FIRST REPORT ON THE PRESENCE OF *BOTANOPHILA FUGAX* (MEIGEN, 1826) (DIPTERA: ANTHOMYIIDAE) IN ROMANIA

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The current study reported the incidence of the *Botanophila fugax* (Diptera: Anthomyiidae) adults in the South East area of Romania (Clătești, Călărași county). Individual insects were sampled from traps mounted on apple trees (*Malus pumila*) during the summer period (June 2021), whilst the fruit was ripen, and identified based on morphological characteristics and COX1-based DNA barcoding. This is the first mentioning of this species on Romanian territory, contributing to extending its reported habitat within the South Eastern part of Europe.

Keywords: Botanophila fugax, Diptera, DNA barcoding, first record, Romania.

INTRODUCTION

Anthomyiidae family as part of Diptera order includes small to medium size fly species characterized by colors varying from yellow and brown to grey and black. As a unique feature, the A1 vein extends to the wing margin (Komzakova *et al.*, 2011).

Botanophila fugax, also known as *Anthomyia fugax*, was first described by Johann Wilhelm Meigen in 1826. This species is a fly that has grey striped thorax with black legs and long hair attached to the thorax (Xue & Song, 2007; Leuchtmann & Michelsen, 2016).

B. fugax has been found in various habitats of temperate and boreal regions of Europe, Asia and North America (Evenhuis & Papne, 2021). This fly is diurnal and thrives as an infestant in rape seed oil fields, cauliflower, savoy cabbage and Brussels sprouts plants, being present mostly in areas dedicated to gardening and agriculture during warm intervals (Buechs & Prescher, 2006). The adult insects preferably visit flowers belonging to the Apiaceae (carrot) family. These Diptera are holometabolous, following all four life stages in a complete metamorphosis. In most cases, the larvae are deposited into the stems of plants (Eilenberg *et al*., 2021).

According to Fauna Europaea, this anthomyiidae fly is widespread in European countries (Austria, Belgium, Britain Isles, Central European Russia, Czech Republic, Danish mainland, East Palearctic, Faroe Islands, Finland, French

mainland, Germany, Greece, Iceland, Ireland, Italy, North and Northwest European Russia, Norway, Poland, Slovakia, Spain, Sweden, Switzerland, The Netherlands) and Near East, Nearctic region, and North Africa (de Jong *et al*., 2014).

In this context, the current morphological and genetic study identifying the presence of *Botanophila fugax* associated with an apple tree habitat in a rural area of the South East region of our country constitute the first report of this Diptera species on Romanian territory.

MATERIAL AND METHODS

Experimental design

Botanophila fugax adult individuals were collected from a rural orchard located in Clătești (Călărași county), Romania (44°08'38.5"N 26°35'54.8"E), during summer (June) 2021 (Fig. 1 A, B). The investigation area is characterized by a continental temperate climate. Insects were sampled from apple trees (*Malus pumila*) using tephry traps, in the presence of banana and vinegar as attractants (Fig. 1 C).

Fig. 1. Sampling site.

(A) Localization on Romanian territory; (B) Localization on Călărași County; (C) Insect trap placed in the apple tree.

The specimens were collected and stored in 1.5 mL Eppendorf tubes for a macroscopic visual analysis, and introduced in 200 µL TE buffer for genetic identification by DNA barcoding (Iancu & Purcarea, 2016).

Morphological identification by direct examination

Morphological characteristics (Meigen, 1826) and bilateral symmetry (Namigai *et al.*, 2014) defining this species were used for the taxonomic classification of the adult insects. Macroscopic elements (Ackland, 2001; Komzáková & Rozkošný, 2009) were identified by optic microscopy analysis using a Stemi 2000 stereomicroscope (Zeiss).

Genetic identification by COX1-based DNA barcoding

Genetic identification of the insects was carried out by DNA barcoding based on cytochrome oxidase I mitochondrial gene (COX1) amplification (Hebert *et al.*, 2003; Mioduchowska *et al*., 2018). The total genomic DNA was extracted from the collected insects using a DNeasy Blood and Tissue Kit (Qiagen). The specimens suspended in 200 µl TE with 0.5 mm Zirconia II /Silica Beads (BioSpec Products) were subjected to 2 series of continuous 50 Hz-power steps, each for 12 minutes at 20^{o}C, using a Cell Homogenizer SpeedMill Plus (Analitik Jena). The PCR amplification was performed in a total volume of 50 µl using a Mastercycler ProS System (Eppendorf). The reaction mixture contained 200 ng insect genomic DNA, 1 unit of Taq DNA polymerase (Thermo Scientific), 1×Taq buffer (Thermo Scientific), 2.5 mM $MgCl_2$, 0.1 mM dNTP (Thermo Scientific), and 10 pmol of the primer pair (LCO1490: 5’ – GGTCAACAAATCATAAAGATATTGG - 3’ and HCO2198: 5’-TAAACTTCAGGGTGACCAAAAAATCA - 3’) specific for these mitochondrial genes (Folmer *et al*., 1994). The amplification reaction included an incubation step of 1 min at 94°C, 5 cycles of 30 s at 94°C, 1.5 min at 45°C and 1 min at 72°, and 35 cycles of 30 s at 94°C, 1.5 min at 51°C, and 1 min at 72°C, with a final extension step of 5 min at 72°C.

The COX1 amplicons were analyzed by electrophoresis on 1% agarose gels, purified using QIAquick PCR Purification Kit (Qiagen), and sequenced (Macrogen) on both strands.

Sequence analyses

Nucleotide sequences of COX1 DNA amplicons were processed with CodonCode Sequence Assembly and Alignment Software (Sequence Assembly and Alignment with CodonCode Aligner). The sequences were further analyzed

using BLAST-NCBI (Nucleotide BLAST: Search nucleotide databases using a nucleotide query (McGinnis and Madden, 2004) screening tool for similarity alignment within nucleotide databases.

RESULTS AND DISCUSSION

Spatial and temporal occurrence of *B. fugax* in Romania

Four *Botanophila fugax* specimens were sampled from tephry traps placed in the apple trees (*Malus pumila*) of the Clătești (Romania) orchard during June 2021 interval (Fig. 1).

The recorded environmental parameters during this summer month (https://www.meteoblue.com/en/weather/historyclimate/weatherarchive/clătești_romania_681342) corresponded to average daily temperatures varying between 31°C during the day and 24°C during the night, relative humidity of 70 ± 6.0%, and precipitation rate of 2.67 mm.

The collected adult specimens were used for taxonomic identification based on morphological key features and on DNA barcoding.

Morpohological description of the species

The morphological description of the *B. fugax* key elements (Fig. 2) evidenced the major elements (Ackland, 2001; Komzáková & Rozkošný, 2009). The specimens presented postocellar setae at least equal to length of antennal postpedicel, the inner margin of the lateral lobe of sternite 5 without hair (Fig. 2 A). A main identification structure is the cerci and the surstyli, where the posterior margin of the surstyli presented large processes bent downwards in a right-angled shape, and the cercal plate presented three processes on the distal margin (Fig. 2 A) (Ackland, 2001). Moreover, the oviscapt had medium width, with basic tergites presenting broad segments 6 and 7 only slightly larger than long on segment 8, each with shallow proximal midincision (Fig. 2 B). Also, the 6 and 7 sternites were narrow, with elongate-oval remnants of sternite 8, and with wider subtriangular epiproct and longer subconical hypoproct (Fig. 2B) (Komzáková & Rozkošný, 2009).

Based on these features, the diptera insects collected from Clătești (Romania) apple tree were identified as belonging to *Botanophila fugax* species.

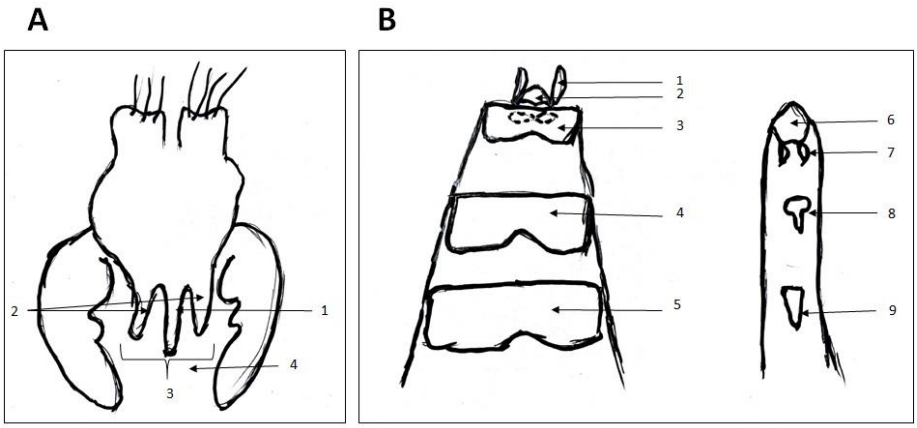

Fig. 2. Morphological identification of *B. fugax* (Meigen, 1826).

(A) Genitalia description-posterior view of the cerci (cercal plate) and surstyli; 1-submedian process; 2-median process; 3-tridentate cercal plate; 4-surstyli (modified from Ackland, 2001); (B) Oviscapts; 1-cerci; 2-epiproct; 3-tergite 8; 4-tergite 7; 5-tergite 6; 6-hypoproct; 7-sternite 8; 8-sternite 7; 9-sternite 6 (modified from Komzáková & Rozkošný, 2009).

DNA Barcoding identification of *Botanophila fugax* specimens

To validate the taxonomy of this species, genetic identification by COX1 gene-based DNA barcoding was further performed on four distinct specimens (M1, M7, M8 and M11). The PCR amplicons obtained from all adult insects corresponded to 710 bp DNA fragments (Fig. 3).

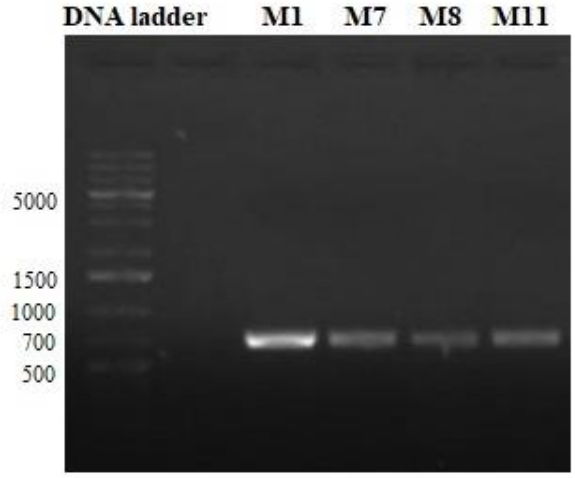

Fig. 3. PCR amplification of COX1 gene fragment from *B. fugax* specimens. Thermo Scientific GeneRuler 1 kb Plus DNA Ladder (DNA ladder); Insect specimens (M1, M7, M8, M11).

After sequencing (Macrogen) and raw data processing, the resulted COX1 DNA sequences of the fly specimens submitted to GenBank were assigned the accession numbers [OK380924], [OK380928], [OK380929], and [OK380932], respectively (Table 1).

Table 1

Insect specimens COX1 gene sequence homology

Insect code [accession number]	**Best match** [accession number]	**Identity %** (bp)	**Best Match Origin**
M1 [OK380924]	*B. fugax* [MT410801.1]	100 (644)	Denmark, woodland, hedges and cropped fields
M7 [OK380928]	*B. fugax* [KP412994.1]	100 (644)	Switzerland, Epichloe-associated
M8 [OK380929]	*B. fugax* [MT410801.1]	100 (636)	Denmark, woodland, hedges and cropped fields
M11 [OK380932]	*B. fugax* [MT410801.1]	100 (636)	Denmark, woodland, hedges and cropped fields

A BLAST analysis of the corresponding nucleotide sequences showed 100% identity with *B. fugax* COX1 gene fragment [MT410801.1] isolated from Denmark and [KP412994.1] from Switzerland (Leuchtmann & Michelsen, 2016), respectively, confirming the taxonomic affiliation of the insects collected from Clatesti, Romania to this Diptera species (Table 1).

While previously reported larvae of *B. fugax* were phytophagous and fed on monocotyledons (Xue & Song, 2007), the adult insects identified in this study were collected from the dicotyledonate *Malus pumila*, suggesting a putative extension of their food source. This Diptera species corresponded to a diurnal fly in the family of root-maggots (Lewis & Taylor, 1965) that is known to also visit flowers of *Solidago Virgaurea*, *Arnica Montana* and *Matricaria Chamomilla* (Poelen *et al*., 2014).

To date, the reported areal of *B. fugax* encompassed eight widespread countries (Germany, Canada, Norway, Belarus, United Kingdom, Russia, Switzerland, Finland) including the neighboring country Bulgaria (Hebert & Ratnasingham, 2007). Thus, the current data identifying this Diptera species for the first time on Romanian territory contributed to expanding the habitat of this species in the South-Eastern part of Europe.

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