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# Molecular identification and culture of *Cordyceps brongniartii*, a teleomorph of *Beauveria brongniartii*: A new record for Morelos, Mexico

# Identificación molecular y cultivo de *Cordyceps brongniartii*, teleomorfo de *Beauveria brongniartii*, nuevo registro para Morelos, México

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## RESUMEN

Antecedentes: El estudio de los hongos alrededor del mundo ha ido en aumento, con nuevas especies descritas, además de novedosas actividades biológicas. *Cordyceps militaris*, después de *C. sinensis*, una de las especies más estudiadas a nivel mundial, han experimentado grandes desarrollos sobre el cultivo para la producción *in vivo* de estromas, sin embargo, se ha dejado de lado otras especies potencialmente cultivables.

Objetivo: El presente estudio pretende identificar especímenes silvestres del género *Cordyceps*, así como, generar datos sobre la producción de estromas cultivados.

Métodos: Para lograr los objetivos se siguieron los siguientes pasos: i. Recolección y aislamiento de las cepas silvestres de *Cordyceps*, ii. Identificación y análisis molecular de las especies aisladas, iii. Producción de estromas sobre sustratos suplementados.

Resultados y conclusiones: El análisis de las secuencias de las regiones ITS confirmó la identificación de *C. brongniartii*, un estado anamórfico de *Beauveria brongniartii*, especie descrita en este trabajo por primera vez para Morelos, México. Además, se describen la producción *in vitro* de estromas fértiles de *C. brongniartii*, obteniendo cuerpos fructíferos con características similares a la de los progenitores recolectados en el campo, obteniendo una eficiencia biológica del 36 %.

Palabras clave: Identificación molecular, producción de estromas, teleomorfo

## ABSTRACT

Background: There is a growing interest in the study of fungi throughout the world, with new species being described, as well as novel biological activities of already known species. The cultivation of the fungus *Cordyceps militaris*, the second most studied species of the genus *Cordyceps* in the world after *C. sinensis*, has seen great advances in the production of stromata *in vivo*. However, much less attention has been paid to other potentially cultivable species.

Objective: This work aims to identify wild specimens of the *Cordyceps* genus and to generate data on the production of cultured stromata.

Materials: To achieve the objectives, the following steps were followed: i. Collection and isolation of wild *Cordyceps* strains, ii. Identification and molecular analysis of the isolated species, iii. Stroma production in supplemented substrates.

Results and conclusions: Sequence analysis of the region ITS confirms the identification of *C. brongniartii*, an anamorphic state of *Beauveria brongniartii*, and this work provides the first report of the species in Morelos, Mexico. In addition, the *in vitro* production of fertile *C. brongniartii* stromata, obtaining fruiting bodies with characteristics like those of field-collected progenitors, with a biological efficiency of 36 %.

Keywords: Molecular identification, stroma production, teleomorph

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## INTRODUCTION

Entomopathogenic fungi of the genus *Cordyceps* Fr. play an important role in ecosystems as biological control agents. In addition, the bioactive metabolites they produce have been attributed to a high medicinal value, thus generating significant economic benefits. Species such as *C. militaris* (L.) Fr. have received considerable attention from the pharmaceutical industry and traditional medicine due to the presence of bioactive compounds with anti-aging, anti-tumor, antioxidant, anti-inflammatory and immunomodulatory effects (Zhao *et al.* 2018, Lou *et al.* 2019, Li *et al.*, 2021, Zhang *et al.* 2021). *Beauveria bassiana* (Bals) (Hypocreales: Clavicipitaceae anamorpha), a fungal insect pathogen (Humber 2000), has been widely used for biocontrol of insect pests in agriculture. In addition, *B. bassiana* is traditionally used for its medicinal value in Asia, where it is administered to patients with cancer, infantile spam, epilepsy, stroke, and sore throat, as well as applied as an external cataplasm and on wounds (Ying *et al.* 1987).

The production of *Cordyceps* sp. ascomata has been reported, especially for *C. militaris* (Kobayasi and Shimizu 1983, Basith and Madeline1968), which now seems to be a routine task. However, the cultivation of other species of the genus *Cordyceps* has not been investigated. In nature, certain ascomycetes may lose the ability to reproduce sexually, mainly due to the loss of mating-type genes (Sharon *et al.* 1996, Turgeron 1998, Yun *et al.* 1999); however, this theory has not been proven, as fungi have the ability to reproduce by heterokaryons, parasexualism, and cloning (Paccola-Meirelles and Azevedo 1991, Bello and Paccola-Meirelles 1998).

*Beauveria brongniartii* (Sacc.) has been described as an anamorph of *C. brongniartii* by Shimazu *et al.* (1988) and Sung *et al.* (2006). However, there seems to be insufficient evidence to support this hypothesis. Recently, *C. bassiana* was described in China by Li *et al.* (2001) on a woodworm larva that is clearly related to *B. bassiana.* The teleomorph-anamorph association of *C. brongniartii* was reported in Korea, based on the original description, confirming the anamorphic nature of *B. bassiana.* In addition, the complete life cycle in artificial culture was described by Sung *et al.* (2006). *Cordyceps militaris* fruiting bodies are relatively easy to obtain from *in vitro* culture, as its culture requirements, including nutrition and controlled environment, are not as demanding as those of *C. sinensis.* The fruiting bodies have been mass-produced for food and medicine by Chinese companies. Different strains of *C. militaris* are often produced using different culture factors, resulting in differences in the size, shape, and color of the stromata. In addition, the ascomata sometimes appear white and do not turn yellow or orange over time (Sung *et al.* 2006, Wang *et al.* 2008, Choi *et al.* 1999).

Currently, there are recent reports on the molecular identification of Cordycipitaceae in Mexico, (López-Rodríguez *et al.* 2022, 2023). So most taxonomic studies are based on morphological characters (Rubio-Bustos *et al.* 1999). The status of the study of entomopathogenic fungi in the world has taken a great leap forward thanks to the use of molecular tools, discovering new variants in the taxonomic order of species and even new species (López-Rodríguez *et al.* 2022). Therefore, in this study, molecular tools were used to identify wild specimens of the genus *Cordyceps* collected in the state of Morelos, Mexico. In addition, data were obtained on the production of artificially grown stromata on a supplemented substrate.

## MATERIALS AND METHODS

### Biological material and strain isolation

Specimens were collected at north of the Universidad Autónoma del Estado de Morelos, within the Chichinautzin Biological Corridor, in a mesophilic mountain forest habitat dominated by pine-oak vegetation. Specimen C-71 (MEMIM C-71) showed characteristics typical of *C. militaris*, such as a yellow stroma 2 to 4 cm long with presence of while those of specimen C-72 (MEMIM C-72) were similar to those of *Ophiocordyceps melolonthae* (Rubio-Bustos *et al.* 1999). Fungal strains were isolated from the collected samples by extracting a fragment of stromal tissue and placing it on potato dextrose agar (PDA). The resulting mycelium was reseeded until all contaminants were removed. Isolated strains were deposited in the strain bank from the Cobioch-UAEMor Mycology Laboratory of the Biological Research Center of the UAEM.

### DNA extraction and amplification of the rDNA region

Total genomic DNA was isolated using a commercial kit (QUIAGENR, Plasmid-Mini-Kit) according to the method described by Challen *et al.* (1995) using lyophilized mycelium from the strains. Amplification of the ITS flanking of the 5.8S ribosomal gene was performed using the ITS-1 and ITS-4 internal transcriptional regions. The polymerase chain reaction (PCR) was performed with the following parameters: denaturation at 94 °C for 30 s, primer annealing at 50 °C for 45 s, and extension at 72 °C for 5 min, for a total of 30 cycles. Amplification products were purified using the PCR Purification Kit (QIAquick) and sequenced by Macrogen Services.

### Phylogenetic analysis

The phylogenetic analyses included 5 taxa and 16 sequences and one outgroup. ITS alignment was performed using MEGA.11 (Tamura *et al.* 2021), 28S using MUSCLE, (Edgar 2004) using *Paraisaria gracilioides* as the outgroup, the access keys to the Genbank for studio strains are: PV276772 for strain C-71 and PV276773 and PV276774 for strains C-72. Subsequently, the Bayesian inference was performed with IQ-TREE v.2.1.2. and MrBayes 3.2.6 in the CIPRES portal (Miller *et al.* 2010) with the Markov B-MCMC algorithm with 10 000 000 generations and four independent chains. The Model Finder algorithm was used to select the best evolutionary model. The analyses were performed with IQ-TREE web service. The trees were sampled every 1000 generations. The first 25 % of trees were discarded as burn-in for the construction of the consensus tree and calculation of the posterior probabilities (PPs). Sequences deposited in the National Center for Biotechnology Information (NCBI) database were used for species identification.

### Stromata production on supplemented sorghum grains

Stromal formation was induced with the two isolated strains. To prepare the inoculum, the strains were inoculated in liquid culture medium with 2 % non-diastase malt for 15 days with continuous agitation at 150 rpm, using 1 % of the working volume inoculum. The liquid inoculum was then transferred to polypropylene circular containers of 500 mL with sterile substrate containing 45 g of Sorghum bicolor (L.) Moench, 333 mg of non-diastase malt, 333 mg of brewer's yeast and one third of a raw egg. The inoculated containers were incubated for six days in the dark at a temperature of 23 °C; they were then transferred to the fruiting room at a temperature and humidity of 16 °C and 62 %. During fructification, the containers were kept under a photoperiod of 12 h of darkness and 12 h of rose-colored light. Biological efficiency was calculated according to Royse *et al.* (2004).

## RESULTS

### Collection of *Cordyceps* stromata and isolation of fungal strains

Two specimens with characteristics similar to *Cordyceps* spp. were collected. The wild specimen C-71 showed several stromata, 25 mm long and 4 mm wide, with a cylindrical to clavate profile. The fertile part is clavate, yellow-orange in color, with perithecia present on top. Perithecia are ovoid, slightly exposed from the stroma. It was found growing on a dead larva. The wild specimen C-72 presented a solitary stroma, 45 mm long and 5 mm wide, with cylindrical to clavate profile. The fertile part is clavate, whitish-beige, with perithecia on top, slightly exposed from the stroma. It was found growing on a dead larva of the genus *Phyllophaga* (Figure 1, a and d). The isolated strains presented septate hyphae and the presence of blastospores (Figure 1, b and e). The perithecia of sample C-71 were oval, with the presence of elongated asci and segmented ascospores. In sample C-72, perithecia were round, with the presence of asci and segmented ascospores (Figure 1, c and f).

### Phylogenetic analysis and species identification

The consensus sequence was used for analysis. Bayesian inference analysis demonstrated the presence of *C. brongniartii* within the strict consensus tree generated (Figure 2), four groups were formed. The four groups showed high statistical support. Within group 2 are the sequences corresponding to *C. militaris*, with posterior probability of 0.98, indicating a high stability of the group. In group 3 are the sequences belonging to *C. brongniartii*, with 0.99, stable supports that ensure the stability of the branch. The fourth group corresponds to *C. mexicana* (López-Rodriguez *et al.* 2022), ruling out the presence of this species in the present study.

A matrix of genetic distances (Chen *et al*. 2004) was computed to correctly identify species in groups. Lower values of 0.00 to 0.02 were used to describe organisms of the same species, and higher values of 0.02 were used to describe organisms of different species. In group 2 of the phylogenetic tree, genetic distances of 0.01, supporting the identification of strain C-71 as *C. militaris.* For group 3, genetic distances of 0.00 to 0.01 were observed for the sequence identified as *C. brongniartii*, and 0.04 for the sequence identified as *Beauveria brongniartii*, showing that strain C-72 is identified as *C. brongniartii*. The distances between group 1 and group 2 were greater than 0.10 and 0.12, respectively, demonstrating separation between the species (Table 1). For group 1, identified as *Ophiocordyceps melolonthae*, the genetic distances between group 2 and group 3 were 0.24 to 0.28. This result excludes the possibility that specimens in group 2 belong to *O. melolonthae*, with which it is often confused due to the morphological characteristics of this species.

**Production of *Cordyceps militaris* (C-71) and *C. brongniartii* (C-72) stromata**

In this study, the isolated strains were cultured *in vitro* with the aim of obtaining fertile stromata. The inoculum was added after 15 days of incubation with agitation and the substrate was completely covered after 6 days. The development of abundant cotton-like mycelium was observed on the substrate, which turned yellowish for strain C-71 and whitish for strain C-72. The appearance of primordia was observed after placing the production units in the presence of light. A higher number of primordia was observed on the top of the substrate after 25 days for C-71 and after 37 days for C-72 (Figure 3, a and c). The presence of orange pigmentation was evident when the containers were placed under pink light with periods of 12 h of light and 12 h of darkness. The mature stage was identified by the presence of small perithecia in the stroma (Figure 3, b and d). The total production time was 76 days for C-71 and 109 days for C-72, with biological efficiencies of 33.7 and 36.13 %, respectively.


Figure 1. Specimens of *Cordyceps* spp. a: Wild C-71 specimen, *Cordyceps af. militaris.* b: Fungal strain of C-71. c: Ovoid perithecia from stroma of specimen 1. d: Wild specimen C-72 *Ophiocordyceps* af. *A. melolonthae*. e: Fungal strain C-72. F: Ovoid perithecia of the stroma of specimen 2.

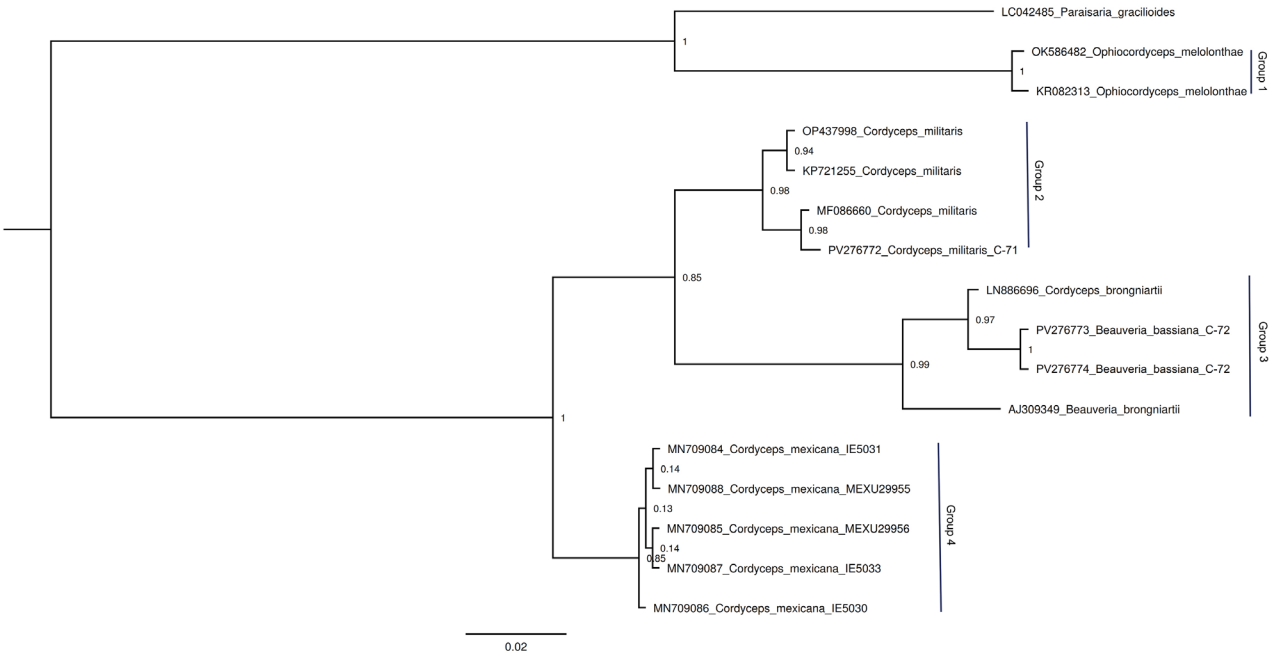


Figure 2. Phylogeny obtained with the Bayesian Inference criterion using *Paraisaria gracilioides* as outgroup. pP values are indicates on the branches.

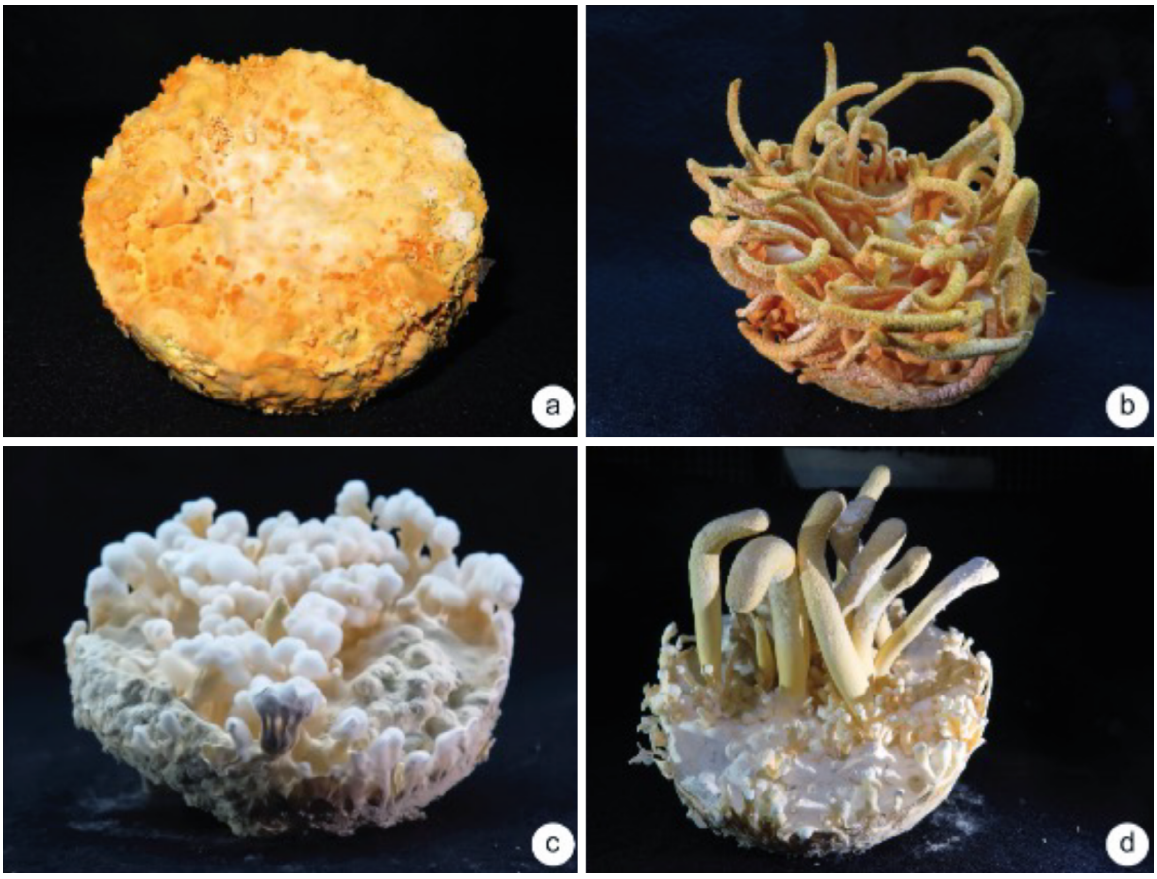


Figure 3. *In vitro* culture of *Cordyceps militaris* (C-71) and *C. brongniartii* (C-72). a: Substrate covered with cotton-like mycelium with orange pigmentation and the presence of primordia (C-71). b: Production of mature stroma with orange pigmentation and the presence of perithecia in the apical part (C-71). c: Substrate covered with cotton-like mycelium without pigmentation. The appearance of stromal primordia and the formation of the anamorphic phase observed in the absence of light (C-71). d: Production of mature stroma with light yellow pigmentation and the presence of perithecia in the apical part (C-71).

Table 1. Genetic distance analysis

| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *CobiochUAEMor-C-71* | GRUPO 1 | *1* | | | | | | | | | | | |
| *OP437998_Cordyceps militaris* | | *2* | *0.02* | | | | | | | | | | |
| *MF086660_Cordyceps militaris* | | *3* | *0.01* | *0.01* | | | | | | | | | |
| *KP721255_Cordyceps militaris* | | *4* | *0.02* | *0.00* | *0.01* | | | | | | | | |
| *CobiochUAEMor-C-72* | GRUPO 2 | *5* | *0.12* | *0.12* | *0.11* | *0.12* | | | | | | | |
| *CobiochUAEMor-C-72* | | *6* | *0.12* | *0.12* | *0.11* | *0.12* | *0.00* | | | | | | |
| *LN886696_Cordyceps brongniartii* | | *7* | *0.11* | *0.10* | *0.10* | *0.10* | *0.01* | *0.01* | | | | | |
| *AJ309349_Beauveria brongniartii* | | *8* | *0.12* | *0.11* | *0.11* | *0.11* | *0.05* | *0.05* | *0.04* | | | | |
| *OK586482_Ophiocordyceps melolonthae* | GRUPO 3 | *9* | *0.27* | *0.27* | *0.26* | *0.27* | *23* | *0.22* | *0.26* | *0.28* | | | |
| *KR082313_Ophiocordyceps melolonthae* | | *10* | *0.24* | *0.24* | *0.23* | *0.24* | *0.20* | *0.20* | *0.24* | *0.27* | *0.01* | | |
| *LC042485_Paraisaria gracilioides* | | *11* | *0.33* | *0.32* | *0.32* | *0.32* | *0.27* | *0.27* | *0.32* | *0.37* | *0.15* | *0.14* | |

Note: Values from 0.00 to 0.02 are considered the same species. Values greater than 0.02 are considered different species.

## DISCUSSION

There is a great diversity of habitats in the state of Morelos, which favors the coexistence of abundant species, including insects and fungi (Burgos-Solorio and Tello-Salgado 2021). This has created an ideal environment for the development and dissemination of entomopathogenic fungi. There are 18 species of the genus *Cordyceps* recorded in Morelos, making it the fourth state with the highest number of species recorded, the first being the state of Mexico (López-Rodríguez *et al.* 2023). More studies are needed, as the state is surrounded by two large natural reserves. There is little information on the molecular identification and genetic diversity of *Cordyceps* in Mexico and the state of Morelos.

Several studies have described the isolation of strains of entomopathogenic fungi. Strains of the genus *Cordyceps* have been isolated by multisporic methods (Shrestha *et al.* 2012, Tasanathai *et al.* 2019). From the resulting fruiting bodies, strains are reisolated using the best characterized and genetically stable stroma for the production of further stromata (López-Rodríguez *et al.* 2022). In this work, strains of *C. militaris* and *C. brongniartii* were obtained through a somatic isolation strategy, taking a fragment of stromal tissue from wild specimens, thus ensuring the dikaryotic character of the mycelium, capable of forming stable fruiting bodies for production without losing the characteristics of the progenitors. Conservation of native biological resources is important because they become valuable genetic resources when domesticated, identified, and produced under controlled conditions (Challen *et al.* 1995). *Cordyceps militaris* and *C. sinensis* have been used as biological resources with anticancer, immunomodulatory, and antihypertensive activities (Castillo *et al.* 2018, Zhao *et al.* 2018, Lou *et al.* 2019, Li *et al.* 2021, Zhang *et al.* 2021).

The analysis of rDNA sequences allowed the identification of *C. militaris* with genetic distance values of less than 0.02, confirming the presence of this species in Morelos, Mexico. Chen *et al.* (1999) showed that there is no genetic variation among specimens of *C. militaris*, since no differences were observed between geographically distant populations using sequences obtained by ITS, obtaining high bootstrap values. Similar results have been reported for *C. sinensis* (Kinjo and Zhang 2001). In addition, Wang *et al.* (2008) confirmed that the globally distributed populations of *C. militaris* can be considered as a single population. Therefore, this study confirms with certainty the identity of the studied species.

For specimen C-72, distance values of 0.01 were obtained with respect to sequence LN886696, which corresponds to *C. brongniartii*. The presence of this species is novel and represents the first record for Mexico. *Cordyceps brongniartii* was first reported in Japan (Shimazu *et al*. 1988), and it was later confirmed by molecular data (Sasaki *et al*. 2007). *Cordyceps brongniartii* has been reported as a teleomorph stage of the anamorph *Beauveria bassiana*; in this work we report for the third time the presence of *C. brongniartii* in the world in the wild, identified by molecular data and the production *in vitro* of stromas. Teleomorph stages of some fungi are very rare; however, environmental conditions and factors favor the expression of the sexual character of *C. brongniartii*.

Group 3 of the phylogenetic tree contains the clade corresponding to *Ophiocordyceps melolonthae*, included in the phylogenetic analysis. The dissimilarity between group 3 and group 2, corresponding to *C. brongniartii*, with genetic distance values from 0.22 to 0.28, shows that the collected specimens do not correspond to *O. melolonthae*, which shares morphological and microscopic characteristics with our specimen, since the presence of this species has been reported in the same area (Pérez-Silva 1977). These results show that C-72 corresponds to *C. brongniartii*. This result emphasizes the need to perform molecular analyses in addition to morphological studies to determine species and avoid misidentifications.

The *in vitro* production of stromata from fungi of the genus *Cordyceps* has been reported, particularly for *C. militaris* (Kobayasi and Shimizu 1983, Basith and Madelin 1968), using rice grains and silkworms as substrates. During the process, environmental factors such as temperature and light are controlled to ensure the production of fruiting bodies (Shrestha *et al*. 2012). In this study, a sorghum-based formulation supplemented with animal and plant proteins was used for the first time with positive results to produce stromata. This substrate could be used for large-scale production of both species, provided that the presence of light and temperature are kept under control. It is important to note that these factors determine the teleomorph stage of the species studied. *C. militaris* was able to produce stromata on supplemented sorghum after 76 days, and *C. brongniartii* did so after 109 days.

The ability to produce stromata of entomopathogenic fungi such as *Beauveria* spp. *in vitro* opens new possibilities for novel studies on ecology, mating systems, developmental biology, biochemical studies, and pathogenesis of these fungi, for which the teleomorph stage is not common and often difficult to study. This finding allows us to obtain useful biological resources to contribute to the generation of knowledge in these disciplines. On the other hand, the production of *C. brongniartii* stromata also opens up new areas of research, such as the production of these fungi for biological control, the cultivation of new isolates and the large-scale production of fruiting bodies, as well as their production in submerged cultures and even the production of fruiting bodies for medicinal purposes.

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