


First Report of a New Isolate of *Metarhizium rileyi* from Maize Fields of Quivicán, Cuba

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Abstract *Metarhizium rileyi* (Farlow) Samson is an important entomopathogenic fungus of more than 30 species of Lepidoptera larvae. The aim of this research was to characterize isolate of *M. rileyi* from Quivicán, Cuba on the basis of morphological and molecular approaches. The fungus was isolated from samples of *S. frugiperda* larvae collected from maize fields of Quivicán municipality, Mayabeque province, Cuba, and it was cultured on PDA + Ampicillin solid media for morphological characterization. The DNA was isolated using CTAB method and internal transcribed spacer (ITS1, ITS4) were used as the primers for the amplification. The amplified products of 1335 bp were purified and sequenced at CINVESTAV-IPN in both the directions using the above primers. A consensus sequence was obtained by alignment of the forward and reverse sequences for this region and deposited in GenBank (MG637450). The fungus produced slightly cottony colony of pale green color and dispersed conidia and septal mycelium were observed under the optical microscope. A BLAST search of the sequence in GenBank revealed a 99% of identity with several strains of *N. rileyi* (e.g., AF368501.1, AB268359.1 and EU553337.1) and *M. rileyi* (e.g., KY436756.1). This is the first report of *M. rileyi*

isolate from maize fields of Quivicán in Cuba and this is important for biodiversity studies and is another possibility for Integrated Pest Management.

Keywords *Metarhizium rileyi* · Entomopathogen fungi · Biological control · Lepidoptera

Introduction

Spodoptera frugiperda (Smith) is a pathogen of many important crops like maize (*Zea mays* L.), which has a great economic importance for being the third most cultivated cereal in the world and the basic food of several hundred million people. This pathogen produce losses from 13 to 60% in maize according to Clark et al. [1] and chemical pesticides are used often for its control. Among the chemical pesticides that have been used for the control of *S. frugiperda* are phosphorates, pyrethroids and carbamates, which present toxicological categories I and II and are toxic for the environment, animal and human health [2]. Biological control is a friendly option for the control of this important pathogen and *Metarhizium rileyi* (Farlow) Samson, previously known as *Nomuraea rileyi* Farlow [3], is an example because it can regulate caterpillar populations in various crops. *M. rileyi* is a cosmopolitan species than can infect numerous noctuids such as *S. frugiperda*, *S. litura*, *Helicoverpa armigera*, *Trichoplusia ni* (Hübner), *Anticarsia gemmatilis*, *Pseudoplusia* sp. [4]. This microorganism is distributed in wide agroecosystems and it frequently can induce natural epizootics on many Lepidoptera species [5].

Morphologically *M. rileyi* is an imperfect and dimorphic fungus in its development and the color of the colony ranges from white-green pale to green intense according to

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the progress of the colony [6, 7]. The hyphae measure between 2 and 3 μm in diameter, septate, hyaline to slightly pigmented and conidiophores are erect and septate; the conidia form divergent chains are smooth, ellipsoidal, and sometimes cylindrical with a pale green color [8].

At present, little is known about molecular identification and characterization of *M. rileyi* isolates and strains. Previous research has shown that RAPD (Randomly Amplified Polymorphic DNA) study can be used to confirm the species level of isolates [9] and also with this analysis some strain have been grouped with a similarity coefficient of 0.76% [10]. Similarly, Boucias et al. [6] with RAPD, internal transcribed spacer (ITS) sequence analysis, amplified fragment length polymorphism (AFLP), and telomeric fingerprinting methods, identified genotypes and as a result ITS-5.8S and 28S regions of *N. rileyi* isolates suggested that this species was more closely related to *Metarhizium anisopliae* and *M. flavoviride* than to *Nomuraea atypicola* and *N. anemonoides*. More recently Patil et al. [11] used rDNA-ITS sequencing to identify *N. rileyi* isolate and also clarifying the taxonomic relationships showing differences in nucleotide sequences of all the species or isolates analyzed. The results from molecular study in this specie may establish a molecular background for further taxonomic, phylogenetic and biological investigations including the use of the fungus in Integrated Pest Management (IPM), also in Cuba there is not any molecular study of this particular specie, for all these reason the aim of this research was to characterize isolate of *M. rileyi* from maize fields of Quivicán, Cuba.

Materials and Methods

Isolation of Entomopathogenic Fungus

Dead infected larvae of *S. frugiperda* were collected from maize fields of Quivicán municipality, Mayabeque province, Cuba. Cadavers showed the symptoms of infection described for the fungus *M. rileyi* like mycelial growth on the surface of the body that were sterilized with 0.5% sodium hypochlorite solution for two minutes and washed with sterile distilled water to remove the traces of sodium hypochlorite. The fungus was isolate by the technique described by Goettel and Inglis [12].

Morphological Characterization

The morphological identification was carried out by recognition of characteristic structures seen in culture, such as, the colony appearance (plate culture), morphology, hyphae color and spore color. A piece of diseased sterilized larvae was transferred to potato dextrose agar medium

(PDA) and incubated for 20 days at 28 °C for the morphological characterization. Once the fungal structures were developed, microcultures were performed on slides with 2% water agar, which were placed in an incubator at 25 °C and after 48–72 h, slides were observed with an optical microscope (Leica model DM500) (400 \times magnification). The isolates were identified using the codes and descriptions of genera and species of entomopathogenic fungi of Samson et al. [13] and Humber [14].

Molecular Characterization

DNA extraction from the isolate was performed from the mycelium of the strain by the method developed by Rajendrakumar et al. [15]. Extracted DNA was visualized in agarose gel at 1% stained with ethidium bromide and stored at $-20\text{ }^{\circ}\text{C}$ for further use.

Amplification of the internal transcribed spacer region (700–1400 bp) was performed using the ITS-1 (TCCGTAGGTGAACCTGCGG)/ITS-4 (TCCTCCGCTTATTGATATGC) primer pair [16]. Polymerase chain reaction (PCR) was performed in a thermocycler (BioRad, CA, USA), with 25 μL of the reaction mixture containing 0.2 mM dNTPs, 2 mM MgCl_2 , 0.5 μM each primer, 1.25 of recombinant *Taq* DNA polymerase (Invitrogen) and the amplification program comprised one cycle at 95 °C for 4 min, 30 cycles at 95 °C for 1 min, 60 °C for 60 min and 72 °C by 2 min, finally one cycle at 72 °C for 5 min. The amplified fragments were visualized by electrophoresis on 1% agarose gel to determine the degree of amplification.

The PCR product was purified with a Wizard SV Gel Kit and PCR Clean-Up System (Promega) and sequenced at the Chemistry DNA laboratory of CINVESTAV-IPN Unit Irapuato using a kit Dye Terminator Cycle Sequencing, Ready Reaction, in an ABI PRISM 377 PERKIN-ELMER (Cetus, Norwalk, CT) sequencer. Forward and reverse sequences were checked and aligned in BioEdit 7.0.9 [17] and ClustalW [18] respectively in order to obtain a consensus sequence for further analyses.

The ITS sequence obtained (AMGSPA) was compared with selected sequences of *M. rileyi* (accession numbers: AB268359.1 (Japan), AF368501.1 (China), AY646390.1 (China), AY646392.1 (China), EU553337.1 (Brasil), KJ728726.1 (India), KY436756.1 (India) available at GenBank® database [19] and using ITS sequences of *Cordyceps* sp. (EF495097.1), *Metacordyceps chlamydosporia* (KC403963.1) and *Paecilomyces* sp. (KU141150.1) as outgroup, all the sequences analysed at the gene bank were reported from different countries, but no report from Cuba was found. Sequences was aligned with ClustalW and the final matrix containing 605 bp was used for a distance analysis performed in PAUP* 4.0b10

[20] using the neighbor-joining method (NJ) [21]. Bootstrap [22] with 1000 replications was performed using 100 random addition cycles each, to evaluate internal branch support. The tree was edited in FigTree 1.4.3 [23].

Results

Morphological Characteristics

One isolate was obtained from the infected larvae of *S. frugiperda*. The colonies on PDA medium grew slowly. In the early stages of their growth they acquired a velvety appearance of white color with irregular borders that turned from pale green to malachite green with sporulation and in the back colonies were pale yellow (Fig. 1).

The vegetative and reproductive structures observed under the microscope had the following characteristics: hyaline hyphae to slightly pigmented, conidiophores with smooth, straight and septate walls. The branches, which formed near the septa, developed in clusters on the same point, with 2–4 phalids, short and rounded, thickened at the base and with a short neck or without it, from which were inserted the conidia, which were smooth, in chains, ellipsoidal and pale green (Fig. 2).

The morphological characteristics of the isolate were similar to those reported for the specie *N. rileyi* [7, 13, 14].

Molecular Characterization

The amplification process with primers ITS-1 and ITS-4 showed a fragment of 1335 pb as discernible in Fig. 3.

Fig. 1 Colony of the fungal isolate. **a** Mycelium of the fungus; **b** sporulated colony

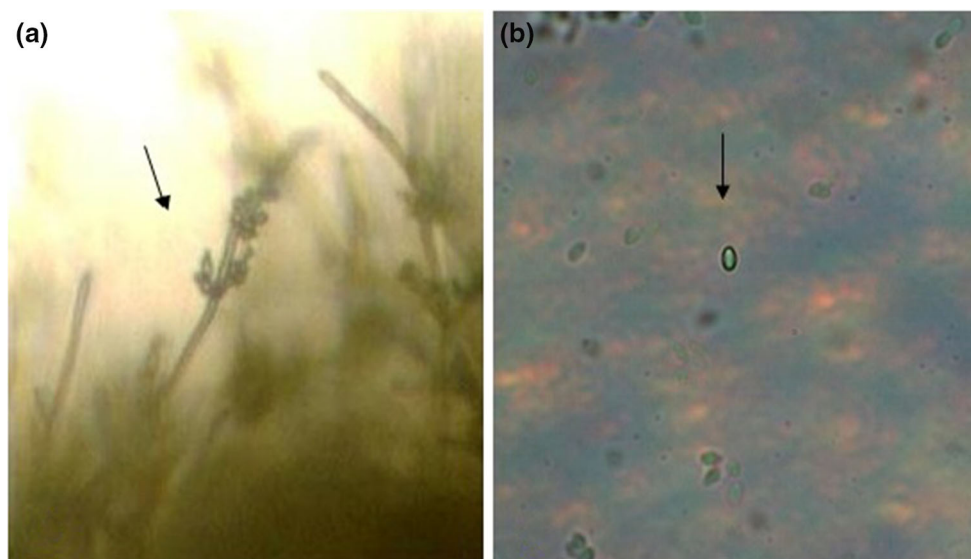
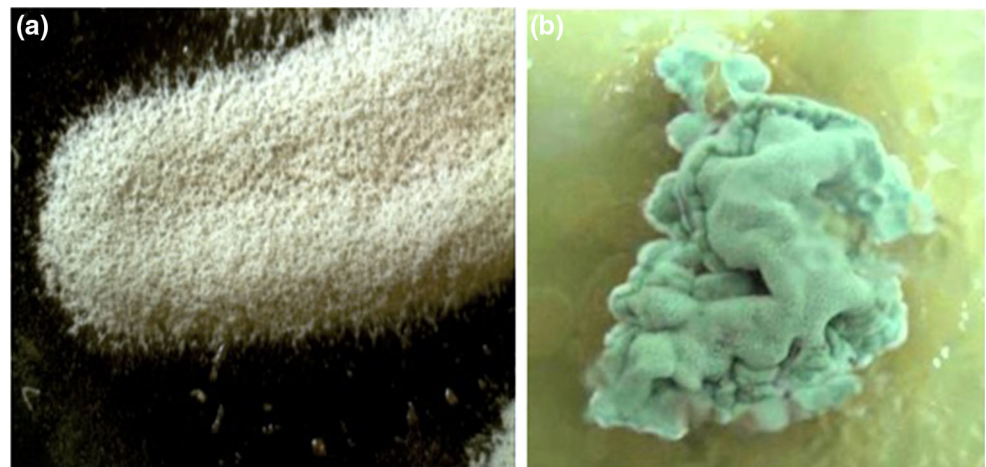


Fig. 2 Microscopic structures of the fungal isolate: **a** conidiogenic cells ($\times 10$); **b** conidia ($\times 40$)

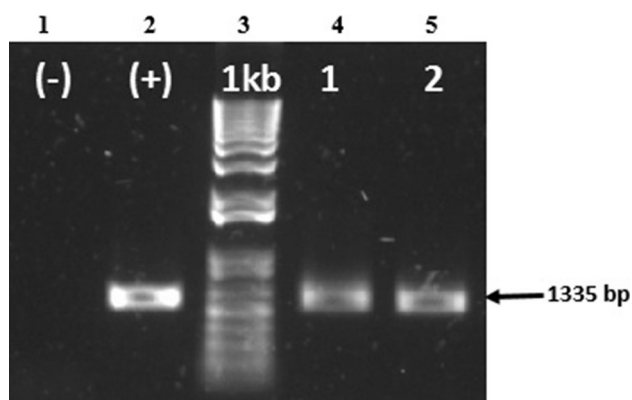


Fig. 3 Amplification of ITS of the fungal isolates PCR. (–): negative control; (+) positive control; (1 kb): 1 Kb marker; (1, 2): isolate fragment

A consensus sequence was obtained by alignment of the forward and reverse sequences for this region and deposited in GenBank with the accession number MG637450.

Molecular characterization of fungal isolate supported with morphological studies allows researchers to identify not only new species but also new isolates and this is especially relevant to generate new knowledge concerning the genetic diversity.

Studies about molecular characterization of *N. rileyi* isolates mostly include molecular marker to determine genetic variability. Swetha and Manjula [24] analyzed 7 isolates of this species using RAPD-PCR and revealed genetic differences and similarities which resulted in three groups of isolates. On the other hand, using β -tubulin gene and ISSR as markers, has been possible to elucidate the phylogenetic relationships between members of genus

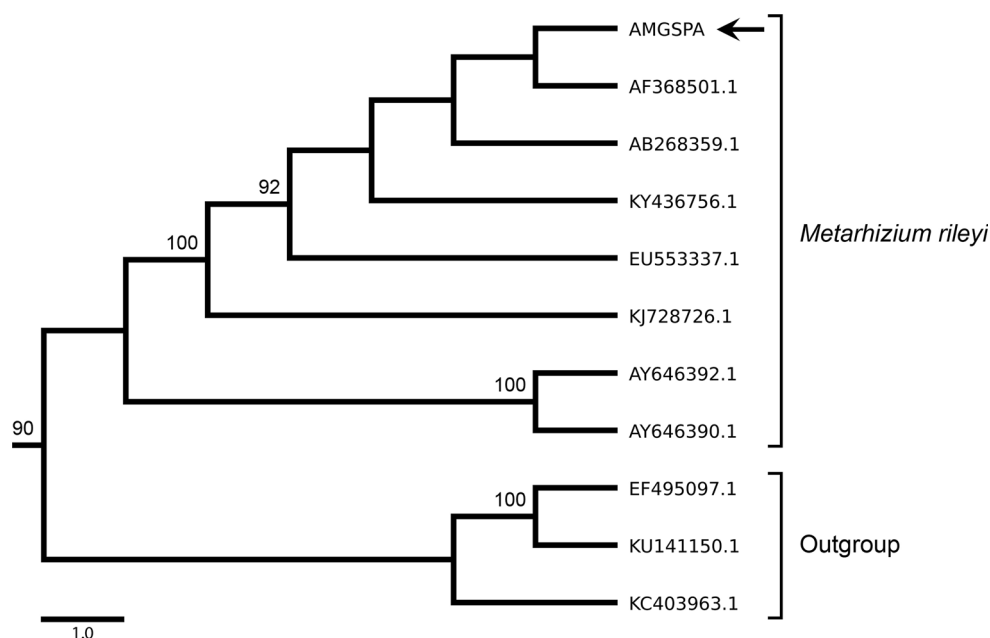
Nomuraea, with an emphasis on *N. rileyi* [25]. Vargas et al. [10] carried out the characterization of five strains of *N. rileyi*, and they found a high degree of homology among them, independently of their place of origin and host where they were isolated. Patil et al. [11] compared *N. rileyi* IOF1 isolate with another four isolates where two of them were similar to IOF1 after sequence using ITS region, however a phylogenetic tree constructed using ITS sequences clearly distinguished those isolates from *N. rileyi* IOF1 isolate. Other filamentous fungi, like *M. flavoviride*, *M. anisopliae* and *Paecilomyces fumosoroseus*, have been used to distinguish between different isolates [26–28].

Two groups can be seen in Fig. 4. According to the phylogenetic analysis, the new isolate forms a monophyletic group (99% bootstrap support) with other accessions of *M. rileyi* available at GenBank database, which confirms its identification based on molecular data. The new isolate was named *Metharhizium rileyi*-Cuba-AMGSPA. All the accessions reported at GenBank are from several countries around the world but none is from Cuba.

According to the phylogenetic tree made in this study (Fig. 4), the isolate *Metharhizium rileyi*-Cuba-AMGSPA shares phylogenetic relationship with *N. rileyi* and *M. rileyi*, showing high homology (99%) with *N. rileyi* (e.g., AF368501.1, AB268359.1 and EU553337.1) and *M. rileyi* (e.g., KY436756.1).

A homology dendrogram of various entomopathogenic fungi demonstrated that the Cuba isolate (AMGSPA) are closely related to *M. rileyi* (*N. rileyi*) (Fig. 4) with 99% of homology. The isolate AY646392.1 and AY646390.1 classified as *M. flavoviride* showed 89 and 90% of homology with Cuban isolate respectively. This result

Fig. 4 Neighbor-joining tree of *M. rileyi* (= *N. rileyi*) and *M. flavoviride* accessions based on ITS sequences. The position of the new strain is indicated (arrow). Sequences of *Cordyceps* sp., *Paecilomyces* clone and *Metacordyceps chlamidosporia* were used as outgroup. Numbers above branches indicate bootstrap support (> 90%). Scale bar indicates nucleotide substitutions per site



demonstrated the high similarity between *M. rileyi* (*N. rileyi*) strains independently of the region or the host from which they were isolated [29]. Bidochka et al. [26] found similar results working with *M. flavoviride* and *M. anisopliae*.

The results of this research demonstrate that, under the natural conditions of Quivicán municipality, Mayabeque province, it is possible to obtain isolates of autochthonous fungi from *M. rileyi*, pathogenic to *S. frugiperda*. This is the first report of *M. rileyi* isolate in Cuba. This can help in the development of massive productions based on these isolates, as well as their introduction in biological control strategies for the management of lepidoptera larvae of agricultural importance.

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