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Identification of Fungi Associated to *Pseudips mexicanus* (Curculionidae: Scolytinae) as a Possible Biocontrol Agent

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Abstract: Entomopathogenic fungi are an important insect biological control group, however most of the fungi described as biological controllers have a broad spectrum of insect hosts. *Pseudips mexicanus* is a bark beetle that infests pine-oak forests in Mexico, causing significant economic and ecologic losses. In addition, the infestation alters the ecology of different organisms that coexist in the forest habitat. The search for bark beetle control methods other than pesticides has led to research into biological control procedures based on naturally occurring beetle pathogens. The goal of this paper was the isolation, morphological and molecular identification of fungi that naturally parasitize the bark beetle *Pseudips mexicanus*, as well as to carry out infection tests to propose a specific biological control alternative to this plague. Associated to this beetle, we have identified four isolates belonging to three genus: *Beauveria*, *Lecanicillium* and *Trichoderma*, within these some species have been used previously as biocontrollers, mainly in agricultural use, in the process of transformation of organic crops. The LVP-2 isolated, which was identified as *Beauveria* was tested for infection of naturally harvested bark beetles and its pathogenicity was proved, at three days post infection (dpi) most of the individuals presented little mobility of the legs and a swelling of the body was noted, however more studies are needed to determine their viability as plague controllers in Mexican forests.

Keywords: Bark Beetle, Forest, Pine Trees, Pest Management, Biological Control

1. Introduction

In Mexico there are approximately 870 bark beetle species, which are involved in the active degradation of plant biomass [1]. Among the most important bark beetle species that have a major impact in the elimination of large extensions areas of tree forests from North to Central america, are the genus *Dendroctonus*, *Scolytus*, *Phloeosinus*, *Ips* and *Pseudips* [2, 3]. Particularly the last two genera are present in the North region of Morelos, Mexico [4]. Bark beetles are among the most destructive of the many threats to forest productivity [5]. Bark beetles are insects that inhabit beneath the bark of pine trees; as a consequence, there is damage over tree tissues such as the phloem, important for the sap conduction, causing tree death and massive infestation of other pine trees. Within the more representative bark beetle species are *Dendroctonus frontalis*, *Dendroctonus adjunctus*, *Dendroctonus mexicanus*, *Pseudips mexicanus* and *Ips* spp. [6, 2]. The last two species are considered secondary bark beetles, meaning that the trees have been colonized previously by other bark beetles' species, usually from the *Dendroctonus* genus. However, when the populations of secondary bark beetles species increase, then these are considered primary pests that can result in forest tree population loss [3].

Besides the bark insects' importance to the ecosystems balance, such as regulating tree populations as a result of the elimination of burned, sick, stressed or week trees, and recycling of organic matter, they have been also considered "forest pests" [7-11]. In the last years the problems caused by these insects have been growing because of increasing

numbers in their populations or migration to new areas [12]. The massive infestation of great forest extensions in Mexico's provincial territories including Chihuahua, Durango, Michoacán, Estado de Mexico and Morelos have had detrimental impact in ecological equilibrium as well as in economical aspects. Among the strategies to control bark insects are the use of pruning sanitization, traps with pheromones, use of insecticides among others [13]. Particularly, organochlorine and organophosphate pesticides have been the more conventional method of bark beetle control.

However, the uncontrolled use of these products has brought barker resistance and the pleiotropic elimination of innocuous organism such as bees or butterflies, which play fundamental roles as pollinators, plus the high cost of these chemicals [14, 15]. Therefore, new strategies to control bark insects have been developed taking in consideration more natural approaches, such is the case of the use of entomopathogenic fungi like *Beauveria bassiana*, *Metarhizium anisopliae* (Ascomycota, Hypocreales), or *Bacillus thuringiensis* among others [16-18]. The use of entomopathogenic fungi as biological control is a regular agricultural and ecological practice that focuses in population reestablishments of insect pests' numbers using their natural controls [19]. This represents an inexpensive and environmental friendly alternative. Furthermore, it may be possible to find biological controls with more specific targets as opposed to the conventional methodologies [20].

The first approach in the characterization of specific biological control microorganisms is the isolation and identification of entomopathogenic fungi infecting bark beetles. The aim of this study was the isolation, as well as morphological and molecular identification of fungi associated with the mortality of *P. mexicanus* in the pine-oak forest in Morelos state, Mexico.

Entomopathogenic fungi are parasitic microorganisms with the ability to infect and kill some arthropods of agricultural interest, they form a monophyletic group, including; Oomycetes, Cytridiomycota, My-crosporidia, Entomophtoromycota, Basidiomycota and more commonly Ascomycota. They are mainly used as biopesticides in organic farming as a safe alternative to toxic chemical insecticides. Mainly species like, *Metarhizium*, *Beauveria* and *Lecanicillium*. In sorghum, tobacco, wheat, soybean, corn, maize, tomato, among others, mainly due to its low toxicity capacity generated from its functional metabolites. [21, 22]

2. Materials and Methods

2.1. Study Site

Fungi were isolated from bark beetles collected from Real Montecasino, Morelos, Mexico, located on the west side of the Neovolcanic Transverse Axis, with the following coordinates 19°00′46.58″ N 99°15′00.21″ O, Altitude 2329 meters above sea level. The location presents temperatures ranging from 8 to 18°C, with average annual precipitation of 1000-2000 mm [21]. The region climate is semi-cold (C (E) (m) (w), C (E) (w2) (w)) and template (C (w2) (w)) according to Köppen system [21, 23]. The location presents pine-oak forests, with *Pinus montezumae*, *Pinus teocote*, *Pinus pseudostrobus* species, among others.

2.2. Isolation of Fungi

Fungi isolates were obtained from death bark beetles invading *Pseudips mexicanus*, that were present in the galleries underneath the bark, showing symptoms of infection (darker color, mycelium or conidiophores growing on the insect cuticle). *P. mexicanus* guished by length 3.6- 5.0 mm; color dark reddish brown; antenna club with sutures 1 an 2 feebly or very strongly, booadly precurved; pronoto the more coarsely puntured pronotal disc, the puntures moderately coarse, pronotal vetiture finer, apparently les abundant; elitra declivity steep the morphological description is based on Wood's criteria [2]. From the beetles' corpses we took ten sample by striating the cuticle and the sample was placed in petri dishes with Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA) medium for eight days. After this period another sample from the isolates was taken with a sterile bacteriological loop and inoculated onto Petri dishes containing medium with yeast extract, followed of six to eight days of incubation at 28°C. Once obtained, the isolated fungal strains were morphologically identified using taxonomic keys [24, 25, 26] for each group of the isolated fungi. All cultures used in this study are maintained in the culture collection of the UAEM (Universidad Autónoma del Estado de Morelos).

2.3. Fungal Morphological Phenotyping

The fungal species were identified based on the macroscopic and microscopic characteristics using identification keys [24-26] Fungi were identified according to colony morphology using a stereomicroscope (×40 magnification) and by further mounting slides with the sample of the specimen and observed by light microscopy (×400 and ×1000 magnifications, using a Microscope Nikon model Eclipse 80i and a digital camera Nikon DS-Fi1). The analysis of dichotomus keys such as size, shape and grouping of conidiophores, morphological aspects of the colony and the fungal hyphae were used for the organism identification. Since dilutions were performed until a pure strain was obtained, a single sample for each isolate was used for microscopic observations [24, 27]. Micromorphological identification was completed with an analysis of a region of the ITS2 ribosomal gene sequence for a polyphasic microbial identification [28].

2.4. Fungal ITS2 rRNA Gene Ribotyping

For identification, we analyzed molecular markers previously described to be distinctive to filamentous fungi. Regions of the internal transcribed spacers 2 (ITS2) were amplified. The primers and conditions used for these PCR

reactions have been previously described by White, et al. 1990 [28], respectively. We performed the following protocol: a plate of PDA medium was inoculated with a representative fungal culture and incubated until a reach layer of mycelium was obtained. PCR reactions contained 1 × PCR buffer, 10 ng of fungal DNA, 10 pmol of the forward and reverse primer 2 mM of dNTP's, 1.5 mM of $MgCl_2$, and 1U of Taq polymerase in a total volume of 20 µl. The cycling conditions were: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s., 55°C for 30 s and 72°C for 45 s. The PCR products were separated by electrophoresis at 100 V for 30 min on a 1% (w/v) agarose gel in 1 × TAE buffer (0.4 M Tris, 0.05 M NaAc and 0.01 M EDTA, pH 7.85) and examined under UV-light [29]. Amplicons were purified from the PCR reactions using a commercial PCR extraction kit (Fermentas Cat. No K0513) and sequenced using the same PCR primers at the Institute of Biotechnology of the UNAM (Universidad Nacional Autónoma de Mexico) in a Terminator Cycle Sequencing Ready Reaction BigDye® terminators v 3.1 (PE Applied Biosystems). The sequencer used was the ABI PRISM Model 3730 Xl genetic analyzer, with data collection software version 3.0. The sequences were analyzed using the online DNA sequence software at the National Center for Biotechnology Information of the National Institutes of Health (NCBI/NIH) website (www.ncbi.nih.gov) using the BLAST similarity search algorithm [30].

2.5. Phylogeny Reconstruction

Phylogenetic analysis was performed online with the server Phylogeny.fr (www.phylogeny.fr) [31, 32]. From different sequences retrieved from the BLAST hits, one of each species was used to construct the phylogenetic trees. The sequences were selected according to the sequencing analysis, with respect to the genus obtained from the preliminary analysis of the sequences. In order to deduce the phylogeny of the fungal isolates, ITS sequences were aligned to sequences from related fungi (the same genera) held in Central bureau voor Schimmelcultures (CBS) only where full length sequences were available.

The workflow setting in Phylogeny~~. fr~~ included "A la Carte" mode step by step. MUSCLE was used for multiple alignments and they were curated in SeaView software (version 4.6) [33]. BioNJ (Neighbour-Joining) [34] with Kimura 2 Parameters (K2P) [35] was utilized as substitution model. The tree topology was bootstrapped 1000 times. Finally, the graphical visualization was performed by TreeDyn [36]. ITS2-based phylogenies were obtained using BioNJ and K2P as a substitution model, which has been previously used to estimate fungal phylogenetic relationships from ribosomal markers [37, 38]. The phylogenetic relationship estimation using the BioNJ method and K2P matrix system is a good strategy for robust and high-quality tree constructions based on ribosomal genes [39]. Having said that, phylogenies obtained using BioNJ/K2P have also been employed to establish taxonomic inferences in bacteria [40], fungi [41, 42] and plants [43].

2.6. Infection Tests on Pseudips Mexicanus

Bark beetles (*P. mexicanus*) were collected from a site located on the west side of the Neovolcanic Transverse Axis, with the following coordinates 19°00′0.8″ N y 99°13′50.7″ E at 2210 meters above sea level. The insects were on fallen branches of *Pinus leiophylla*, and transported to the laboratory where they were put on quarantine on disinfected plastic containers (washed with 98% ethanol) for 20 days to ensure no fungal development showed before inoculation.

The LVP-2 isolate (*B. bassiana*) was used for the tests chosen by the pathogenicity criterion registered for this species, used for biological control in agricultural crops and was streaked on PDA medium at 25 ± 2°C for 12 days until conidiation occurred. An inoculum of 2.4375×10^{10} conidia/g was used to inoculate the beetles after a 30 S wash with a 0.1% tween solution in 10 ml of buffer. [44]. Six biological replicates each with ten insects were inoculated. Three biological replicates were left without inoculation as a negative control.

2.7. Experimental Design and Statistical Analysis of Bioassay

A survival analysis was performed, which consisted of statistical methods using non-parametric data, using the Kaplan-Meier method calculating the survival of each infected organism, this analysis measures time in intervals and calculates the survival of each interval.

3. Results

We obtained 19 samples from *P. mexicanus* corpses that were propagated and subcultured several times to obtain pure isolates (see Figure 1A for an example). We continued with the characterization of four fungal isolates, since those were the more representative according with distinctive colony morphology. These isolates were named LPV-2 to 5 that stands for Laboratorio de Parasitología Vegetal.

Once isolated, the fungi were identified by morphological and molecular criteria to the species level. Only three fungal genera were found in four isolates: *Lecanicillium* (1) (Clavicipitaceae), *Trichoderma* (2) (Hipocreaceae), and *Beauveria* (1) (Clavicipitaceae).

3.1. Morphological Description of the Isolates

3.1.1. Isolate LPV-2 (Beauveria bassiana)

The colonies of this isolate had a diameter of 10-16 mm after 1 week of growth, these were woolly with dust like shape due to abundant conidia. In the initial growth the colony was white but it turned yellow in PDA medium (Figure 1B). Occasionally the conidial apparatus was clustered; more often the conidiogenous cells were arranged in small groups or solitarily along the hyphae. Conidiogenous cells were observed consisting of a subglobose or flask-shaped, sometimes subcylindrical in the basal part with the following measurements in our sample (3-) 4-15 (-28) × (1.5-) 2-3.5 (-4) µm, and a well developed rachis, up to 25 µm

long and mostly 1-1.5 µm wide. Conidia were hyaline, smooth, ellipsoidal (rarely subglobose), widest near or below the middle, sometimes with a pointed or apiculate base, (2-) 2.5-4.5 (-6) × (1.5-) 2-2.5 (-3) µm. These observations are coincident with the morphological description assigned to Beauveria genus [45] (Figure 1C).

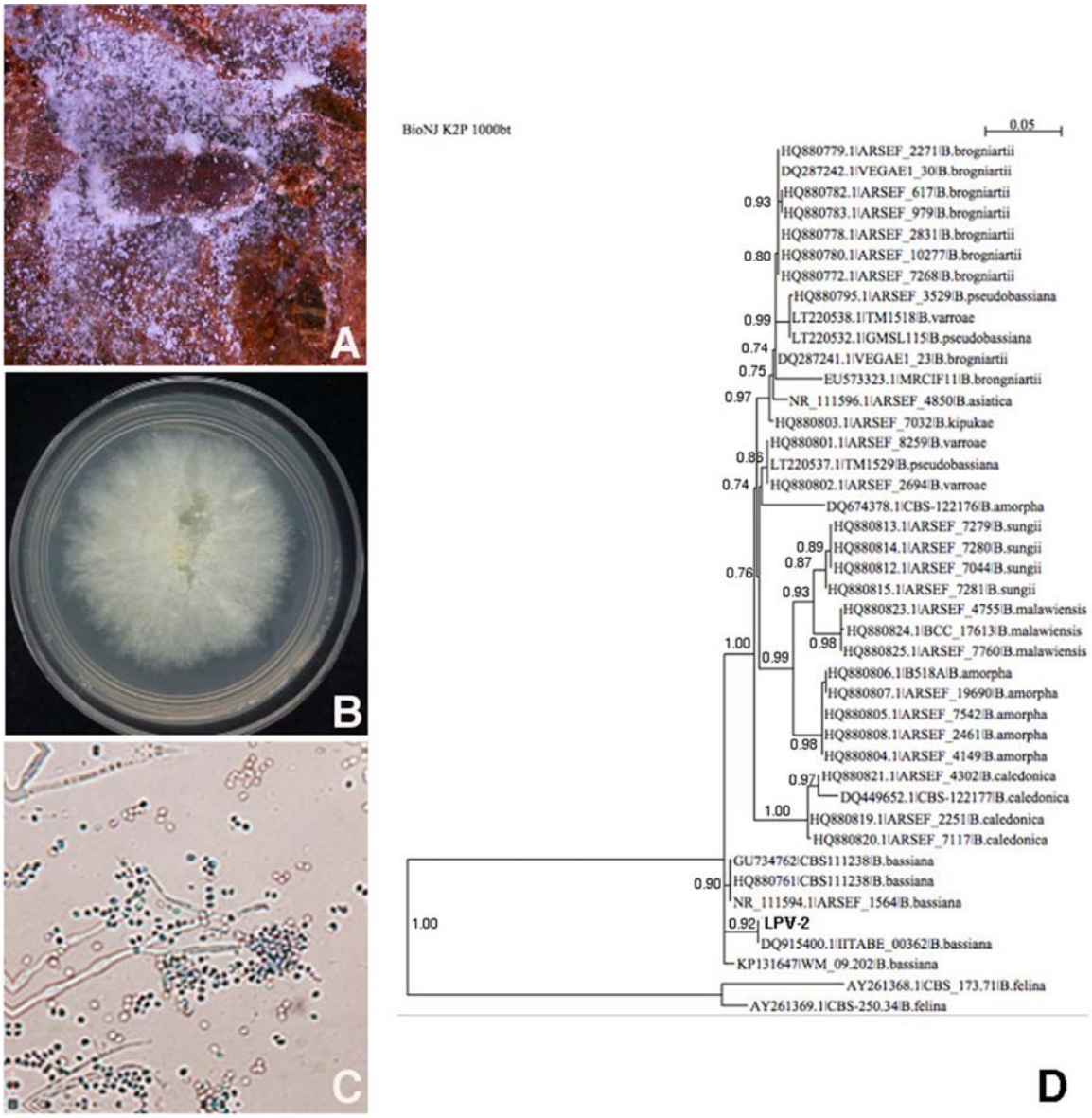

Figure 1. *Colony morphology of LPV-2 fungi isolated from bark beetle Pseudips mexicanus. A) Bark beetle corpse from where strain LPV-2 was isolated. B) Colony morphology of the LPV-2 isolate. C) Conidia of isolate LVP-2. D) Phylogenetic reconstruction for strain LVP-2. ITS2 sequences for LPV2 were deposited in NCBI under accession number KY432692.*

3.1.2. Isolate LPV- 3 (Lecanicillium Aphanocladii)

Isolate LPV-3 grew similarly on two cultivation media (PDA and MEA) forming white and deeply woolly colonies, yellow pigmentation was observed which was time-depending, starting from the 7th day of cultivation at 25°C in the dark and on dependency on the medium composition (Figure 2A). The culture medium influences also the intensity of conidia formation (data not shown). This fungus turned media to a reddish color, a characteristic that has been reported for *L. aphanocladii* Zare & W. Grams [46]. The mycelium is septated and the aphanophialides were scattered or in verticillium with variable size with range of 12-40 × 0,8-3,0 µm. In our samples, the conidia are cylinders with the following measurements: 2-10 × 1,0-2,6 µm, which could be present in groups and without presence of chlamydospores. The fungus formed short, basally swollen, with narrow tip, rapidly collapsing into inconspicuous denticles conidiophores (aphanophialides in the sense of Gams, 1971) [47], which bear single ellipsoidal conidia; aphanophialides scattered laterally along the procumbent or prostrate aerial hyphae. The mycelium was septated, presenting verticilate or solitary phialides with variable size between 12-40 × 0,8-3,0 µm. According to [27], the morphological analysis of colonies suggests that the microorganism is from the genus *Lecanicillium* (Figure 2B).

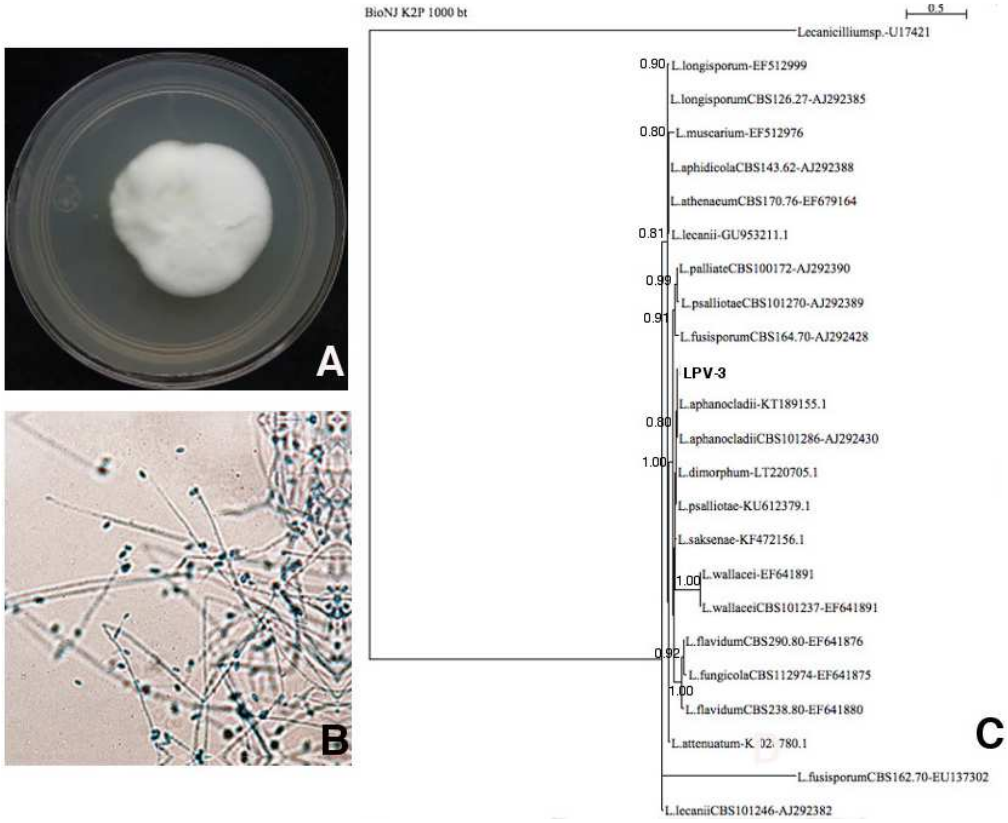

Figure 2. *Characteristics for strain LVP-3. A) Colony morphology of the LPV-3 isolate. B) Conidia of isolate LVP-3. D) Phylogenetic reconstruction for strain LVP-3. ITS2 sequences for LPV3 were deposited in NCBI under accession number KY433313.*

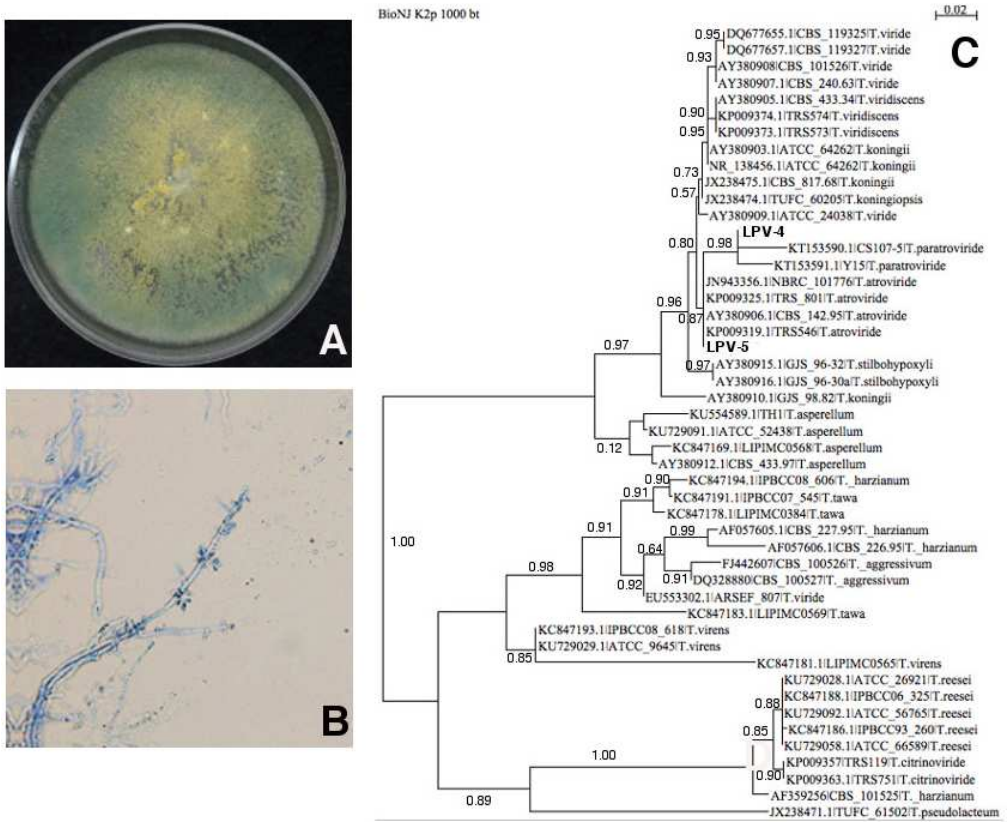

Figure 3. *Characteristics for strain LVP-4. A) Colony morphology of the LPV-4 isolate. B) Conidia of isolate LVP-4, C) Phylogenetic reconstruction for strains LVP-4 and 5. ITS2 sequences for LPV4 and LPV5 were deposited in NCBI under accession numbers KY433314 and KY433315.*

3.1.3. Isolates LPV-4 and LPV5 (Trichoderma Paratroviride and T. Atroviride, Respectively)

Other fungi obtained from the beetle presented the following morphology: they grew forming concentric rings (Figure 3A), with septated a hyaline hyphae and the following measurements in our sample: intricate conidiophores with 50.0–60.0 × 2.0–3.0 µm, forming *verticillium* with five phialides ampule shaped with dimensions of 10.5 × 3.3 µm and 2.2 µm to the base. They did not present intercalated phialides. Conidia were globose to subglobose, with smooth walls and green olive color, ranging in size of (3.5 × 3.1) µm. The characteristics of this isolates were in accordance to the description given by [22] for the genus *Trichoderma* (Figure 3B).

3.2. Phylogenetic Analysis

A first phylogeny was constructed with the three isolates that allowed us to situate the fungi with their respective genera confirming the morphological identification (Figure 4). Also this tree indicated the probable species to which the isolates belonged. To confirm this, more refined trees were constructed for each isolate using specific sequences of well-identified fungi from databases. (Figures 1D, 2C and 3C).

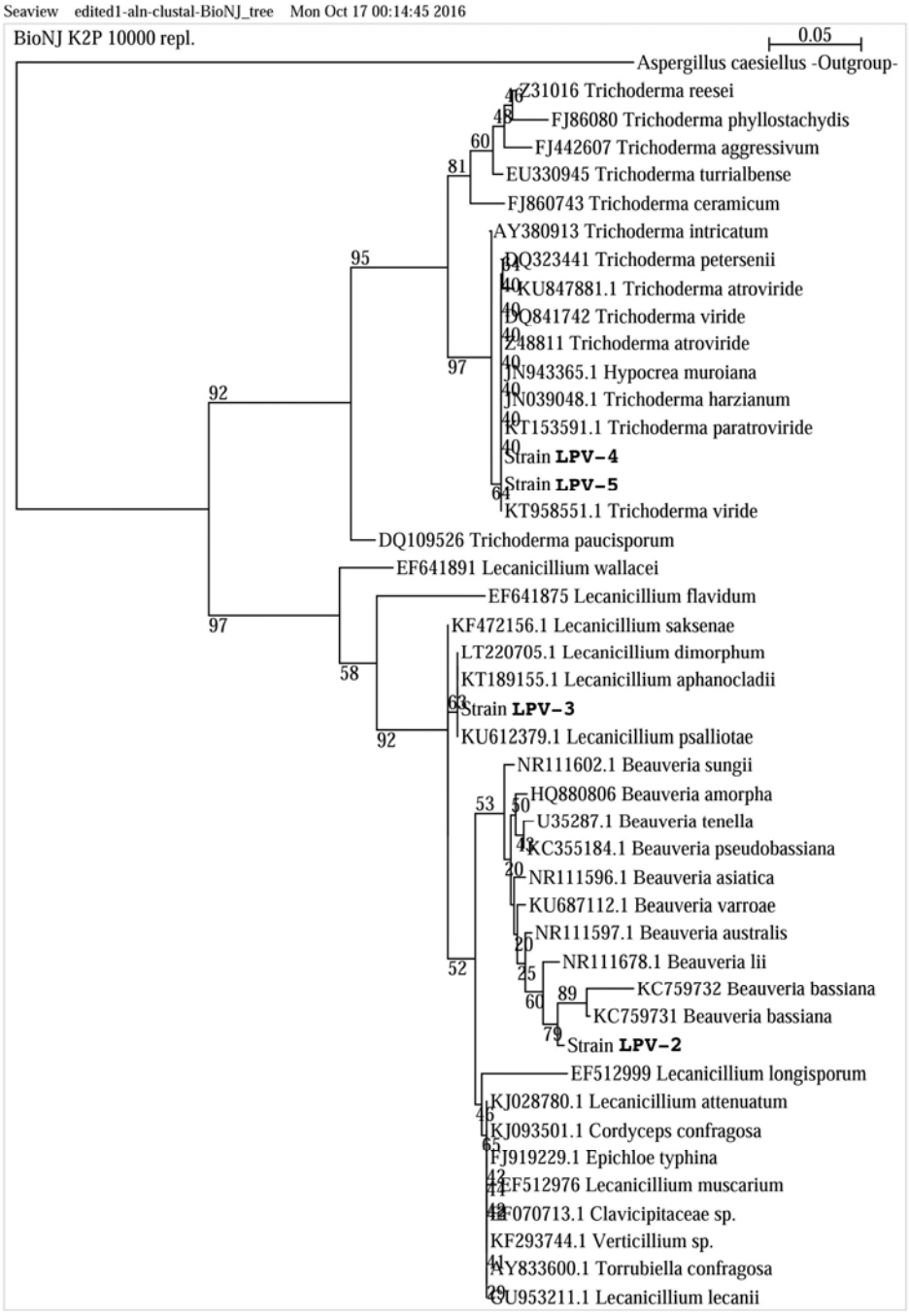

Figure 4. *Phylogenetic Tree for the four Isolates from Pseudips mexicanus. The phylogeny was obtained using BioNJ and K2P as a substitution model.*

Strain LPV-2 was clustered with Beauveria species, being B. bassianna grouped in exactly the same position as other B. bassianna species, confirming its identity. A high bootstrap value (92%) supports this result (Figure 1D).

Strain LPV-3 was grouped with Lecanicillium genera, being L. aphanocladii the most phylogenetically related species, grouping in the same position with reference species, thus allowing its identification. Bootstrap values strongly support our identification (80%) (Figure 2C).

Regarding the tree topology of strains LPV-4 and LPV-5, they were clustered with Trichoderma paratroviride and T. atroviride, respectively. This clade showed a strong support (97% branch support) all the Trichoderma´s clade shows high bootstrap values indicating the robustness of the tree (Figure 3C).

Finally, these results are consistent with the morphological classification for these four fungal isolates.

3.3. Infection of Bark Beetles by Isolate LVP-2

After 24 hours the experimental population showed a little lethargy. Two days post infection (dpi) lethargy was evident in most of the individuals and the treated insects showed clumsy movements. At three dpi most of the individuals presented little mobility of the legs and a swelling of the body was noted. At four dpi, inflammation of the abdomen was evident and the intersegment unions were slightly separated. At five dpi, none of the individuals moved and from six to seven dpi a mucilaginous secretion was observed in the intersegment unions between the thorax and the abdomen. At 12 dpi mycelia covered completely the body of the bark beetles, all treated individuals died after six days of infection with LPV-2 isolate. (100% of the treated individuals). The control insects did not show this behavior, only a few of them died (16%) (Table 1) and none of them developed mycelial growth. Conidia recovered from the dead infected bark beetles were identical to those initially described for this isolate.

Table 1. Mortality percentage of individuals treated or untreated with LPV-2 isolate (*B. bassiana)* at different days' post inoculation (Dpi) Letters indicate statistical significance using an ANOVA test.

Table 1. *Percentage of mortality of Pseudips mexicanus.*

Time (Days)	% of mortality of *Pseudips mexicanus*	
	Infected	Non-infected
1	0 (±0.0)	0 (±0.0)
2	6.66 (±5.77)^{e}	0 (±0.0)
3	16.66 (±5.77)^{d}	0 (±0.0)
4	36.66 (±5.77)^{c}	0 (±0.0)
5	66.66 (±5.77)^{b}	0 (±0.0)
6	100 (±0.0)^{a}	6.66 (±5.77)^{e}
7	100 (±0.0)^{a}	16.66 (±5.77)^{d}

4. Discussion

The bark beetles, specially the genus *Ips* and the species *Pseudips mexicanus*, are considered "forest pests" that cause major economic losses and ecologic alterations in Mexico and other countries such as Canada, USA and some European countries [48, 49]. Fungi associated with to bark beetles represent a potential to develop specific strategies of biological control. For example, it has been described that the anamorphic taxa *B. bassiana* and *M. anisopliae*, (Hypocreale, Ascomycota), are among the natural enemies of pests, such as; *Hypothenemus hampei*, *Helicoverpa zea*, *Aphis gossypii* and *Ostrinia nubilalis*. [50-53]. Both are natural enemies of a wide range of insects and arachnids particularly in agriculture, however less is known about their relevance in forest ecosystems [54, 59]. In this work we showed morphologically and phylogenetically that an isolate of *B. bassiana* obtained from a bark beetle corpses was capable of acting as an entomopathogenic agent on *P. mexicanus*, killing the insects in only six days. To the best of our knowledge this is the first report of a *B. bassiana* strain that infects *P. mexicanus* and could represent a potential biocontrol to this pest in pine forests. However, previously [55], report the isolation of *B. bassiana* was previously reported to be isolated from *Ips sexdentatus* and *Ips typographus* both collected from Austria. Also, it has been reported the use of *B. bassiana*, *M. anisopliae* to control bark beetles of the genus *Ips*, particularly species such as *I. typogrpahus*, *I. sexdentatus*, *I*ps *grandicollis* and *Ips calligraphus* mostly in Europe, however those strains were from commercial sources [56-59].

Furthermore, other fungi such as genera *Lecanicillium*, *Paelomyces*, and *Nomurae rileyi* have been reported to control aphids and other pests [60]; however most of these reports focused the attention to agroeconomic important pests.

In this paper, we also report the presence of different fungi belonging to distinct genera which were found present in association with bark beetles' corpses that have not been studied in such detail as other genera as *Beauveria* or *Metharizium*: *Lecanicillium aphanocladii* and *Trichoderma paratroviride* and *atroviride*. The fact that these fungi were isolated from bark beetle corpses found inside tree galleries and showed other symptoms of sickness such as darker color and mycelia emerging from the inside of the body, suggests their pathogenic activity in those insect. Experiments are under way to test their infection capabilities. Furthermore, these genera have been reported to be entomopathogens for other invertebrate orders such as Lepidoptera, diptera as well as nematodes (see below). To the best of our knowledge, this is the first report in Mexico of the association of these entomopathogenic fungi with a bark beetle. In 2014, [61] showed the efficient use of Trichoderma harzianum as biological control for Aedes aegypti, reporting a 96% of mortality. There are also recent reports of Trichoderma hamatum that infect aphids [62]. Nevertheless, we have found no reports of any Trichoderma species infecting Ips or Pseudips Bark beetles, which would be a novel and interesting finding, especially because T. atroviride has proven to be also a mycoparasite [63] and has the ability to

colonize the roots of certain plants [64], features that would enhance its environmental potential as a biocontroller.

Lecanicillium spp. has been used as biological control against dipters, such as *A. aegypti*, the carrier of the malaria parasite, and some mites with impact in human health. For example, [65] identified adult mosquitoes of *A. aegypti*, *Anopheles arabiensis* and *Culex quinquefasciatus* naturally infected with *Lecanicillium* [65]. The use of these fungi as biological controllers shows high efficiency since the mortality of mosquitoes reaches the 87%. From these results a trap methodology has been established to inoculate domestic mosquitoes and control their populations. We have also not found any report of any *Lecanicillium* species infecting bark beetles. Our findings thus open a new perspective to study this genus to apply these fungi as biocontrollers for forest plagues.

An effective biocontrol strategy against bark beetles of forest importance requires the selection of fungal isolates that combine desirable characteristics such as pathogenicity and conidia production to control the bark beetles, in particular *P. mexicanus*.

This study proposes the bases to develop proper strategies to control and manage of forest important pests in Mexico, as is *P. mexicanus*.

5. Conclusion

The alternative to control bark insects with entomopathogenic organisms is essential, since today agrochemicals are not specific and harm beneficial organisms for crops. The control Biological is a viable alternative for the control of barking insects of forest importance, since that these may be specific to certain pests.

A correct identification of pest control organisms isolated from specific and natural environments is essential to design strategies to reduce the damage caused by pest insects. Modern biotechnological identification methods combined with classical identification methods lead to correct identification, which is useful to obtain genetic resources with unique properties.

The biological tests carried out with *Pseudips mexicanus* using the fungus *B. bassiana*, presented a high mortality in a time of 96 to 120 hours, which is essential for the control of insects of forest importance. In the tests carried out, it was found that the control method for debarkers is appropriate for a good integrated pest management.

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Conflict of Interest

The authors declare that they have no conflict of interest and we agree to the publication of these results.

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