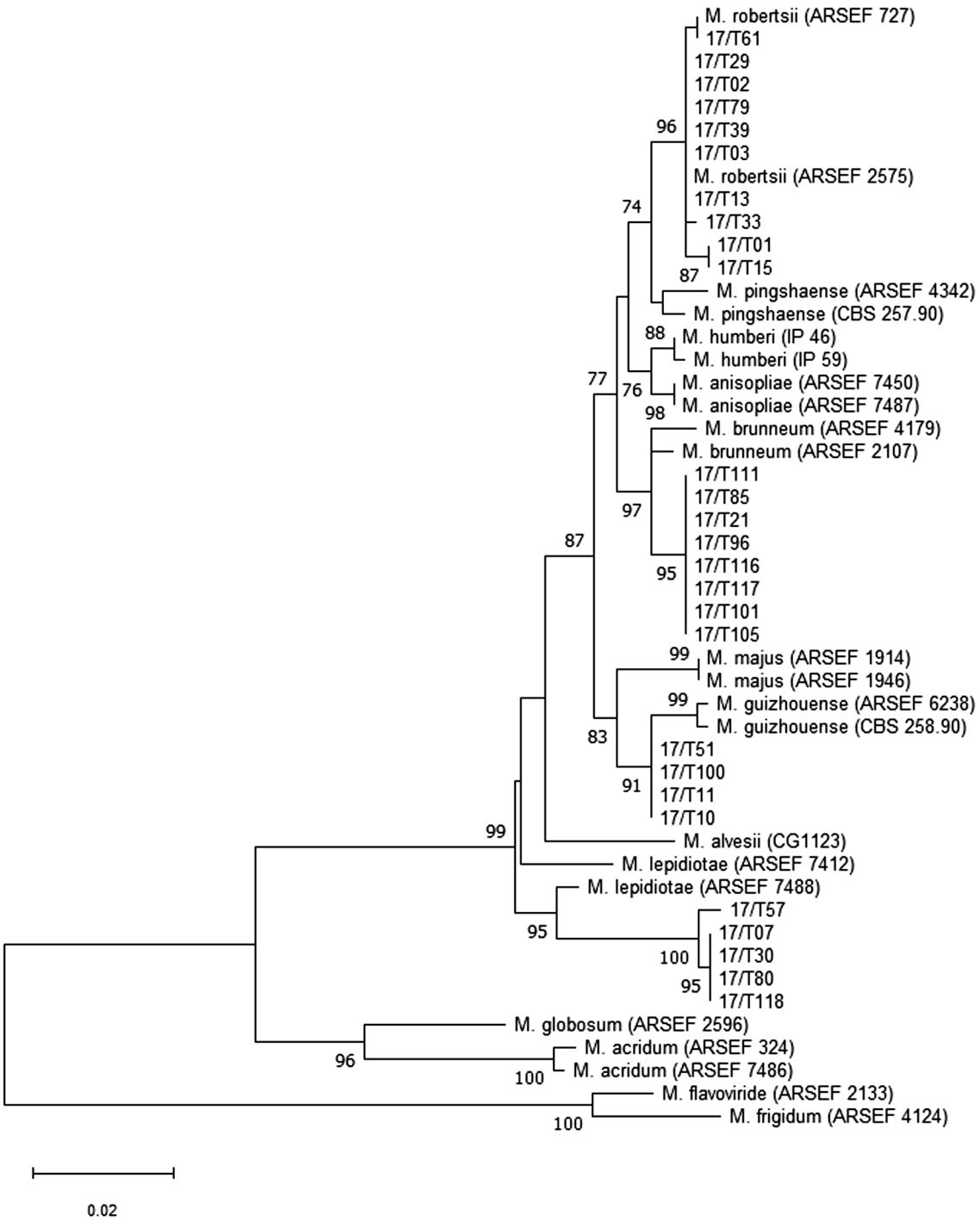

Fig. 2. Maximum likelihood phylogenetic tree based on the alignment of 5′-TEF-1α sequences of *Metarhizium* isolates representing the 27 different multilocus genotypes (MLG) and 22 reference strains. Bootstrap values >70 %, calculated with 1000 replicates, are shown. The bar scale indicates 0.02 changes per nucleotide.

2021), but also with different types of plants (such as grasses, shrubs, and trees) or crops (Fisher et al., 2011; Wyrebek et al., 2011; Kepler et al., 2015; Steinwender et al., 2015; Cabrera-Mora et al., 2019). Some studies also report that in some cases specific *Metarhizium* genotypes have been found linked to or predominant in a particular environment (Steinwender et al., 2014; Kepler et al., 2015; Fernández-Bravo et al., 2021).

The number of species recovered in our study was similar to that reported by several other authors performing comparable studies (Wyrebek et al., 2011; Steinwender et al., 2014; Garrido-Jurado et al., 2015; Kepler et al., 2015; Keyser et al., 2015; Steinwender et al., 2015; Hernández-Domínguez and Guzmán-Franco, 2017). However, species prevalence and abundance vary according to different authors (Keyser et al., 2015; Steinwender et al., 2015; Hernández-Domínguez and Guzmán-Franco, 2017; Inglis et al., 2019). *Metarhizium robertsii* was the most common species in our survey, as reported also by Kepler et al. (2015) and Wyrebek et al. (2011). In our study *M. brunneum* was correlated with woody environments and only a single isolate was obtained from another environment, i.e., grasslands. *Metarhizium robertsii* was instead present in all the environments sampled. In contrast to our findings, Fernández-Bravo et al. (2021) reported a presence of *M. brunneum* in arable land, grasslands, and forest, and *M. robertsii* and

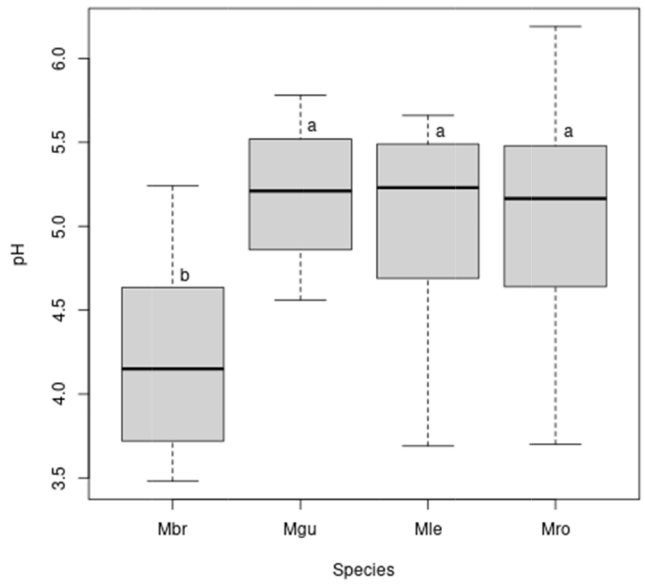

Fig. 3. Box plot illustrating the pH values of the soil samples from which isolates of the four different *Metarhizium* species were recovered. The lower and upper side of the boxes represent the first and third quartile, respectively, the line inside the box is the median, and the whiskers represent the minimum and maximum value. Boxes indicated with the same letter are not significantly different. *Metarhizium* species: Mro = *M. robertsii*, Mbr = *M. brunneum*, Mle = *M. lepidiotae*, Mgu = *M. guizhouense*.

M. guizouhense were present in arable land and grasslands only. In our study, *M. guizouhense* isolates were mostly collected from meadows, while the seven *M. lepidiotae* isolates were equally distributed between woodlands and meadows. Taken together, *Metarhizium* species distribution and abundance are highly variable among different regions, habitats, and crops. Several studies have reported correlations of specific ecological and/or human factors, such as clay content, soil pH, organic matter, C:N ratio, or soil disturbance due to cropping activities with the distribution and population structure of *Metarhizium* spp. (Quesada-Moraga et al., 2007, Meyling and Eilenberg, 2007, Schneider et al., 2012, Fernández-Bravo et al., 2021). However, a comprehensive understanding of how such factors in the context of different habitats and crops may drive *Metarhizium* abundance and diversity is still missing. Future research performing systematic studies at local and regional levels including different environmental conditions, crops as well as insect hosts combined with *meta*-analyses may allow further steps to a better understanding of the factors driving the population structure of this important fungal genus.

Microsatellite marker-based typing revealed substantial genotypic diversity among our *M. robertsii* isolates (10 MLGs among 48 isolates), which was comparable to the results reported by Kepler et al. (2015) but lower than that reported by Steinwender et al. (2015). On the other hand, we found a higher variability among our *M. brunneum* isolates compared to the variability reported by Kepler et al. (2015) and Steinwender et al. (2015). In accordance with Steinwender et al. (2014) and Kepler et al. (2015), we found that few genotypes prevail in the entire community, both for *M. robertsii* and *M. brunneum*. As shown in Supplementary S3, the two prevalent *M. robertsii* genotypes have been isolated from all the habitat types sampled and no habitat association was detected. This suggests that other factors may dominate and affect the abundance and prevalence of specific genotypes together with the habitat type. Further studies are needed to clarify these aspects, which could be very important in the perspective of the use of these *Metarhizium* genotypes as BCAs in the future.

In our lab tests, we found a high variability between and within species as regards the virulence of *Metarhizium* MLGs against 3rd instar *Popillia* larvae (Table 2). Indeed, the most effective isolate resulted to belong to an MLG of *M. robertsii* (MLG no. 2, isolate 17/T02), but within the same species, we also found isolates with low efficacy (1.2 %), as

Table 2
Virulence test results. Mean percent mortalities are reported, together with their Standard Error. Mean mortality values sharing the same letter are not significantly different. Percent efficacy has been calculated according to the Schneider-Orelli formula. The codes of the isolates used in the virulence test, the corresponding MLG number they represent, and the number of isolates sharing the same MLG are reported.

Isolate code	Genotype n°	Number of isolates	*Metarhizium* species	Mean mortality (%) ± SE		Efficacy (%) (Schneider-Orelli)
17/T02	2	10	*M. robertsii*	83.4 ± 13.2	a	80.3
17/T29	6	3	*M. robertsii*	81.5 ± 10.5	ab	78.1
17/T33	7	5	*M. robertsii*	71.0 ± 0.0	abc	65.6
17/T03	3	18	*M. robertsii*	66.7 ± 0.0	abc	60.5
17/T15	5	2	*M. robertsii*	64.5 ± 27.8	abc	57.9
17/T61	9	5	*M. robertsii*	60.0 ± 2.0	abc	52.6
17/T13	4	1	*M. robertsii*	41.7 ± 8.3	abc	30.8
17/T79	10	1	*M. robertsii*	37.5 ± 20.8	abc	25.9
17/T01	1	1	*M. robertsii*	29.2 ± 12.5	abc	16.0
17/T39	8	2	*M. robertsii*	16.7 ± 0.0	bc	1.2
17/T96	13	10	*M. brunneum*	72.9 ± 16.0	abc	67.9
17/T85	12	1	*M. brunneum*	64.7 ± 8.3	abc	58.1
17/T117	18	1	*M. brunneum*	58.3 ± 0.0	abc	50.6
17/T116	17	3	*M. brunneum*	41.7 ± 8.3	abc	30.8
17/T105	15	1	*M. brunneum*	33.3 ± 0.0	abc	20.9
17/T21	11	1	*M. brunneum*	29.2 ± 4.2	abc	16.0
17/T101	14	1	*M. brunneum*	25.0 ± 0.0	abc	11.1
17/T111	16	1	*M. brunneum*	8.3 ± 0.0	c	−8.7
17/T57	21	1	*M. lepidiotae*	75.0 ± 8.3	abc	70.4
17/T30	20	1	*M. lepidiotae*	75.0 ± 0.0	abc	70.4
17/T07	19	3	*M. lepidiotae*	50.0 ± 0.0	abc	40.7
17/T118	23	1	*M. lepidiotae*	49.5 ± 8.5	abc	40.1
17/T80	22	1	*M. lepidiotae*	37.5 ± 4.2	abc	25.9
17/T11	25	1	*M. guizouhense*	50.0 ± 4.0	abc	40.7
17/T10	24	1	*M. guizouhense*	41.7 ± 16.7	abc	30.8
17/T100	27	1	*M. guizouhense*	16.7 ± 0.0	bc	1.2
17/T51	26	1	*M. guizouhense*	12.5 ± 4.2	bc	−3.8
			Control	15.7 ± 7.5	c	

well as observed in all the other species. In accordance with our results, Bidochka et al. (2001) have reported no consistent patterns of virulence within the clonal groups they examined.

The high EPF biodiversity in this limited territory gave us the opportunity to find an interesting candidate for the microbiological control of *P. japonica* grubs in the soil. *Metarhizium robertsii* genotype no. 2 proved to be the best-performing EPF in our lab trials and, among the four *Metarhizium* species found.

The possibility of controlling an insect pest in the soil with a natural control agent is highly interesting, especially in the current perspective of quickly abandoning or limiting the use of chemicals for such purposes. To date, there are only very few *Metarhizium* strains available that are commercialized as products for pest control (e.g., *M. brunneum* strain CB15-III, against *Agriotes* spp. larvae, *M. brunneum* strain BIPESCO5, against *Popillia japonica*, *Phyllopertha horticola*, *Amphimallon* spp., *Otiorhynchus* spp.). The adaptation and use of existing products to control new and emerging pests allow to optimize and reduce efforts and costs, e.g., for registration purposes. However, due to biosafety issues, the use of indigenous EPF is preferred in the eco-friendly management of invasive alien species (e.g., Lockwood, 1993) such as *P. japonica*. Moreover, indigenous EPF are more adapted to the habitat and environment they originate from and therefore are supposed to have the best capability to survive and compete in that environment (Bidochka et al., 1998; Jackson et al., 2010). This aspect is pivotal since fungal cycling and survival are key aspects to be preserved (Bidochka et al., 2001) in the perspective of maintaining EPF active in the environment for a longer time. Furthermore, the use of indigenous isolates minimizes risks for adverse effects on non-target organisms. For instance, Mayerhofer et al. (2017) have reported that the use of an indigenous EPF strain did not affect soil microbial communities.

In conclusion, assessing local EPF diversity and testing for efficient new isolates as performed in this study is an important step to provide new resources for biological control and increase the number of available control strains.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2023.107891.

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