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Article

Identificación y distribución del mosquito fungoso negro, *Bradysia impatiens* Johannsen, 1912 (Diptera: Sciaridae) en viveros de clima templado

Identification and distribution of the black fungus gnat *Bradysia impatiens* Johannsen, 1912 (Diptera: Sciaridae) in temperate climate nurseries

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Abstract

The black fungus gnat is a dipteran that causes severe damage to ornamental plants and forest nurseries with *Pinus* species in central Mexico, with losses over 30 %. The most recent reports of the black fungus gnat are from 2013 and 2015; the current distribution of the genus *Bradysia* sp. is unknown. Therefore, the objectives of this study were to identify the black fungus gnat morphologically and molecularly, as well as to know its distribution on pine species on five regions of Mexico. Sampling was carried out from April to June 2019 in 20 nurseries with pine species. The collected samples were placed in emergency chambers for 45 days under photoperiod conditions of 12:12 h and relative humidity (*RH*) of 75 % for obtaining the adults. 934 adults of black fungus gnat were obtained, and the species identified by morphology and molecularly through a fragment of the mitochondrial COI gene corresponds to *Bradysia impatiens* (Johannsen, 1912) (Diptera: Sciaridae), present in 17 pine species, including *Pinus arizonica*, *P. ayacahuite*, *P. cembroides*, *P. cooperi*, *P. douglasiana*, *P. durangensis*, *P. engelmannii*, *P. hartwegii*, *P. leiophylla*, *P. oaxacana*, *P.* oocarpa, *P. patula* and *P. teocote*, as new hosts. In addition, the fungus gnat is recorded for the first time in nine Mexican states: *Chiapas*, *Chihuahua*, *Durango*, *Hidalgo*, *Jalisco*, *Oaxaca*, *Querétaro*, *Veracruz* and *Zacatecas*. Currently, *B. impatiens* is known in 15 states of Mexico.

Key words: *Bradysia impatiens* (Johannsen, 1912), COI, black fungus gnat, *Pinus*, pest, forest nurseries.

Resumen

El mosquito fungoso negro es un díptero que causa severos daños en plantas ornamentales y en viveros forestales que cultivan especies de *Pinus* en el centro de México, con pérdidas superiores al 30 %. Los

informes más recientes del mosquito fungoso negro son de 2013 y 2015; se desconoce la distribución actual del género *Bradysia*. Por lo anterior, los objetivos del presente estudio consistieron en identificar morfológica y molecularmente a las especies del mosquito, conocer su distribución y las especies de pino que ataca en cinco regiones de México. Se realizó un muestreo de abril a junio de 2019 en 20 viveros con especies de pino. Las muestras recolectadas se colocaron en cámaras de emergencia durante 45 días en condiciones de fotoperiodo 12:12 h y humedad relativa (*HR*) de 75 %. Se obtuvieron 934 adultos, y la especie identificada por caracterización morfológica y un fragmento del gen mitocondrial COI correspondió a *Bradysia impatiens* (Johannsen, 1912) (Diptera: Sciaridae), presente en 17 especies de pino y, como nuevos hospederos, en *Pinus arizonica*, *P. ayacahuite*, *P. cembroides*, *P. cooperi*, *P. douglasiana*, *P. durangensis*, *P. engelmannii*, *P. hartwegii*, *P. leiophylla*, *P. oaxacana*, *P. oocarpa*, *P. patula* y *P. teocote*. Además, el mosquito fungoso se registró en nueve estados mexicanos: Chiapas, Chihuahua, Durango, Hidalgo, Jalisco, Oaxaca, Querétaro, Veracruz y Zacatecas. Actualmente, *B. impatiens* se conoce en 15 estados de la república mexicana.

Palabras clave: *Bradysia impatiens* (Johannsen, 1912), COI, mosquito fungoso negro, *Pinus*, plaga, viveros forestales.

Introduction

At present, 90 % of coniferous plants in Mexico are produced in forest nurseries under a container production system (Conafor, 2016). At these nurseries, the black fungus mosquito of the Sciaridae family is considered a pest. Around 2 400 species have been described in the world (Mohrig and Menzel. 2009). In the Nearctic region, which includes Canada, the United States of America and Northern Mexico, there are an estimated 166 species of 25 genera (Mohrig *et al.*, 2012). Despite their economic and ecological importance, cyariids have been poorly studied due to their small size, way of life and the difficulty to determine their taxonomic identity (Mohrig and Menzel, 2009; Villanueva-Sánchez *et al*., 2013).

At nurseries in central Mexico, *Bradysia impatiens* Johannsen, 1912 and *Lycoriella ingenua* Dufour, 1839 cause economic losses of up to 30 % in the production of pine seedlings, even when cultural, biological, chemical and physical control measures are applied (Cibrián *et al.*, 2008; Marín-Cruz *et al*., 2015a; García, 2017). In Italy, *B.*

impatiens is responsible for damage to eucalyptus plants (Mansilla *et al*., 2001) and in South Africa it attacks pine seedlings (Hurley *et al.*, 2007, Hurley *et al.*, 2010).

Both Diptera species are common in greenhouses, nurseries, potted plants, edible mushroom cultivation, and decaying organic matter (Menzel *et al.*, 2003; Mohrig and Menzel, 2009; Shin *et al*., 2012). *Bradysia* Winnertz is the largest genus in the family with 433 species compared to 85 species of *Lycoriella* (Menzel and Mohrig, 1999).

Ciarid larvae cause direct damage by feeding on the root and indirect damage from their ability to transmit phytopathogenic fungi such as *Botrytis cinerea* Pers., 1797, *Pythium* sp. nov., *Fusarium oxysporum* Schltd., 1824, *Verticillium alboatrum* Reinke & Berthold, 1879, *Verticillium fungicola* (Preuss) Hassebr., 1936 and *Fusarium circinatum* Nirenberg & O'Donnell, 1998 (Hurley *et al*., 2007; Shamshad *et al.*, 2009; Cloyd, 2015; Marín-Cruz *et al.*, 2015b). These insects disperse to new areas through the movement of plants by humans and substrates such as peat (Marín-Cruz *et al.*, 2015b); they have a cosmopolitan presence (Mohrig *et al.*, 2012; Shin *et al.*, 2012). The current extent of the fungus mosquito and what species of ciarids are in the pine species are unknown in Mexico. Therefore, the objectives of the following study were to identify the species of black fungus mosquito by morphological and molecular characterization, to know their distribution and the pine species that attack in five regions of Mexico.

Materials and Methods

Sample collection

The samples were collected in 20 forest nurseries (April to June 2019), which produce pines for reforestation; 19 nurseries have the container production system and one uses polypropylene bag (*La Gloria*). The nurseries are located in 12 Mexican states (Table 1), which belong to five geographical regions (I=North, II=West, III=Center, IV=Gulf, V=South). In each nursery samples were collected by species in production at the time of the visit, whose selection was made by directed sampling.

Table 1. Location of forest nurseries in the 2018-2019 production cycle in five regions of Mexico.

Region	State	Municipality	Nursery	Coordinates	Altitude masl
I=North	*Chihuahua*	*Delicias*	*El Forestal*	28°08'42'' N; 105°31'04'' W	1 193
		Bocoyna	*El Ciruelo*	28°02'08'' N; 107°36'12'' W	2 451
	Durango	*Durango*	*Francisco Villa*	23°58'22'' N; 104°35'53'' W	1 875
		Santiago Papasquiaro	*Cielo Azul*	25°02'09'' N; 105°15'40'' W	2 077
	Zacatecas	*Tlaltenango*	*Tlaltenango*	21°46'12'' N; 103°17'29'' W	1 723
II=West	*Jalisco*	*Gómez Farías*	*MASVI*	19°48'53'' N; 103°28'53'' W	1 527
		Sayula	*Provincia de Avalos*	19°52'46'' N; 103°35'50'' W	1 370
	Michoacán	*Zinapécuaro*	*Atzimba*	19°51'23'' N; 100°51'53'' W	1 845
III=Center	*Querétaro*	*Amealco de Bonfil*	*Ignacio Pérez*	20°32'00'' N; 100°11'01'' W	1 912
	Hidalgo	*Zimapán*	*Zimapán*	20°44'38'' N; 99°23'15'' W	1 799
	Estado de México	*Jilotepec*	*Teoran Productores*	19°59'00'' N; 99°33'10'' W	2 520
		Temamatla	*Temamatla*	19°11'12'' N; 98°52'23'' W	2 289
	Puebla	*Chignahuapan*	*El Rincón*	19°46'15'' N; 98°01'05'' W	2 423
			Pueblo Nuevo	19°57'35'' N; 98°06'26'' W	2 603
		Vicente Guerrero	*Plantaciones Teotlalco*	18°27'54'' N; 98°46'32'' W	1 027
IV=Gulf	*Veracruz*	*Perote*	*Perote*	19°34'51'' N; 97°13'30'' W	2 409
			La Gloria	19°36'20'' N; 97°12'18'' W	2 426
V=South	*Oaxaca*	*Tamazulapan*	*Tamazulapan*	17°41'06'' N; 97°35'08'' W	1 965
		Monjas	*Los Pocitos*	16°21'55'' N; 96°37'43'' W	1 523
	Chiapas	*Cintalapa*	*San Agustín*	16°28'06'' N; 93°59'57'' W	699

The samples consisted of four plants with root by species. Only specimens were chosen with wilting signs, yellowish green foliage, leaf loss, root rot and little growth, all caused by the larvae of the fungus gnat in pines. Each sample was saved in Ziploc® hermetic bags to avoid the possible contamination of other samples. In total, 20 samples of the *Pinus genus* (80 trees) were collected. The samples were processed at the insectary of the Forest Parasitology Lab in the Forest Science Division of the *Universidad Autónoma Chapingo* for their analysis.

Processing of collected plants and adults emergence

The plants collected with the root ball were placed in adult emergency chambers under 12:12 h photoperiod conditions and 75 %relative humidity (*RH*). The chambers consisted of one-liter containers, made of transparent plastic with a lid, with a 3 cm diameter circle perforation in the center (to collect fungus mosquito adults, as well as to supply water and food); this perforation was covered with a plug of gauze and cotton. The emergence cameras were reviewed every 24 h for 45 days. From each of the samples placed in the breeding chambers, adult specimens of the fungus mosquito were taken with a manual aspirator, which were sacrificed and preserved in 70 % and 100 % ethanol for their morphological and molecular identification.

Morphological identification

The identification of the specimens was made with a EZ4 Leica® stereoscopic microscope; in the first instance, they were separated by sex. Subsequently, 20 males were cut from the genitalia, head, palps, tibias and wings, structures that were prepared in slides with cotton blue to lighten the tissues (Poinar and Thomas, 1984). A M80 Leica® compound optical microscope and a DFC295 camera of the same brand were used, with which photographs of the structures were taken for species determination purposes. Thus, the taxonomic keys of the Sciaridae family of Menzel *et al.* (2003), Mohrig and Menzel (2009), Mohrig *et al.* (2012), Shin *et al.* (2012) and Marin-Cruz *et al.* (2015a) were also used.

In order to confirm the taxonomic identification, a sample of eight adults (4 ♂ and 4 ♀) per nursery was sent to M. Sc. Herón Huerta, head of the Collection of Arthropods with Medical Importance (CAIM, for its acronym in Spanish) of the Institute of Epidemiological Diagnosis and Reference (InDRE). The material was deposited in the entomological collection of the Division of Forest Sciences (DiCiFo, for its acronym in Spanish) of the *Universidad Autónoma Chapingo* (UACh) with registration number 28x+48.

DNA extraction, COI gene amplification, PCR and analysis

Genomic DNA was extracted from complete adults (one adult per nursery) by the CTAB method (Stewart and Via, 1993). The DNA was quantified in a ND 1 000 Nanodrop (Thermo Scientific, USA). From each of the DNA samples, 20 ng dilutions were prepared for amplification of the Oxidated Cytochrome I (COI) gene by means

of PCR with oligonucleotides LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al.*, 1994). The amplified products were cleaned with kit ExoSAP-IT ™ (Thermo Fisher Scientific, USA), according to the manufacturer's instructions, and sent to the Institute of Biology of the *Universidad Nacional Autónoma de México* (UNAM).

Sequences were assembled and edited with Bioedit 7.0.5 (Hall, 1999) and compared with sequences deposited in NCBI GenBank (National Center for Biotechnology Information, www.ncbi.nih.gov) using the BLAST program. To confirm the morphological identification, a phylogenetic analysis was carried out with reference sequences from GenBank (KX538548, MW798234, OM421642) and together with those obtained from this study they were aligned with the Clustal W method and processed with the Maximun Likelihood method based on the three-parameter Tamura model with 5 000 bootstrap replications (Tamura, 1992) with the MEGA 11 program (Tamura *et al.*, 2021).

For the phylogenetic tree, a sequence of *Psychoda alternata* Say, 1824 from GenBank with access number LC422861 was used as root. All the sequences of this study were deposited in GenBank to obtain their accession number.

Results and Discussion

934 black fungus mosquito adults in total were collected, with an average of 46.7 adults per forest nursery and 100 % belonged to the *B. impatiens* species, considered the main pest in the five sampling regions (Figure 1).

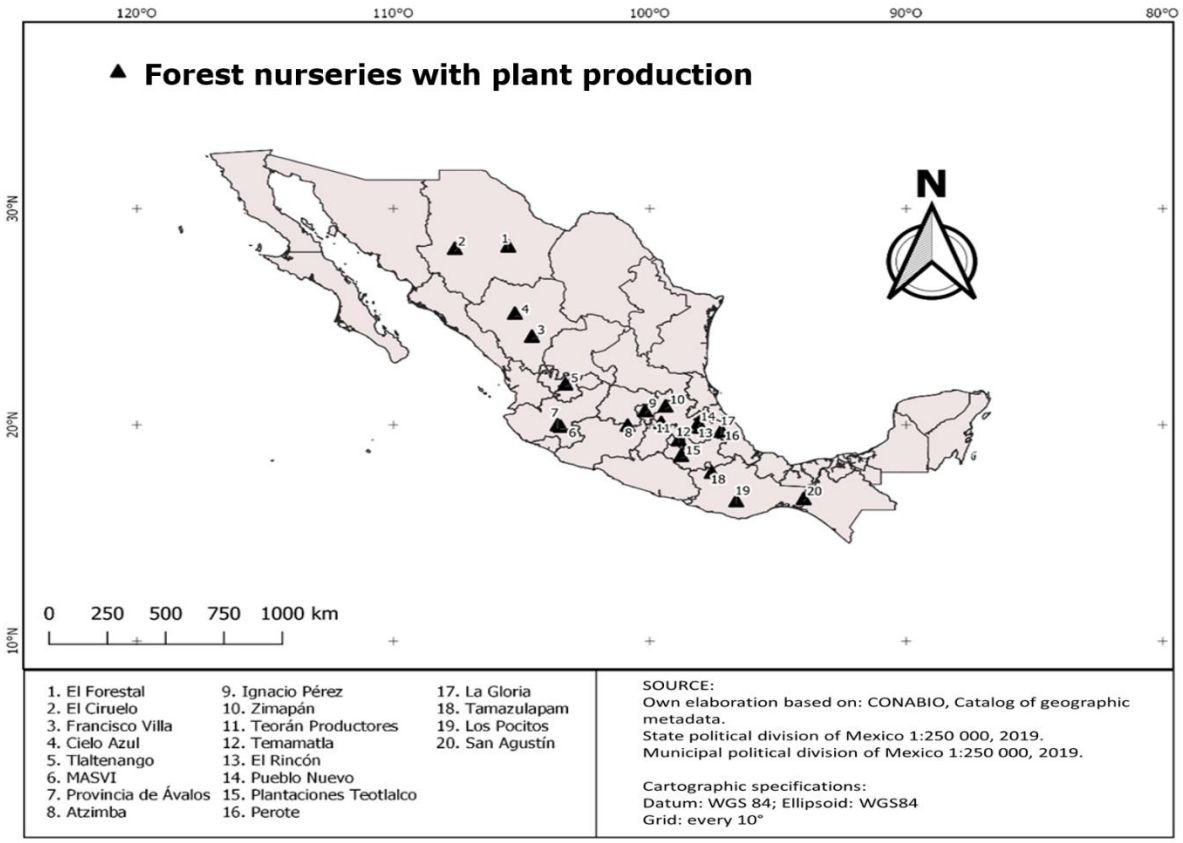

Figure 1. Distribution of *Bradysia impatiens* Johannsen, 1912 in Mexico.

In order to make easier the identification of *B. impatiens*, its characteristics are described below: Adults dark gray to black, 2 to 2.5 mm long, female larger than male. Round to ovoid head with moderately prominent eyes with slightly elongated mouthparts (Figure 2A). Antenna: 14 flagellomeres, fourth flagellomere 1.6 times as long as wide, surface slightly rough (Figure 2B-C). Palp: moderately long, yellow to light brown, three-segmented, basal segment with deep sensory fossa, long, slightly curved sensillae with blunt tip (Figure 2D). Tibia: Inner side of the anterior tibia with a row of 10 setae; middle and posterior tibia with two thin setae in the form of spurs, subequal (Figure 2E). Genitals: compacted, no basal lobe or group of setae in ventral view. Short gonococcyte, covered with dark setae, as well as thick and long setae, mainly at the base (Figure 2F). Tergite 9 short, trapezoidal, slightly

emarginate apically with several long setae. Tegmen slightly wider than long, rounded apically; aedeagus with sclerotized base, length 0.1 mm. Gonostyle: 2.5 times as long as wide, eight subequal spines curved ventromedially and one thick apical spine (Figure 2F). Wing: with total length 1.95 mm, width 0.80 mm, greyish-brown infused; posterior veins without macrotrichia; base of M longer than bifurcation of M (Figure 2G).

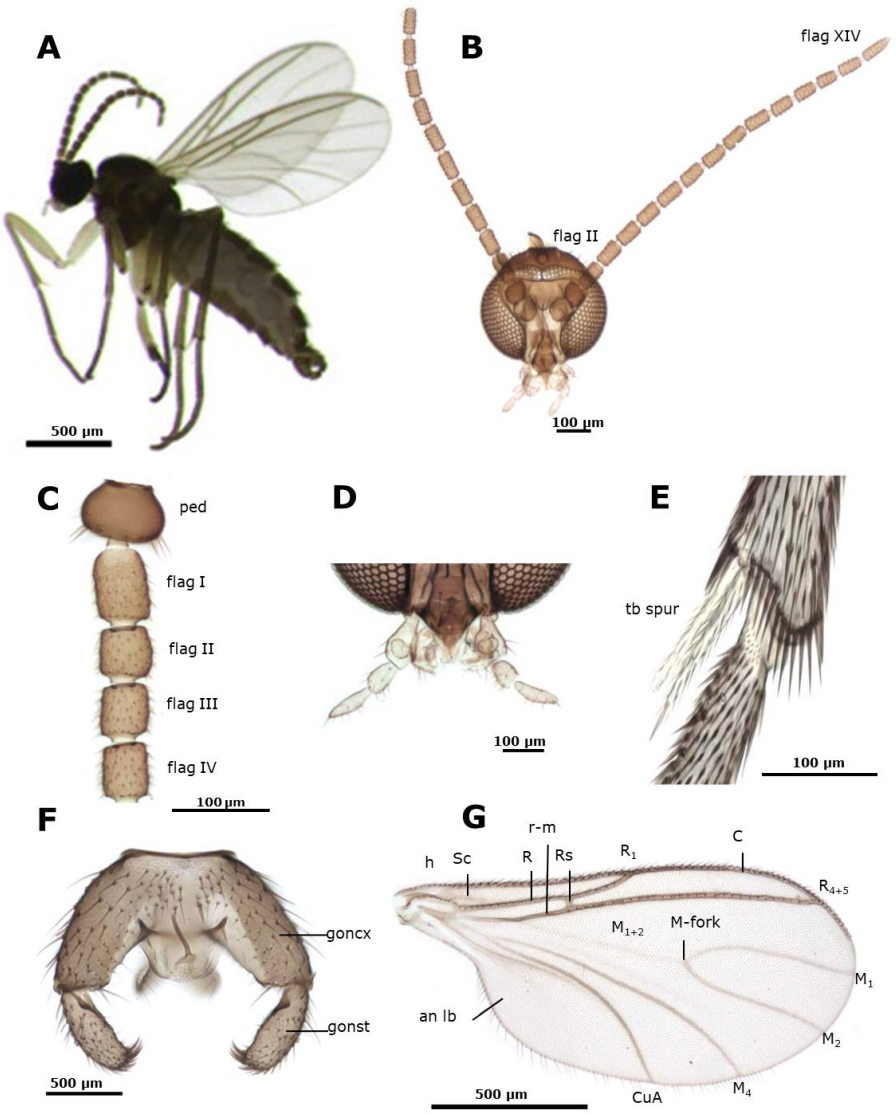

Figure 2. Lateral view of adult *Bradysia impatiens* Johannsen, 1912. A) Habit (♂); B) Head frontal view; C) Lateral view of antennal flagellomeres; D) Feel; E) Tibial spur; F) Genitalia; ventral view; G) To. Abbreviations: flag I, flag II, flag III, flag IV, flag; XIV: first, second, third, fourth, fourteenth antennal flagellomere; tb spur: tibial spur; goncx: gonocoxyte; gonst: gonostyle; h: brachial vein; Sc: subcostal vein; R: radial vein; r-m: transverse radiomedial vein; Rs: radial sector; R_1: first longitudinal vein; C: costal vein; R_{4+5}: third longitudinal vein; M_1: first branch of the median vein; M_2: second branch of the median vein; M_4: fourth branch of the median vein; M_{1+2}: fusion of the first and second branches of the median vein; M-fork: bifurcation of the vein medial; CuA: anterior cubital vein; an lb: anal lobe.

The genetic identification confirmed the morphological identification: the dipterans that emerged from the breeding chambers belong to *B. impatiens*, with 99.6 % identity with those available in Genbank (Figure 3, Table 2). The phylogenetic tree shows the formation of two groups: Group 1, made up of adults of the fungus mosquito from 16 forest nurseries in the Northern, Western, Central and Gulf regions; Group 2, formed by a nursery from the center (*Teoran Productores*) and three from the southern region (*Chiapas*: *Los Pocitos* and *San Agustín*; *Oaxaca*: *Tamazulapan*).

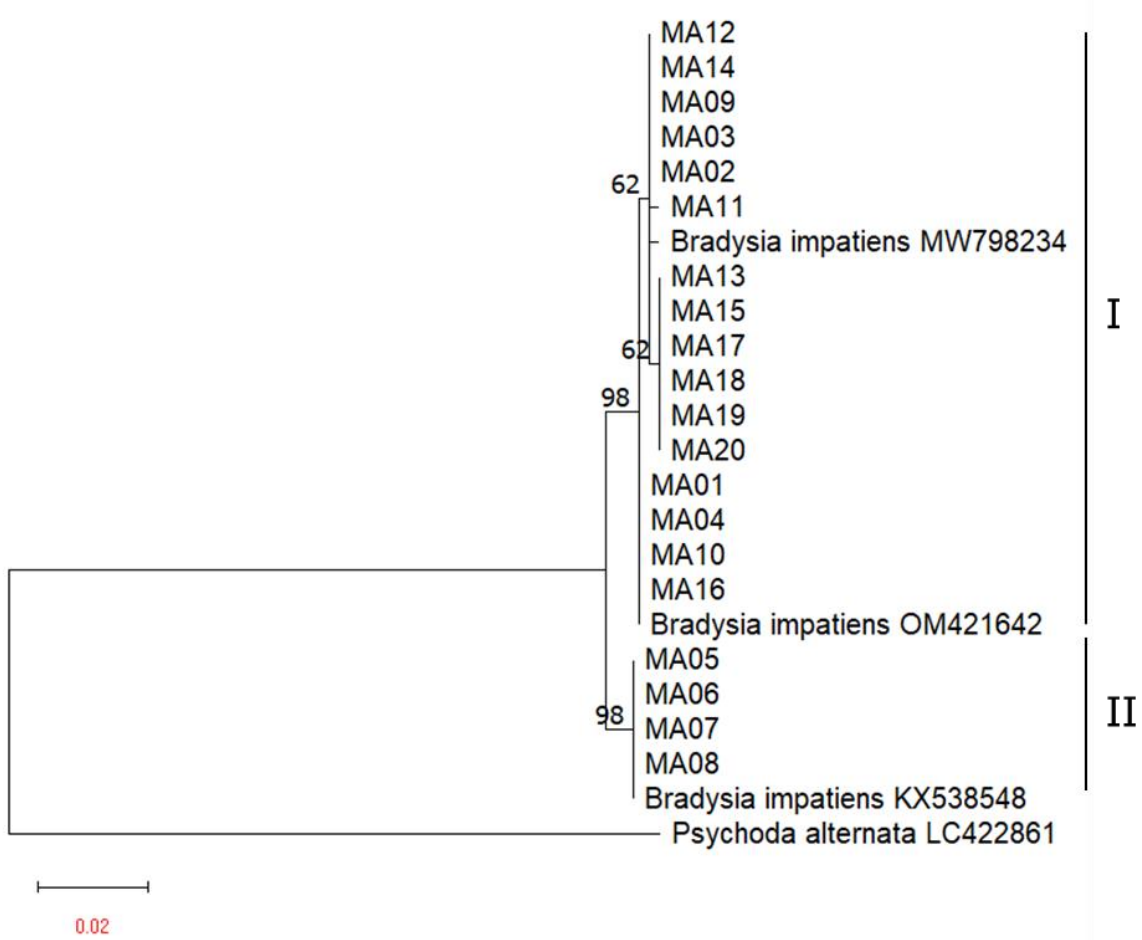

Figure 3. Phylogenetic tree constructed with sequences corresponding to Cytochrome oxidase I (COI) of *Bradysia impatiens* Johannsen, 1912 with the maximum likelihood method based on the three-parameter Tamura model (T92). The percentages were calculated with 5 000 bootstrap replications.

Table 2. Nurseries with pine species with the presence of *Bradysia impatiens Johannsen, 1912*, collection key and access number in GenBank**.**

Nursery	Species	Key	Genbank number
El Forestal	*Pinus engelmannii* Carrière	MA17	MT827936
El Ciruelo	*Pinus durangensis* Martínez	MA04	MT827923

Francisco Villa	*Pinus cooperi* C. E. Blanco	MA02	MT827921
Cielo Azul	*Pinus arizonica* Engelm.	MA11	MT827930
Tlaltenango	*Pinus leiophylla* Schiede ex Schltdl. & Cham.	MA03	MT827922
MASVI	*Pinus douglasiana* Martínez	MA12	MT827931
Provincia de Avalos	*Pinus devoniana* Lindl.	MA13	MT827932
Atzimba	*Pinus pseudostrobus* Lindl.	MA01	MT827920
Ignacio Pérez	*Pinus greggii* Engelm. ex Parl.	MA09	MT827928
Zimapán	*Pinus cembroides* Zucc.	MA14	MT827933
Teoran Productores	*Pinus ayacahuite* C. Ehrenb. ex Schltdl.	MA07	MT827926
Temamatla	*Pinus hartwegii* Lindl.	MA16	MT827935
El Rincón	*Pinus teocote* Schltdl. & Cham.	MA10	MT827929
Pueblo Nuevo	*Pinus montezumae* Lamb.	MA18	MT827937
Plantaciones Teotlalco	*Pinus oaxacana* Mirov	MA15	MT827934
Perote	*Pinus patula* Schltdl. & Cham.	MA19	MT827938
La Gloria	*Pinus montezumae* Lamb.	MA20	MT827939
Tamazulapam	*Pinus devoniana* Lindl.	MA08	MT827927
Los Pocitos	*Pinus devoniana* Lindl.	MA06	MT827925
San Agustín	*Pinus oocarpa* Schiede ex Schltdl.	MA05	MT827924

Sequence alignment yielded an array of 540 bp per sequence. This molecular marker, Oxidized Cytochrome I (COI), is useful for species-level identification from immature stages of *Bradysia* sp. (Shin *et al.*, 2015). In addition, the design of a pair of specific oligonucleotides for this species of black fungus mosquito should be considered from the mitochondrial Cytochrome Oxidase I (COI) gene in combination with oligonucleotides LCO1490 and HCO2198, which would help to identify faster. A similar case was used to identify *B. odoriphaga* Yang and Zhang in Japan (Arimoto, 2022).

The location of the black fungus gnat in Mexico is as follows: Mexico City, State of Mexico, *Michoacán*, *Morelos*, *Puebla* and *Tlaxcala* (Villanueva *et al*., 2013; García, 2017), which states that *Chiapas*, *Chihuahua*, *Durango*, *Hidalgo*, *Jalisco*, *Oaxaca*, *Querétaro*, *Veracruz* and *Zacatecas* are new reports for the country (Figure 1). This species has a wide range of distribution, since the localities where it is found stand from 699 to 2 603 masl and distances of 800 to 1 800 km between each nursery, a fact that makes it impossible for *B. impatiens* to migrate naturally (Table 1, Figure 1).

Steffan (1981), Frouz and Nováková (2001) and Marín-Cruz *et al.* (2015a) mentioned that the insect has little ability to fly, its life span as adults is 4 to 6 days and its habits prevent it from moving long distances. Mohring *et al.* (2012) consider that the spread of *B. impatiens* is mainly by humans.

The species has been recorded in Europe (Germany, Azerbaijan, Spain, Finland, Netherlands, Ireland, Italy, Latvia, United Kingdom, Czech Republic, Switzerland and the Ukraine), Asia (China, South Korea, Japan and Russia), America (Brazil, Canada, United States and Venezuela), Africa (South Africa) and Oceania (Australia) under cover crops and in nurseries (Menzel *et al.*, 2003; Mohring *et al.*, 2012; Shin *et al.*, 2012). This Diptera species causes considerable economic losses in the center of the country in the cultivation of *Pinus montezumae* Lamb., *P. greggii* Engelm. ex Parl., *P. devoniana* Lindl. (Syn.: *P. michoacana* Martínez 1948) and *P. pseudostrobus* Lindl. (López-Pérez *et al.*, 2009; Marín-Cruz *et al.*, 2015b; García *et al.*, 2017; Marín *et al.*, 2017).

In addition, there are reports that *B. difformis* (like *B. impatiens*) lodges and feeds on the stem and root of Christmas Eve (*Euphorbia pulcherrima* Willd. ex. Klotzch) (Villanueva-Sánchez *et al.*, 2013) and other ornamentals such as cyclamen (*Cyclamen* sp. L.), carnation (*Dianthus* sp. L.), gerbera (*Gerbera* sp. L.), lily (*Lilium* L.), rose (*Rosa* sp. L.), African violet (*Saintpaulia* sp. H. Wendl.) and geranium (*Pelargonium* sp. L'Hér.) (García, 2008; García-Pérez *et al.*, 2021). However, the presence of other species of the

genus should be considered, mainly in the southern part of Mexico, as it forms part of the neotropical region, where there are few studies related to this group of insects.

For the neotropical region made up of South America, Central America and the Caribbean, about 62 *Bradysia* species are recorded (Amorim, 1992). Broadley *et al.* (2018) have recorded *B. impatiens*, *B. ocellaris* Comstock, 1882, *B. tilicola* Loew, 1850, *Cosmosciara* sp. Frey, 1942, *Lycoriella agraria* Felt, 1898, *L. ingenua* Dufour, 1939, *L. sativae* Johannsen, 1912 and *Pnyxia scabiei* Hopkins, 1895 as pests in the Holarctic region, for feeding on living plant tissue.

Obtaining adults from plants with symptoms of fungus gnat damage indicates that it can inhabit and reproduce in species of the genus *Pinus*, regardless of growth habit, moisture requirements, production system (containers or polyethylene bag) and the kind of substrate (compost, mountain soil, precomposted bark, sawdust, peat, vermiculite or agrolite).

Finally, this study provides evidence that *B. impatiens* has great plasticity to adapt to different heights, environmental conditions and hosts. In this study, the fungus fly is reported as the cause of plant loss in 17 pine species in 12 states of the Mexico and, as new hosts, 13 species of the *Pinus* genus (*P. arizonica*, *P. ayacahuite*, *P. cembroides*, *P. cooperi*, *P. douglasiana*, *P. durangensis*, *P. engelmannii*, *P. hartwegii*, *P. leiophylla*, *P. oaxacana*, *P. oocarpa*, *P. patula* and *P. teocote*).

Conclusions

The results of morphological characterization and molecular analysis of the black fungus gnat confirmed that the cyariids obtained from pine seedlings correspond to

Bradysia impatiens, which is the cause of the loss of specimens in 17 pine species from five regions of Mexico. Of the 17 species, 13 are new records as hosts of it (*Pinus arizonica*, *P. ayacahuite*, *P. cembroides*, *P. cooperi*, *P. douglasiana*, *P. durangensis*, *P. engelmannii*, *P. hartwegii*, *P. leiophylla*, *P. oaxacana*, *P. oocarpa*, *P. patula* and *P. teocote*). In addition, the fungus mosquito is recorded for the first time in nine states of Mexico: *Chiapas*, *Chihuahua*, *Durango*, *Hidalgo*, *Jalisco*, *Oaxaca*, *Querétaro*, *Veracruz* and *Zacatecas*. Currently, *B. impatiens* is known in 15 states of the country.

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Conflict of interests

The authors declare no conflict of interests.

Contribution by author

Víctor Hugo Marín Cruz: collection in the field and identification, writing and correction of the manuscript; David Cibrián Tovar and Silvia Edith García Díaz: writing and correction of the manuscript; Omar Alejandro Pérez Vera: phylogenetic analysis, writing and correction of the manuscript; Herón Huerta Jiménez: identification and photography; Edgar Magdaleno Hernández: writing of the manuscript.

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