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MICROBIOLOGY

Studies on new isolates of native entomopathogenic fungi from the Argentinean Pampas region affecting grasshopper pest

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Abstract: Fungi are the most important entomopathogens in the natural regulation of insect populations. They have a wide distribution and significant advantages as biological control agents. Prospecting for new native strains of entomopathogenic fungi in the Pampas region has not yet been carried out. The main objectives of this study were isolating and identifying strains of different species of entomopathogenic fungi that naturally infect grasshopper species in the Pampas region and determining, through laboratory tests, their virulence and pathogenicity on nymphs of the pest grasshopper *Dichroplus maculipennis*. Furthermore, whether the phylogenetic relationships between *Beauveria bassiana* isolates would be associated with virulence and pathogenicity or geographical distribution. Thirty-four isolates of entomopathogenic fungi were obtained from different grasshopper species corresponding to eleven sites in three counties in the center of Buenos Aires province. There were 31 isolates of *B. bassiana* and 3 of Metarhizium genus. These isolates scored 66.6% (LPSc 1443) and 100% (LPSc 1441, LPSc 1445, and LPSc 1476) of mortality against third-instar nymphs of *D. maculipennis*, and these last cause death at 4.86 ± 0.20, 4.80 ± 0.17 and 5 ± 0.15 MST days, respectively. An association between phylogenetic relationships and geographical distances in *B. bassiana* was found.

Key words Biocontrol, entomopathogenic fungi, *Beauveria bassiana*, Metarhizium sp., native isolates, pathogenicity.

INTRODUCTION

Several grasshopper species are native insects of the grassland ecosystems in the Pampas region, and other grasslands. They have an important ecological role as primary consumers, as components of the trophic network, and in the cycling of nutrients and energy (Guo et al. 2006, Song et al. 2018). Nevertheless, some species constitute real agricultural pests, and during outbreak years, they can destroy various crops and compete with livestock for available forage (Branson et al. 2006, Mariottini et al. 2012, Lecoq & Zhang 2019, Carbonell et al. 2024). One of the most severe grasshopper pests is *Dichroplus maculipennis* (Blanchard 1851), mainly in areas of the Pampas and Patagonia regions, where it constitutes a significant problem to several crops and natural pastures (Carbonell et al. 2024). This species has experienced outbreaks of historical magnitude that occurred between 2008 and 2010 years in ten counties of the South of Buenos Aires province and in different areas of Patagonia (Mariottini et al. 2012, 2013, 2022). In Argentina, chemical insecticides are

still the most common tool used to control pest grasshoppers, without considering the adverse effects it causes on the environment, such as their high toxicity, low selectivity, and bioaccumulation (Gonzalez et al. 2010, Álvarez et al. 2013). The requirement for non-chemical alternatives has become increasingly important in recent years (Foster et al. 2010) due to the more significant environmental awareness, food safety concerns, and less chance of developing resistance compared to modernized chemicals (Ilondu & Enwemiwe 2020). Fungi are probably the most important entomopathogens in the natural regulation of insect populations with a wide distribution; around 700 species of entomopathogenic fungi are known worldwide (Goettel et al. 1995). However, relatively few of them have been found to affect grasshoppers (Prior & Greathead 1989). The most common of these insects are *Beauveria bassiana* (Ascomycota: Hypocreales) (Bals.-Criv.) Vuill., *Metarhizium anisopliae* (Ascomycota: Clavicipitaceae) (Metschn.) Sorokin., *Metarhizium flavoviride* (Ascomycota: Clavicipitaceae) (Gams & Rozsypal) and *Entomophaga grylli* (Zygomycota: Entomophthorales) (Fresen.) A. Batko (Goettel et al. 1995, Lacey & Brooks 1997). Entomopathogenic fungi have great importance in the biocontrol of insects because they have advantages such as high pathogenicity and specificity, adequate virulence contact transmission, *in vitro* culture capacity maintaining pathogenicity, harmless to invertebrates, the possibility of maintaining lasting biocontrol once established in the environment (Vega et al. 2012). These organisms offer benefits as sustainable biocontrol agents protecting biodiversity, soil, and human health while contributing to the reduction of the carbon footprint and mitigating the climate change (Garrido-Jurado et al. 2023). Besides, the ability of different genera of entomopathogenic fungi to colonize various host plants as endophytes provides an exciting opportunity to improve their effectiveness (Vega 2018) since pest insects can be affected by ingesting infected plants. In Argentina, various studies have been carried out in recent years on different aspects of this microorganism to reaffirm its potential as biocontrol agents (Mariottini et al. 2019, 2022, Pelizza et al. 2017, 2019, 2023). However, prospecting for new native strains of entomopathogenic fungi in the Pampas region has not yet occurred. In this sense, the main objectives of this study were isolating and identifying strains of different species of entomopathogenic fungi that naturally infect grasshopper species in the Pampas region and determining through laboratory tests, their virulence and pathogenicity on nymphs of the pest grasshopper *Dichroplus maculipennis*. Furthermore, it was analyzed whether the phylogenetic relationships between *B. bassiana* isolates would be associated with virulence and pathogenicity or geographical distribution.

MATERIALS AND METHODS

Collection of insects

Adult males and females of *D. maculipennis*, were collected with sweep nets in native grasslands of Tandil, Olavarría, Rauch, and Benito Juarez counties in the South Central of Buenos Aires province, Argentina (Fig. 1).

Samples were immediately taken to the laboratory where they were kept in groups in wire-screened cages in a rearing room under controlled conditions (30°C; 14:10. L:D photoperiod; 40% relative humidity). These insects were kept in quarantine under high-density conditions to stimulate the expression of diseases or latent infections that they could bring from the field (Madelin 1963, Shah et al. 1997).

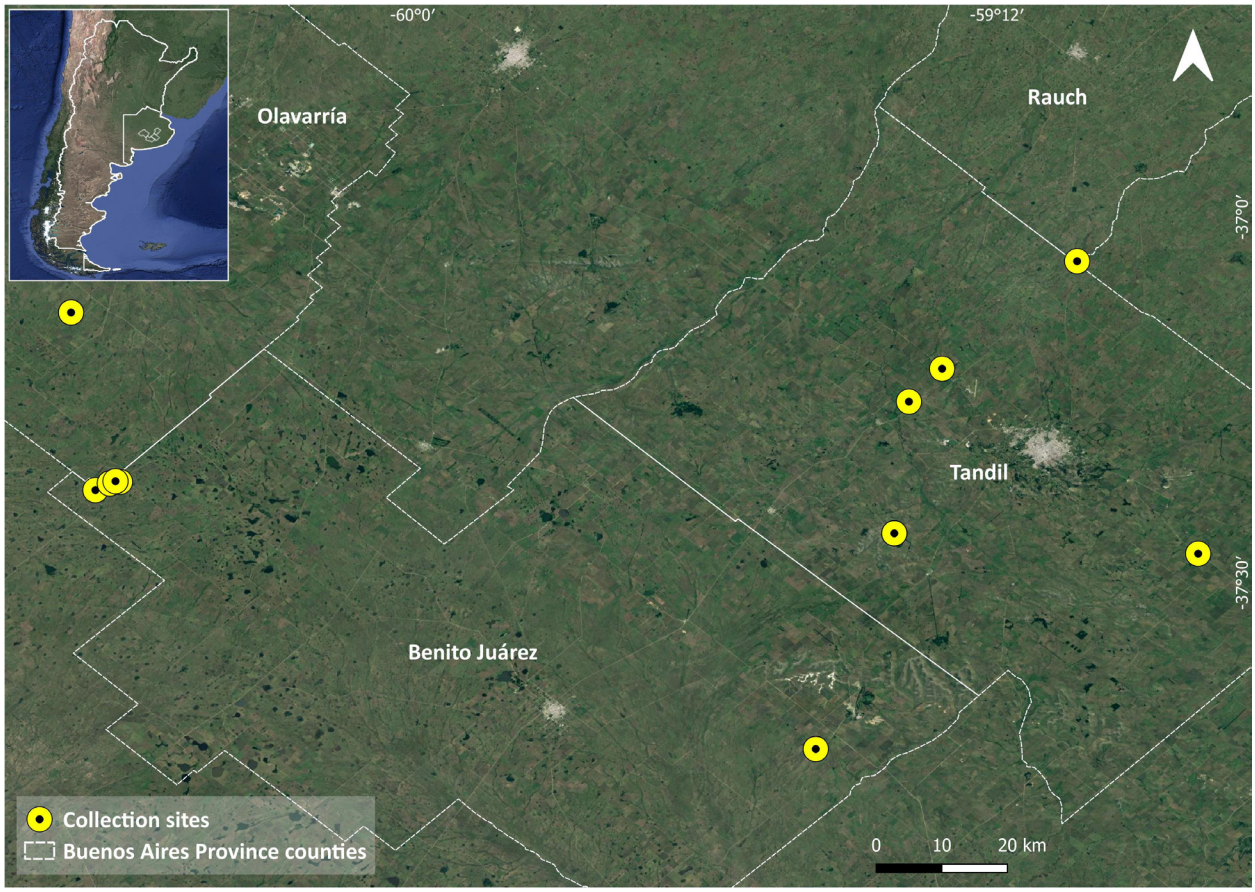

Figure 1. Location of collection sites in Olavarria, Benito Juárez, Tandil y Rauch counties, Buenos Aires province, Argentina

Fungi isolation

The entomopathogenic fungi obtained from specimens of grasshoppers collected in the field and kept in quarantine were isolated and deposited in the Spegazzini Institute culture collection. Morphological species identification was corroborated by extracting DNA of the monosporic cultures, according to Stenglein & Balatti (2006). DNA extraction was performed starting from mycelium ground with liquid nitrogen using a nucleic acid extraction kit (Qiagen. Germany) following the manufacturer's protocol. The polymerase chain reactions (PCR) were performed to amplify two genes widely used in phylogenetic analyses, the translation elongation factor-1α (TEF) and the nuclear intergenic region Bloc (Rehner et al. 2011, Khonsanit et al. 2020). PCR reactions were verified by means of agarose gel electrophoresis, and the band corresponding to each region was purified using an extraction kit (Qiagen) for subsequent sequencing through a third-party service with the company Macrogen Ltd. (Sanger Sequencing, Macrogen Ltd. South Korea).

Pathogenicity assays

Conidia from the different isolates were obtained from cultures on potato-dextrose-agar medium after incubation for ten days at 25°C in the dark. Conidia were harvested with disposable cell scrapers (Fisherbrand™) and placed in test tubes containing 0.01% (v/v) Tween

80™ (polyoxyethylene sorbitan monolaurate) (Merck). Suspensions were vortexed for 2 minutes, filtered through four layers of sterile muslin, and adjusted to $1x10^8$ conidia/ml after cell counting in a Neubauer hemocytometer. The viability of the conidia from each isolate used in the tests was determined after 24 h, as Goettel & Inglis (1997) described. This germination test was repeated for each stock suspension to maintain the constancy of the viability assessments. In all cases, the average viability of the conidia was over 95%.

Nymphs of *D. maculipennis* used in this study belonged to the first laboratory generation [F1] of specimens initially collected in the southern Pampas region, Argentina. The nymphs were kept at (30°C, photoperiod 14–10 h L-D, 40% RH) according to Mariottini et al. (2011). Three replicates (on different dates) of 10 third-instar *D. maculipennis* nymphs each were sprayed with 1 ml of a suspension containing $1x10^8$ conidia/ml (in 0.01% [v/v] Tween 80TM) through the use of a 35-ml glass atomizer. Three additional replicates of 10 grasshoppers, each to be used to control were sprayed with 1 ml of 0.01% [v/v] Tween 80™ only. The grasshoppers were kept in groups of 10 individuals in 50 x 9-cm acetate tubes with screened ends (Henry 1985) and fed with lettuce, cabbage, and wheat bran. Treated and controlled grasshoppers were maintained at 30°C, 60% relative humidity, and 14:10-h light: dark photoperiod. The cumulative mortality was recorded daily for ten days. Mycosis was confirmed by microscopic examination of dead grasshoppers.

Phylogenetic analysis

The phylogenetic analysis was conducted only on the most frequently found species *B. bassiana*. Analyses were conducted using sequences from TEF and Bloc genes for the 31 isolates, combined with reference sequences from five specimens of *B. bassiana* (Rehner et al. 2011). Detailed taxon information and GenBank accession numbers can be found in Table II. Sequences for the two gene regions were examined, trimmed, and aligned using BioEdit software (Hall 1999). The JModelTest v2.1.7 (Posada 2008) was used to infer the most appropriate model of molecular evolution for each dataset (TEF: TrN + G; Block: K80 + I) based on the Bayesian information criterion (BIC) (Schwarz 1978). The phylogenetic analysis was performed using the Metropolis-coupled Markov chain Monte Carlo (MC3) algorithm as implemented in BEAST2 v2.7.5 (Bouckaert et al. 2014). An input file for BEAST was generated using the program BEAUti v2.7.5 (Bouckaert et al. 2014). The two genetic regions were simultaneously analyzed; each partition was treated as unlinked for substitution models but as linked for clock models and trees. The analysis was run for 15.000.000 generations to ensure convergence; trees were sampled every 5000 generations, and the Coalescence Exponential Population was chosen as the tree prior. Convergence was inferred through the effective sample sizes, which were all above 200. The first 1.500.000 trees (10%) were discarded as burn-in after ensuring the likelihood scores reached a plateau, which was determined with the program Tracer 1.7.1 (Rambaut et al. 2014). The maximum clade credibility tree was calculated with TreeAnnotator v2.7.5 (Drummond & Rambaut 2007). The tree was visualized in FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/), displaying posterior probabilities as branch support.

The phylogenetic signal was evaluated for the percentage of mortality and virulence. The analyses were performed using the "phylosignal" function of the "picante" package in R, evaluating its significance through 9999 permutations.

In addition, the phylogenetic signal was analyzed by comparing the phylogenetic

relationships with the geographic distance through Mantel's test. The phylogenetic distance matrix was calculated between pairs of species using the 'ape' package versión 5.7-1 (Paradis & Schliep 2019). A distances matrix with distm {geosphere} function was generated depending on the latitude and longitude to evaluate the geographical distance between collect sites of the specimens.

Statistical analysis

For all analyses, the three replicates of each isolate were considered together. First, the summary measures associated with the mortality and the media survival time for each one of thirty-four isolates were calculated (Table I). The Kaplan-Maier curves about each isolate were estimated and analyzed.

In order to describe the behavior of the thirty-four isolates, the survival curves of each of them were analyzed. The curves were compared through the same behavior groups of isolates by means of the logarithmic ranges test of Peto & Peto (1972); this comparison is about the complete distribution.

Media survival time (MST), 95% confidence interval of the survival times per treatment, and survival curves were calculated through the Kaplan–Meier survival analysis (Surfvfit function of R). In this sense, a response was obtained for each isolate.

Since comparing so many curves is complex, the number of deaths of each of the ten days was considered for each of the forty-four isolates, using the Bray-Curtis distance. To describe and compare multivariately the isolates, we perform a Principal Coordinate Analysis (PCoA) with the mortality values of Table I.

Table I. Number of individuals of *D. maculipennis* deaths per day for each of the thirty-four isolates used. The three replicates of each isolate were considered together.

Treatment	Day									
	1	2	3	4	5	6	7	8	9	10
1411	0	0	3	4	8	11	2	1	0	0
1412	3	0	2	9	9	0	0	0	0	0
1415	0	0	1	1	5	4	8	5	0	0
1417	2	0	5	9	9	0	0	0	0	0
1428	0	0	0	2	7	7	5	3	0	0
1429	1	0	1	6	8	3	2	2	0	0
1430	0	0	1	2	10	6	2	0	1	0
1431	0	0	0	2	0	9	7	3	2	0
1432	0	0	1	5	15	5	1	0	0	0
1433	2	0	3	13	4	1	0	0	0	0
1434	0	0	1	2	13	8	5	0	0	0
1435	0	1	1	1	4	14	7	0	0	0
1436	0	0	0	3	1	8	9	0	1	0
1437	0	0	2	0	11	10	2	0	0	0
1438	0	0	1	1	1	3	8	3	4	1
1439	0	0	0	1	4	14	4	2	0	0
1440	0	0	0	3	12	4	3	0	0	0
1441	0	1	2	8	9	9	1	0	0	0
1442	0	0	1	1	3	8	6	1	1	1
1443	0	0	1	0	4	2	2	5	4	2
1444	1	0	2	6	6	4	6	2	0	0
1445	0	0	2	10	10	8	0	0	0	0
1471	0	0	0	7	5	7	5	0	1	0
1473	0	0	2	10	11	4	0	0	0	0
1474	1	0	6	17	4	0	0	0	0	0
1475	2	0	3	11	7	0	0	0	0	0
1476	0	0	0	8	16	4	2	0	0	0
1477	1	0	2	12	12	0	0	0	0	0
1478	0	0	1	4	16	6	0	0	0	0
1479	0	0	5	10	7	1	1	0	0	0
1480	0	0	3	12	7	0	0	0	0	0
1481	0	0	3	4	11	6	0	0	0	0
1487	0	0	0	0	0	1	3	9	2	8
1488	1	0	2	6	7	12	0	0	0	0

RESULTS

In this study, thirty-four isolates of entomopathogenic fungi were obtained from different grasshopper species (Table II), corresponding to eleven sites in three counties in the center of Buenos Aires province.

Thirty-one of these isolates of entomopathogenic fungi correspond to *Beauveria bassiana* species, and three of them are from the genus Metarhizium (Fig. 2).

These isolates were deposited in the Spegazzini Institute culture collection with different accession numbers (Table II).

Mortality test

In assessing the pathogenicity of the thirty-four isolates on third-instar nymphs of *D. maculipennis*, we observed that the isolates LPSc 1441, LPSc 1445, and LPSc 1476 produced 100% of mortality (Table III).

In contrast, the fungal isolate that showed the lowest mortality was LPSc 1443 with 66.7 ± 13.3%, and according to the confidence interval, they began to cause death on the eighth day (Table III). No mortality was observed in the controls.

Table II. Entomopathogenic fungi species with their respective LPSc numbers and GenBanck accession numbers, grasshopper species and geographical location from which they were isolated.

Isolate Number LPSc	Fungal isolates	*GenBank (accession numbers)	Grasshoppers' species	Location (geographical coordinates)
1411	*Beauveria bassiana*	OQ621392- OQ621360	*Dichroplus pratensis*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1412	*Beauveria bassiana*	OQ621393- OQ621361	*Dichroplus elongatus*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1415	*Beauveria bassiana*	OQ621394-OQ621362	*Scotussa lemniscata*	Don Fermín, Est. Sta. Luisa, Olavarría (37°7'50.05"S; 60°28'4.20"W)
1417	*Beauveria bassiana*	OQ621383 - OQ621347	*Borellia bruneri*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1428	*Beauveria bassiana*	OQ621396 - OQ621364	*Dichroplus elongatus*	Don Pelusa(1), Pje. El Luchador, B.Juárez(37°21'43.7"S; 60°24'03.0"W)
1429	*Beauveria bassiana*	OQ621397 - OQ621365	*Dichroplus maculipennis*	Yatay, Pje. El Luchador, B.Juárez(37°22'24.3"S; 60°26'04.3"W)
1430	*Beauveria bassiana*	OQ621378 - OQ621353	*Scotussa lemniscata*	Don Fermín, Est. Sta. Luisa, Olavarría (37°7'50.05"S; 60°28'4.20"W)
1431	*Beauveria bassiana*	OQ621398 - OQ621366	*Scotussa lemniscata*	Don Fermín, Est. Sta. Luisa, Olavarría (37°7'50.05"S; 60°28'4.20"W)
1432	*Beauveria bassiana*	OQ621399 - OQ621367	*Dichroplus pratensis*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1433	*Metarhizium guizhouense*	OQ621342	*Orphulella punctata*	El Cacique, Villa Cacique, B.Juárez (37°43'38.4"S; 59°26'52.8"W)
1434	*Beauveria bassiana*	OQ621400 - OQ621368	*Dichroplus elongatus*	Don Fermín, Est. Sta. Luisa, Olavarría (37°7'50.05"S; 60°28'4.20"W)
1435	*Beauveria bassiana*	OQ621401 - OQ621369	*Dichroplus elongatus*	Don Fermín, Est. Sta. Luisa, Olavarría (37°7'50.05"S; 60°28'4.20"W)
1436	*Beauveria bassiana*	OQ621384 - OQ621350	*Dichroplus pratensis*	El Cacique, Villa Cacique, B. Juárez(37°43'38.4"S; 59°26'52.8"W)
1437	*Beauveria bassiana*	OQ621402 - OQ621370	*Dichroplus elongatus*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)

Table II. Continuation.

Isolate Number LPSc	Fungal isolates	*GenBank (accession numbers)	Grasshoppers' species	Location (geographical coordinates)
1438	*Beauveria bassiana*	OQ621389 - OQ621357	*Dichroplus pratensis*	Las Chilcas, Tandil(37°27'37.20"S; 58°55'27.40"W)
1439	*Beauveria bassiana*	OQ621403 - OQ621371	*Dichroplus pratensis*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1440	*Beauveria bassiana*	OQ621404 - OQ621372	*Dichroplus pratensis*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1441	*Beauveria bassiana*	OQ621405 - OQ621373	*Baeacris pseudopunctulatus*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1442	*Beauveria bassiana*	OQ621386 - OQ621348	*Dichroplus pratensis*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1443	*Beauveria bassiana*	OQ621406 - OQ621374	*Borellia bruneri*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1444	*Metarhizium guizhouense*	OQ621343	*Borellia bruneri*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1445	*Beauveria bassiana*	OQ621376 - OQ621355	*Dichroplus elongatus*	Sierra Alta, Tandil (37°25'56.91"S; 59°20'25.67"W)
1471	*Beauveria bassiana*	OQ621391 - OQ621359	*Dichroplus pratensis*	La Elena, Pje. El Luchador, B.Juárez (37°21'51.4''S; 60°24'53.4''W)
1473	*Beauveria bassiana*	OQ621382 - OQ621352	*Scotussa lemniscata*	Don Pelusa (2), Pje. El Luchador, B.Juárez (37°21'51.4''S; 60°24'53.4''W)
1474	*Beauveria bassiana*	OQ621381 - OQ621356	*Dichroplus pratensis*	La Elena, Pje. El Luchador, B. Juárez (37°21'51.4''S; 60°24'53.4''W)
1475	*Beauveria bassiana*	OQ621387 - OQ621349	*Dichroplus elongatus*	Don Pelusa (2), Pje. El Luchador, B. Juárez (37°21'51.4''S; 60°24'53.4''W)
1476	*Beauveria bassiana*	OQ621388 - OQ621351	*Baeacris pseudopunctulatus*	Don Pelusa (2), Pje. El Luchador, B. Juárez (37°21'51.4''S; 60°24'53.4''W)
1477	*Beauveria bassiana*	OQ621379 - OQ621345	*Dichroplus pratensis*	La Elena, Pje. El Luchador, B. Juárez (37°21'51.4''S; 60°24'53.4''W)
1478	*Beauveria bassiana*	OQ621380 - OQ621346	*Dichroplus elongatus*	Don Pelusa (2), Pje. El Luchador, B. Juárez (37°21'51.4''S; 60°24'53.4''W)
1479	*Metarhizium robertsii*	OQ621341	*Dichroplus pratensis*	La Elena, Pje. El Luchador, B. Juárez (37°21'51.4''S; 60°24'53.4''W)
1480	*Beauveria bassiana*	OQ621390 - OQ621358	*Dichroplus elongatus*	Don Pelusa (2), Pje. El Luchador, B. Juárez (37°21'51.4''S; 60°24'53.4''W)
1481	*Beauveria bassiana*	OQ621377 - OQ621344	*Dichroplus elongatus*	Base Aérea, Tandil (37°12'26.8"S; 59°16'30.1"W)
1487	*Beauveria bassiana*	OQ621385 - OQ621354	*Dichroplus pratensis*	Gardey, Tandil (37°15'08.8"S; 59°19'13.6"W)
1488	*Beauveria bassiana*	OQ621407 - OQ621375	*Scotussa lemniscata*	La Clelia, Rauch (37°3'37.53"S; 59° 5'24.26"W)

***Access numbers providedby GenBankfor the nuclear intergenic region (Bloc)and the translation elongation factor 1-alpha (EF intron region) respectively for *Beauveria bassiana* strains.**

Figure 2. Nymphs of ***D. maculipennis*** **affected by a)** ***Metarhizium robertsii*** **and b)** ***Beauveria bassiana*****.**

The LPSc 1441, LPSc 1445, and LPSc 1476 isolates were the most efficient, with media survival time (MST) of 4.86 ± 0.20, 4.80 ± 0.17, and 5 ± 0.15 days after starting the trial, respectively (Table III, Fig. 3).

The isolates that caused the lowest MST were LPSc 1474 with 3,82 ± 0,15 days and LPSc 1433 with 3,87 ± 0,24 days after starting the test. The isolates LPSc 1438, LPSc 1443, and LPSc 1487 showed the highest MST values with 7.09 ± 0.36, 7.25 ± 0.43, and 8.57 ± 3.3 days, respectively (Table III).

Phylogenetic analyses

The phylogenetic data matrix included two loci of 37 isolates and 1775 characters after removal of ambiguously aligned and gaps (Bloc: 816, TEF: 959). The analysis evidenced two major groups, one encompasses all the local isolates except for the LPSc 1438, which is associated with the outgroups (Fig. 4). Among the local group, we can observe four highly supported clades, first the LPSc1434 isolate sister to the rest of this species, the second groups would contain the sister isolates LPSc 1436 and LPSc1471, and the third grouping would be composed of LPSc1431 and LPSc1440 isolates. The fourth group comprises all the remaining isolates with very low internal support values (Fig. 4).

The percentage of mortality and virulence did not evidence a phylogenetic signal for the mean and standard deviation values. Mantel's test showed a significant positive correlation between the phylogenetic and geographic distances (p= 0.0062).

Table III. Entomopathogenic fungi species, mean mortality in percentage with standard errors (SE), Media Survival Times (MST) and lower and upper confidence limit 95% (LCL-UCL) of all treatments.

Isolate Number	Fungal isolate	Percent mortality (means ± SE)	Media survival time (means ± SE)	0.95LCL	0.95UCL
LPSc 1411	*Beauveria bassiana*	96.7 ± 3.3	5,25 ± 0,23	5	6
LPSc 1412	*Beauveria bassiana*	76.7 ± 13.3	3,91 ± 0,27	4	5
LPSc 1415	*Beauveria bassiana*	80.00 ± 11.5	6,3 ± 0,28	6	8
LPSc 1417	*Beauveria bassiana*	83.3 ± 12	3,92 ± 0,23	4	5
LPSc 1428	*Beauveria bassiana*	80.00 ± 5.8	6,00 ± 0,24	6	8
LPSc 1429	*Beauveria bassiana*	76.7 ± 8.8	5,04 ± 0,33	5	8
LPSc 1430	*Beauveria bassiana*	73.3 ± 8.8	5,45 ± 0,26	5	9
LPSc 1431	*Beauveria bassiana*	76.7 ± 3.3	6,65 ± 0,26	6	9
LPSc 1432	*Beauveria bassiana*	90.00 ± 0.00	5,00 ± 0,16	5	6
LPSc 1433	*Metarhizium guizhouense*	76.7 ± 18.6	3,87 ± 0,24	4	5
LPSc 1434	*Beauveria bassiana*	96.7 ± 3.3	5,48 ± 0,18	5	6
LPSc 1435	*Beauveria bassiana*	93.3 ± 3.3	5,79 ± 0,23	6	7
LPSc 1436	*Beauveria bassiana*	73.3 ± 8.8	6,23 ± 0,25	6	9
LPSc 1437	*Beauveria bassiana*	83.3 ± 8.8	5,40 ± 0,19	5	6
LPSc 1438	*Beauveria bassiana*	73.3 ± 3.3	7,09 ± 0,36	7	NA
LPSc 1439	*Beauveria bassiana*	83.3 ± 12	6,08 ± 0,18	6	7
LPSc 1440	*Beauveria bassiana*	73.3 ± 3.3	5,32 ± 0,19	5	7
LPSc 1441	*Beauveria bassiana*	100.00 ± 0.00	4,86 ± 0,20	4	6
LPSc 1442	*Beauveria bassiana*	73.3 ± 14.5	6,32 ± 0,32	6	10
LPSc 1443	*Beauveria bassiana*	66.7 ± 13.3	7,25 ± 0,43	8	NA
LPSc 1444	*Metarhizium guizhouense*	90.00 ± 10	5,30 ± 0,32	5	7
LPSc 1445	*Beauveria bassiana*	100.00 ± 0.00	4,80 ± 0,17	4	5
LPSc 1471	*Beauveria bassiana*	83.3 ± 8.8	5,56 ± 0,27	5	7
LPSc 1473	*Beauveria bassiana*	90.00 ± 10	4,63 ± 0,16	4	5
LPSc 1474	*Beauveria bassiana*	93.3 ± 3.3	3,82 ± 0,15	4	4
LPSc 1475	*Beauveria bassiana*	76.7 ± 18.6	3,91 ± 0,23	4	5
LPSc 1476	*Beauveria bassiana*	100.00 ± 0.00	5,00 ± 0,15	5	5
LPSc 1477	*Beauveria bassiana*	90.00 ± 5.8	4,26 ± 0,17	4	5
LPSc 1478	*Beauveria bassiana*	90.00 ± 5.8	5,00 ± 0,14	5	6
LPSc 1479	*Metarhizium robertsii*	80.00 ± 10	4,29 ± 0,20	4	5
LPSc 1480	*Beauveria bassiana*	73.3 ± 3.3	4,18 ± 0,14	4	5
LPSc 1481	*Beauveria bassiana*	80.00 ± 10	4,83 ± 0,20	5	6
LPSc 1487	*Beauveria bassiana*	76.7 ± 3.3	8,57 ± 0,26	8	10
LPSc 1488	*Beauveria bassiana*	93.3 ± 3.3	4,93 ± 0,24	5	6

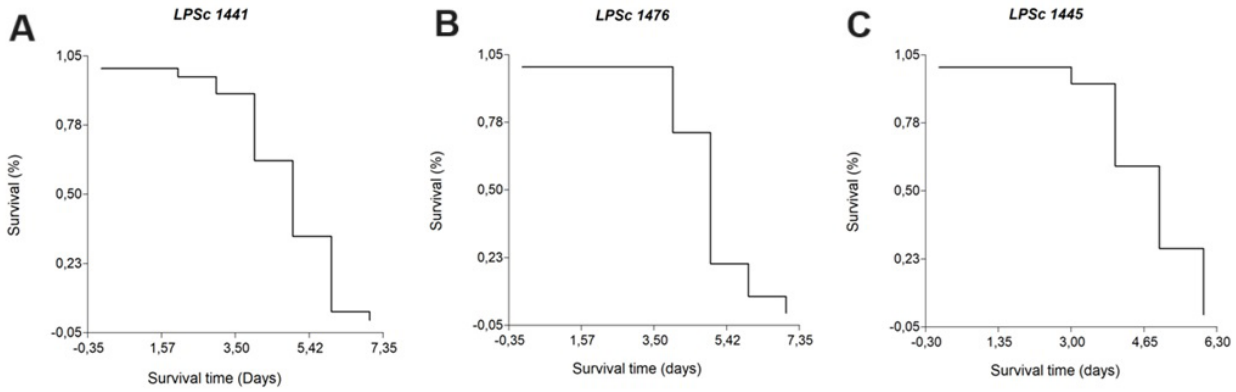

Figure 3. Survival curves (Kaplan-Meier) of most efficient isolates: a) LPSc 1441, b) LPSc 1476, and c) LPSc 1445.

Bray Curtis multivariate analysis

Principal Coordinates analysis allowed us to compare the behaviors of fungal isolates. We identified three groups with clear behaviors. Firstly, a group composed of isolates LPSc 1474, LPSc 1433, LPSc 1479, LPSc 1475, LPSc 1480, LPSc 1417, LPSc 1412, and LPSc 1477 that causes the highest mortality on the first days of the trial until the sixth day. Secondly, a group composed of isolates LPSc 1487, LPSc 1438, LPSc 1431, LPSc 1443, and LPSc 1415 presented extreme behaviors, causing the death of the third-instar nymphs of *D. maculipennis* since the fifth day of the trial.

The rest of the isolates presented an average behavior. Therefore, they were analyzed separately to find a pattern. We observed that the isolates LPSc 1428, LPSc 1442, LPSc 1439, LPSc 1436, and LPSc 1437 could be a third group with similar behaviors causing the highest mortality from day 4 to day 7 of the trial (Fig. 5).

DISCUSSION

Entomopathogenic fungi (especially *M. anisopliae* and *B. bassiana*) are the main microorganisms used to control different species of grasshoppers and locust pests (Lomer et al. 2001, Mariottini et al. 2022, Pelizza et al. 2023). So far, eleven fungal formulations are commercially available to control grasshoppers and locusts in different regions of the world (de Faria & Wraight 2007). Green Muscle and Green Guard on the basis of *Metarhizium acridum* (Driv. & Miln.) Bisch., Rehn. and Humb, are the most popular formulations. Recently, antilocust mycoinsecticides Mycolar B (*Beauveria bassiana* (Bals.-Criv.) Vuill.) and Mycolar M (*Metarhizium anisopliae* (Metschn. Sorok.) have been developed by All-Russian Institute of Plant Protection in collaboration with colleagues from Kazakhstan. In this work, it was possible to find 34 new isolates of entomopathogenic fungi that were found in different grasshopper species in several sites in Buenos Aires province. There have been few records in Argentina of entomopathogenic fungi that naturally affect different species of grasshopper pests (Pelizza et al. 2010, 2018).

For the first time, this work prospected entomopathogenic fungi species that naturally infect grasshoppers in the Pampas Region. We found entomopathogenic fungi isolates in each of the eleven sampling sites, which suggests that they could be common natural enemies of grasshoppers in this region.

Isolates LPSc 1441, LPSc 1445, and LPSc 1476 of entomopathogenic fungi *B. bassiana* ended

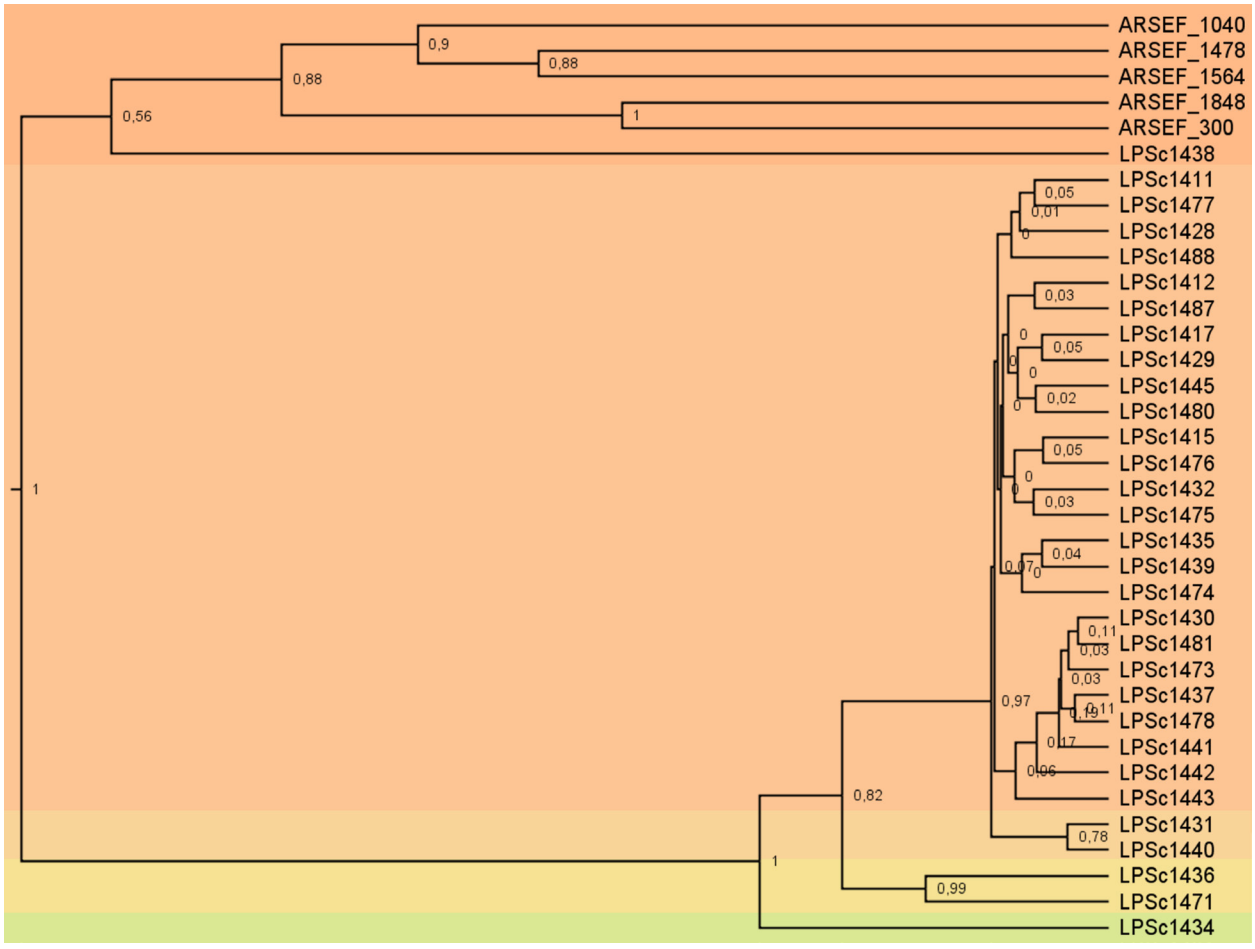

Figure 4. Coalescence Exponential Population tree based on the combined translation elongation factor 1-α (TEF) and intergenic Bloc region of the *Beauveria bassiana* isolates. Reference sequences (ARSEF_1040, ARSEF_1478, ARSEF_1564, ARSEF_1848, ARSEF_300) proceed from Serna-Domínguez et al. (2019).

up being the most efficient to control the third-instar nymph of *D. maculipennis*, since they caused a 100% mortality with MST values around the fifth day after starting the trial. The isolate LPSc 1441 is the most virulent due to its lower MST (Fig. 3).

Likewise, the isolates LPSc 1474 and LPSc 1477 caused less mortality (ca. 90 %), nevertheless they also had good potential since they presented the lowest MST. They belong to the most efficient group obtained from Coordinates Principal analyses (Fig. 5). They are followed by isolated LPSc 1479 of the entomopathogenic fungi species *M. robertsii* which caused high mortality (ca. 80 %) and recorded a lower MST.

Similar results were recorded by Pelizza et al. 2023 which obtained 100% of mortality in the same species of grasshopper and with the same dose of *B. bassiana* (isolate LPSc 1067). As regards the MST, they registered 5.96 ± 0.26 days. However, in our study, the most efficient *B. bassiana* isolates (LPSc 1441, LPSc 1445 and LPSc 1476), all recorded lower MST (ca. 4.89 days).

The most efficient isolates (LPSc 1441, LPSc 1445, and LPSc 1476) were found within the group with low internal support values since no synapomorphy was found in its bases. In this

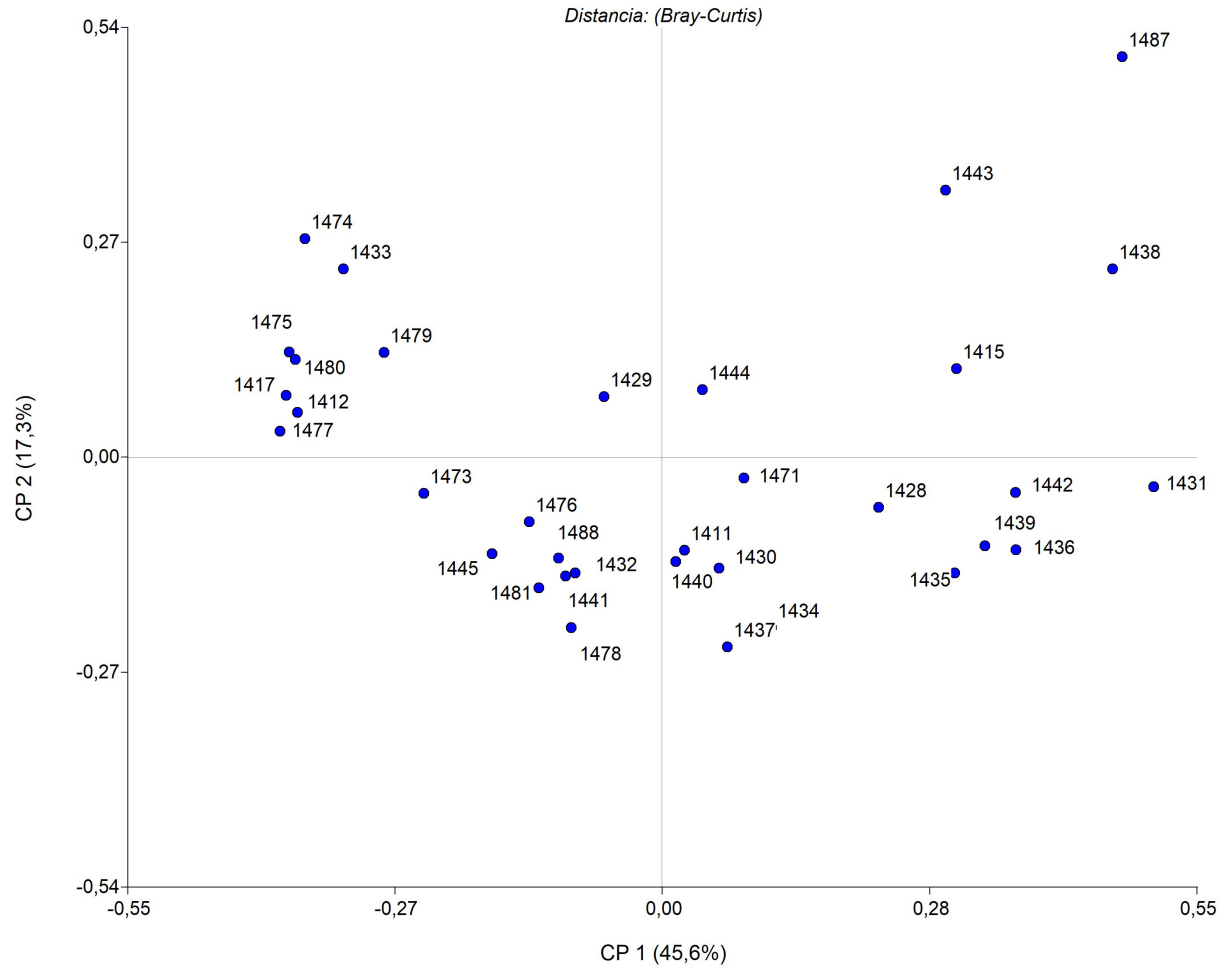

Figure 5. Principal Coordinates analysis of all isolates using the Bray Curtis distances.

sense, the isolates LPSc 1441, and LPSc 1445 were found in the same sampling site (Sierra Alta, Tandil), but they were isolated from a different grasshopper species (*Beacris pseudopunctulata* and *Dichroplus elongatus* respectively). However, LPSc 1476 was isolated from a different sampling site (Paraje El Luchador, Benito Juárez) from 90 km away and isolated from *B. pseudopunctulata*. The *B. bassiana* isolates that composed the most efficient group obtained from the Principal Coordinates analysis (LPSc 1474, LPSc 1475, LPSc 1480, LPSc 1417, LPSc 1412, and LPSc 1477) presented very low internal support values, which suggests that they could be different isolates belonging to the same strain of *B. bassiana*.

We found an association between phylogenetic relationships and geographical distances in *B. bassiana*, a pattern that has been previously mentioned in several studies (Bidochka et al. 2002, Aquino de Muro et al. 2003, Wang et al. 2005, 2013, Estrada et al. 2007, Rehner et al. 2006, Fernandes et al. 2009, Garrido-Jurado et al. 2015). However, a relationship between the phylogeny and the mortality and virulence

capacity was not evident; similar results were found by Aynalem et al. (2021).

The discovery of these native entomopathogenic fungi strains enhances our understanding of the biodiversity in the ecosystems of the Pampas region.

Due to the high mortality and short time of action, we consider that three isolates would have the potential to be biological control agents of the grasshopper pest *D. maculipennis* in the Pampas.

The knowledge obtained in this study could contribute to the development of an alternative to the commonly used chemical insecticides for pest grasshopper control. We demonstrated that the entomopathogenic fungi are effective as biological control agents for these insects. Furthermore, the pest insects used in this study and the entomopathogens that we found are naturally present in the same ecosystems.

Nevertheless, further studies are needed on the virulence of these isolates on other harmful grasshopper species such as *Dichroplus elongatus* Giglio-Tos (Melanoplinae), *Bufonacris claraziana* Saussure (Tristiridae), or *Tropidacris collaris* Stoll (Romaleidae) is necessary, and their potential applicability in other regions of Argentina where the mentioned pest grasshopper species are present. In addition, their efficiency in semi-field conditions must be tested to evaluate their potential as bio input.

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Author contributions

All authors contributed to the conception and design of the study. YM led the field sampling and biotery studies, FA led the molecular analysis, CM and RC led the statistical analysis, MCS led the phylogenic analysis, and SAP led the funding acquisition. MAM participated in all studies, wrote the first draft, and curated the data. All authors commented on and approved the final version of the manuscript.

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