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Scientific Notes

Insights into the microbes and nematodes hosted by pupae of the arundo leaf miner, *Lasioptera donacis* (Diptera: Cecidomyiidae)

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The arundo leaf miner *Lasioptera donacis* Coutin (Diptera: Cecidomyiidae) is a biological control agent of the invasive bamboo-like weed, *Arundo donax* L. (Poaceae), also known as giant reed or carrizo cane, that is severely threatening riparian habitats throughout the southern half of the United States from California to Maryland and south into Mexico (Dudley 2000; Goolsby et al. 2016). This agent was imported from the Mediterranean region of Europe to the USDA-APHIS Arthropod Quarantine Laboratory, Edinburg, Texas, USA, for evaluation (Thomas & Goolsby 2015; Goolsby et al. 2017a, b) after which the agent was approved for release in the United States and Mexico (USDA-APHIS 2016). Pupae and third instar larvae are the preferred stages for transatlantic shipment of living material, and knowledge of the basic biology and parasitism of pupae is of some importance owing to the potential mutualistic relationship of the agent with fungi, as first reported by Coutin & Faivre-Amiot (1981). One partner in this relationship is the cosmopolitan grass saprophytic fungus *Arthrinium arundinis* Corda (Xylariales: Apiosporaceae), that was shown to be associated with oviposition and larval feeding of *L. donacis* (Cristofaro et al. 2014; Goolsby et al. 2017a). However, it is still unclear as to whether the newly emerged females, in order to successfully achieve their development, would carry forward conidia of this fungus or another fungus from the pupa, or would have to acquire the conidia exclusively from the plant. The second strategy would correspond with the findings of Rohfritsch (2008) on *L. arundinis* Schiner (Diptera: Cecidomyiidae) on common reeds. We hypothesized that conidia would not be transferred via the immature stage to the adult. The purpose of the present study is to test this hypothesis by artificially smearing arundo leaf sheaths with one local strain of *A. arundinis* (strain EGG3) in order to ensure that conidia are available to midge females by allowing their eggs to develop and eventually attain the pupal stage, and by screening for fungi in pupae by polymerase chain reaction (PCR). The choice of the universal primers of the ITS region spanning 2 intergenic transcribed spacers (ITS1 and ITS2) and 5.8S rRNA in this PCR approach also would allow identification of any potentially transferred fungus other than *A. arundinis*. If *A. arundinis* is found inside pupae, the strain would be further characterized using a multi-locus approach. In addition, we tested for the presence of parasitic nematodes in these pupae using universal primers of the SSU rRNA gene (18S), and of a fragment of the D1/D2 domains of the large subunit rRNA genes (28S) owing to the fact that pupae of this midge were shown to be infected by *Tripius gyraloura* (Aphelenchoidea: Sphaerulariidae) (Poinar & Thomas 2014).

Giant reed canes infested with *L. donacis* were collected near the European Biological Control Laboratory in Southern France, which was one of the sites where *T. gyraloura* was historically collected (Poinar & Thomas 2014). Sections of these canes were set up in a 40 × 30 × 35 cm clear plastic box with 2 windows covered with fine white mesh fabric containing about 4 cm of moistened sand to await emergence of the adults needed for conducting the assay. Meanwhile, rhizomes of the giant reed were dug locally and planted in 5 L pots to develop stems up to 1 m tall. Following the emergence of adult midges, the leaf-sheaths of these stems were smeared with a fungal conidia suspension (10^6 cfu per mL) of EGG3. Strain EGG3 was isolated by Guy Mercadier from the surface of the ovarioles of dead *L. donacis* females and was maintained in pure culture at the European Biological Control Laboratory collection. This strain was characterized by multi-locus sequence typing including ITS (GenBank Accession number MF627422), the Elongation factor 1 (GenBank Accession number MF627423), and the Beta Tubulin (GenBank Accession number MF627424). Then each cane was pricked (10–12 times per sheath) following Goolsby et al. (2017a). Treated plants were placed inside 29 × 55 × 79 cm insect-proof cages where about 50 midge adults were released. The cages were maintained in a culture room at 25 °C with a photoperiod of 13:11 h (L:D) for 3 to 4 wk. Pupae were dissected from leaf sheaths of these plants. Pupae of *L.*

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donacis are enclosed within a silken cocoon that potentially contains microbial contaminants. Therefore, pupae were surface disinfected with a 0.5% solution of sodium hypochlorite for 15 min and rinsed extensively with sterile water. Pupae were transferred onto malt extract agar medium supplemented with 400 mg per L of chloramphenicol in Petri dishes. Dishes were incubated at 25 °C for 2 d and transferred at 4 °C for 3 more d. Pupae with no sign of contamination about 5 d after inoculation verified under a phase contrast microscope at 40× and 100× magnification (Olympus BH2; Olympus Corporation, Tokyo, Japan) were frozen, whereas those with microbial contaminants were disinfected again. Genomic DNA of individual pupa was extracted from ground frozen tissue using the DNeasy Blood and Tissue Kit (Qiagen, Courtaboeuf, France) following the manufacturer's recommendation. ITS was amplified in all DNAs using fungal primers ITS1F and ITS4 (Gardes & Bruns 1993). The amplification of the 18S was tested in all DNAs using the universal primer 988F and the nematode specific primer 1912R (Holterman et al. 2006). Then the 28S was amplified in all DNAs testing positive for nematodes using universal primers NL1 and NL4 (O'Donnell 1993). PCR for each region or gene was performed with a 23 µL reaction mixture and 2 µL of diluted DNA in a Perkin Elmer 9700 Thermocycler (Perkin Elmer, Villebon sur Yvette, France). The reagent concentrations were 1× PCR buffer (Qiagen), 1 unit Qiagen *Taq* Polymerase, 200 µM dNTPs, and 0.3 µM of each primer. PCR amplifications were carried out as follows: initial denaturation at 94 °C for 3 min, followed by 5 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, plus 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s (ITS), or 40 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 55 °C (28S) or 54 °C (18S), elongation at 72 °C for 1 min, and final extension at 72 °C for 10 min. The purified PCR products were sequenced on both strands by Genoscreen (Genoscreen Company, Lille, France) using the PCR primers. The chromatograms were edited and aligned using Seaview software, version 4 (Gouy et al. 2010). The resulting consensus sequences obtained for each region or gene were Blasted in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.gov) with the Basic Alignment Search tool (BLASTn) for homology in order to identify the probable taxa in question. In case of fungi, sequences were blasted against the rRNA_typestrains/ITS_RefSeq_Fungi (Schoch et al. 2014). Sequences were deposited in GenBank (Table 1). All of the DNAs extracted in this study had previously given rise to a positive amplification band of about 700 bp for the insect host barcode using Folmer primers (Folmer et al. 1994) attesting to their amplifiability. In the time course of this study, we obtained voucher specimens of *T. gyraloura* for which DNA extraction and 18S and 28S amplifications were conducted as mentioned earlier.

Following the disinfection treatment and culture, no microbial growth was observed in 40 out of 69 pupae, representing only 57.9% of the pupae initially dissected. These 40 pupae were screened for the presence of fungi and yeast-like fungi using the ITS primer set. Of the 40 DNAs, 9 (22.5%) gave rise to a 600 bp ITS amplicon. Eight were confirmed after sequencing to be of fungal origin and 1 was a yeast-like fungus (Table 1). We identified 5 Ascomycota sequences from these 9 ITS products, and taxa were identified with similarity scores ranging from 97 to 99% (Table 1). The predominant phylotypes belonged to the genus *Sarocladium* of the order Hypocreales. This result is not surprising since *Sarocladium* are reported as plant pathogens, saprobes, and grass endophytes (Giraldo et al. 2015), and often are associated with Poaceae (Yeh & Kirschner 2014). We did not detect *A. arundinis* in pupae, which suggests that the newly emerged females are free of conidia and will have to acquire *A. arundinis* conidia present on the *A. donax* leaf-sheath to start the oviposition process. The 40 pupae also were screened for the presence of parasitic nematodes using the 18S primer set. Of the 40 DNAs, 17 (42.5%) gave rise to a single approximately 960 bp amplicon which was confirmed to belong to *T. gyraloura* (Table 1). All these 17 DNAs gave rise to a single approximately 780 bp fragment of 28S that was sequenced for species confirmation to *T. gyraloura* (Table 1). Dual infection of pupae by a fungus or a yeast and a nematode was not detected. The 18S and 28S sequences obtained in our voucher specimens of *T. gyraloura* represent the first reference sequences of this taxa in public databases. Our PCR screening confirmed that there is only 1 nematode species infecting the pupae, which infected 42.5% of the pupae at the field site in France. Owing to such high infection rate, this parasite would need to be eliminated during the quarantine screening process or a nematode-free population would have to be identified in the native range. A logical follow up would be to develop a *Tripius gyraloura* specific PCR in order to confirm the absence of this parasite. A prerequisite would be to collect additional populations of this nematode in order to capture the intraspecies variation before designing primers.

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Table 1. Sequences and phylotypes recovered in this study, detailing top-scoring hit amongst published sequences in GenBank.

Sequences (GenBank accession number, this study), (size in bp)	Frequency of the sequence recovered %	Sequence identity %	Gaps	E value	Query Coverage %	Phylum: Taxonomic identity
18S (MG193547), (932 bp)	100% (17/17)	100	0/932	0	100	Nematoda: *Tripius gyraloura* (MG902913), this study
28S LSU (MG193548), (754 bp)	100% (17/17)	100	0/754	0	100	Nematoda: *Tripius gyraloura* (MG902912), this study
ITS (MG193549), (568 bp)	45% (4/9)	97	4/552	0	96	Ascomycota: *Sarocladium subulatum MUCL 9939* (NR145047.1)
ITS (MG193550), (568 bp)	22% (2/9)	98	4/552	0	96	Ascomycota: *Sarocladium subulatum MUCL 9939* (NR145047.1)
ITS (MG193551), (581 bp)	11% (1/9)	99	1/581	0	99	Ascomycota: *Sarocladium strictum* CBS346.70 (NR111145.1)
ITS (MG193552), (568 bp)	11% (1/9)	98	3/545	0	94	Ascomycota: *Penicillium sumatrense* (NR119812.1)
ITS (MG193553), (447 bp)	11% (1/9)	99	0/444	0	99	Ascomycota: *Galactomyces candidum* CBS 11176 (JN974290.1)

Summary

The leaf miner *Lasioptera donacis* Coutin (Diptera: Cecidomyiidae) is a biological control agent of the invasive weed, *Arundo donax* L. (Poaceae), that was approved for release in the U.S. and Mexico. Pupae are preferred for shipment of living material to quarantine facilities. There is a question of whether emerged females would carry conidia of a potential mutualist fungus, and in particular the saprophyte *Arthrinium arundinis*, from the pupa or if they would have to acquire the conidia exclusively from the plant to start the oviposition process. We artificially smeared leaf-sheaths of growing plants with *A. arundinis* before being exposed to female midges, and maintained these host plants until the pupal stage of the midge developed. Polymerase chain reaction methods were applied to detect *A. arundinis* and any other potential fungi in these pupae. Only 9% of the pupae were infested by fungi or yeast, predominantly belonging to the genus *Sarocladium*, but not *A. arundinis*, confirming that the newly emerged females are free of this fungus and will have to acquire conidia present on the leaf-sheath for successful oviposition. We also tentatively tested by PCR for the presence of parasitic nematodes in these pupae. More than 42% of the pupae were shown to be infested specifically by *T. gyraloura*. Such high infection rate calls for developing methods to eliminate this parasite or to find a parasite-free native population prior to release of *L. donacis* adults in North America for biological control of *A. donax*.

Key Words: biological control; invasive; behavior; leaf miner

Sumario

El minador de hojas *Lasioptera donacis* Coutin 2001 (Diptera: Cecidomyiidae) es un agente de control biológico de la maleza invasora, *Arundo donax* L. 1753 (Poales: Poaceae), que fue aprobado para su liberación en los Estados Unidos y México. Las pupas son preferidas para el envío de material vivo a instalaciones de cuarentena. Existe la cuestión de si las hembras emergentes llevarían los conidios de un hongo mutualista potencial, y en particular el saprofito *Arthrinium arundinis*, sobre de la pupa o tendrían que recolectar los conidios exclusivamente de la planta. Para comenzar el proceso de oviposición pusimos artificialmente *A. arundinis* sobre las hojas de las plantas en crecimiento, antes de exponerlas a los mosquitas hembra, y mantuvimos estas plantas hospederas hasta que se desarrolló el estadio de pupa de la mosquita. Se aplicaron los métodos de PCR para detectar *A. arundinis* y otros hongos potenciales en estas pupas. Solo el 9% de las pupas fueron infestadas por hongos y/o levaduras, que pertenecen predominantemente al género *Sarocladium*, pero no a *A. arundinis*, lo que confirma que las hembras recién emergidas están libres de este hongo, y tendrán que recolectar conidios presentes en la cubierta foliar para una oviposición exitosa. También probamos tentativamente por PCR para detectar la presencia de nematodos parásitos en estas pupas. Se ha demostrado que más del 42% de las pupas están infestadas específicamente por *T. gyraloura*. Tal alta tasa de infección requiere el desarrollo de métodos para eliminar este parásito y/o encontrar una población nativa libre de parásitos antes de la liberación de adultos de *L. donacis* en América del Norte para el control biológico de *A. donax*.

Palabras Clave: control biológico; invasor; comportamiento

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