

DNA Barcode sequence of *Necremnus tutae* Ribes & Bernardo, 2015 (Hymenoptera, Eulophidae), parasitoid of the tomato leaf miner, and new record for the Canary Islands

Secuencia de código de barras de ADN de *Necremnus tutae* Ribes & Bernardo, 2015 (Hymenoptera, Eulophidae), parasitoide de la polilla del tomate y nueva cita para Canarias

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Recibido: 07-10-2021. Aceptado: 16-06-21.
ISSN: 0210-8984 (versión impresa).

Publicado online: 30-06-2022.
ISSN: 2792-2456 (versión online).

ABSTRACT

The tomato leaf miner, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae), is a pest that causes significant losses to tomato production all over the world. Recently, it has been reported that the eulophid parasitoid, *Necremnus tutae* Ribes & Bernardo, 2015 (Hymenoptera, Eulophidae), has great potential as a biocontrol agent of this pest. Herein, we obtained and make available the reference DNA barcode sequence of *N. tutae*. and report on its occurrence in the Canary Islands for the first time. The possible consequences that this new record could have on the biological control of the tomato leaf miner in tomato producing greenhouses in the Canary Islands are also discussed.

Key words: Lepidoptera, *Tuta absoluta*, pest, parasitoid, eulophid, identification, COI, Canary Islands.

RESUMEN

La polilla del tomate, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera, Gelechiidae), es una plaga que causa importantes pérdidas en la producción de tomate en todo el mundo. Publicaciones recientes han descrito el potencial del parasitoide eulófido, *Necremnus tutae* Ribes & Bernardo, 2015 (Hymenoptera, Eulophidae), como agente de biocontrol de esta plaga. En este trabajo obtuvimos y publicamos la secuencia de código de barras de ADN de referencia de *N. tutae*, y citamos por primera vez su presencia en las Islas Canarias. También se discuten las posibles consecuencias que este organismo podría tener sobre el control biológico de la polilla del tomate en invernaderos productores de tomates en Canarias.

Palabras claves: Lepidoptera, *Tuta absoluta*, plaga, parasitoide, eulófido, identificación, COI, Islas Canarias.

INTRODUCTION

The tomato leaf miner, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera, Gelechiidae), is a devastating pest of tomato (*Solanum lycopersicum* L.) crops, which is native to South America (MIRANDA *et al.*, 1998). It was detected outside its native continent for the first time in 2006 in Europe, specifically in Eastern Spain. Since then, it has spread quickly throughout the Mediterranean Basin, the Middle East, Africa and Western Asia (BIONDI *et al.*, 2018). The first report of this pest in the Canary Islands was in December 2008 in Tenerife. Subsequently, it was found to have affected all the tomato producing areas of the islands of Gran Canaria, Tenerife, and Fuerteventura (POLASZEK, 2012). To date, this pest is considered the greatest threat to the islands' tomato production. In 2019, the impact of *T. absoluta* was reported to have caused up to 40% of tomato crop losses (PÉREZ, 2019).

Up to now, chemical control has been the main strategy to control this pest. However, extensive insecticide use has been reported to increase the risk of insecticide resistance in *T. absoluta* populations (CAMPOS *et al.*, 2014, 2017; RODITAKIS *et al.*, 2018; GUEDES *et al.*, 2019). Therefore, the development of biological control strategies using parasitoid species should be adopted to reduce chemicals use and increase the sustainability of tomato production (URBANEJA *et al.*, 2012; ZAPPALÀ *et al.*, 2013; GIORGINI *et al.*, 2019; GERVASSIO *et al.*, 2019). Among the autochthonous parasitoids of *T. absoluta* in the Mediterranean region, *Necremnus tutae* Ribes & Bernardo, 2015 (Hymenoptera, Eulophidae), previously referred to as *Necremnus artynes* or *N. sp. nr artynes*, is considered the most abundant and active (GEBIOLA *et al.*, 2015), therefore, it is a promising candidate in biological control strategies (CALVO *et al.*, 2013; FERRACINI *et al.*, 2012). It's an idiobiont ectoparasitoid of the family Eulophidae, which belongs to a complex of closely related species called the artynes group with *Necremnus cosmopterix* Ribes & Bernardo, 2015, *N. artynes* (Walker, 1839) and *Necremnus navonei* Ribes, 2015 (GEBIOLA *et al.*, 2015). It has been recorded in Spain, Italy, Tunisia (DESNEUX *et al.*, 2010; ZAPPALÀ *et*

al., 2012; ABBES *et al.*, 2014) and recently in Morocco, Algeria (GEBIOLA *et al.*, 2015) and Libya (KISSAYI *et al.*, 2019).

DNA barcoding is a tool for species identification using a short, standardized DNA sequence. In animals, the selected region is the 648 bp region from the 5'-end of the cytochrome c oxidase I (HEBERT *et al.*, 2003). Recently, DNA barcodes have been successfully employed in eulophid parasitoid studies as they can prevent erroneous identification, mostly caused by the presence of species complexes or by species with slight morphological differences (HERNÁNDEZ-LÓPEZ *et al.*, 2012; VAN NIEUKERKEN *et al.*, 2012; ŠIGUT *et al.*, 2017). In the present study, DNA barcoding was applied for the first time on the parasitoid species *N. tutae*. We also report, for the first time, its presence in the Canary Islands and discuss its potential application in the biological control of *T. absoluta*.

MATERIALS AND METHODS

Sampling

In early June 2019, many parasitized tomato leaf miner larvae were observed in a tomato greenhouse in the Telde municipality of the province of Las Palmas on the island of Gran Canaria. Parasitized leaf miners were transferred to the laboratory and kept at room temperature until parasitoid emergence. The emerged specimens were then collected and conserved in 90% ethanol or in absolute ethanol for molecular identification.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from two male and two female specimens using a Chelex non-destructive protocol (CASQUET *et al.*, 2012). Subsequently, the standard DNA barcode region at 5' end of the cytochrome c oxidase I gene (COI-5P) was amplified using

the universal primer pair, LCO-1490 and HCOI-2198 (FOLMER *et al.*, 1994). As there are no sequences of the DNA barcode gene for *N. tutae*, the 3' region of the COI (COI-3P) was also amplified using the primers Jerry/Pat (SIMON *et al.*, 1994). PCR amplification was performed in a 25 µl final reaction volume containing 0.4 µM of each primer, 3 mM MgCl₂, NH₄ buffer (1×), 0.2 mM of each dNTP, 0.4 mg/ml of acetylated bovine serum albumin (BSA), 0.02 unit/µl of Taq-polymerase (Bioline) and 2 µl of DNA extract (concentration not determined). Polymerase chain reactions (PCRs) were carried out in Swift™ Maxi Thermal Cyclers (ESCO Technologies) applying the following thermal step: initial denaturation for 4 min at 94 °C, followed by 39 cycles of 30 s at 94 °C, 30 s at annealing temperature of 48 °C (for LCO-1490 and HCOI-2198) and 53 °C (for Jerry and Pat), and 45 s at 72 °C, then a final extension step of 10 min at 72 °C. PCR products were enzymatically cleaned with 0.025 unit/µl rApid alkaline phosphatase (Roche) and 50 unit/ml exonuclease I (BioLabs) for 15 min at 37 °C followed by 15 min at 95 °C. Then the purified products were sequenced in both directions at Macrogen Inc. (Madrid, Spain). Sequences were checked, edited, and assembled with CLUSTALW within the MEGA 7 software (TAMURA *et al.*, 2011). The sequences obtained were then compared using BLAST and the identification engine tool (IDS) (https://boldsystems.org/index.php/IDS_OpenIdEngine) against the known sequences in the Genbank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the Barcode of Life Data System (BOLD Systems), respectively. As the barcode regions of *N. tutae* are not publicly available, the sequences of COI-3P gene fragments were used for the phylogenetic analysis. The COI-3P sequences were aligned with those of the other species of the artynes group analyzed by GEBIOLA *et al.* (2015) by codon using MUSCLE implemented in MEGA7 (EDGAR, 2004). Neighbor-joining algorithms were then conducted using Kimura-2-parameter (K2P) substitution model with 1000 bootstrap replications in MEGA version 7 (KUMAR *et al.*, 2016). The sequences obtained in this study were deposited in BOLD Systems (ICIA001-21,

ICIA002-21) and Genbank database (MW996443, MW996444 for COI-5P gene fragment and for MW965791 COI-3P gene fragment).

Morphology identification

After DNA extraction, specimens from which DNA was extracted, were transferred to 70% ethanol for morphological identification. For the observation of the sensory pores on the scape, the antennae were detached from the specimen and mounted in Canada balsam following the protocol of NOYES (1982). The identification was then carried out using the recent illustrated key of the *Necremnus* European species (GEBIOLA *et al.*, 2015). The diagnostic characters of slide-mounted and 70% ethanol-preserved specimens were photographed with a NIKON DFC450 camera coupled with a NIKON Eclipse 80i Microscope or a Nikon SMZ1270 binocular stereo microscope, using the imaging software NIS ELEMENT D. The material examined was deposited in the Entomological Collection of the Museum of Nature and Archaeology (MUNA) located in Santa Cruz de Tenerife (Catalogue numbers: TFMC/HY-7415, TFMC/HY-7416).

RESULTS

Molecular identification

The four specimens analyzed were successfully sequenced for the COI-3P and barcode regions. The comparison of COI-3P sequence obtained in this study with public sequences available in the Genbank database showed absolute homology (100%) with a specimen of *Necremnus tutae* collected in Morocco (accession number: KJ846120). The phylogenetic tree of the species of the artynes group was paraphyletic with two distinct branches (Fig. 1). The first branch separated into two clusters. The first cluster grouped together the COI-3P sequence for the specimens obtained from this study and those of *N. tutae* specimens collected from France, Italy, Tunisia, Morocco, and Al-

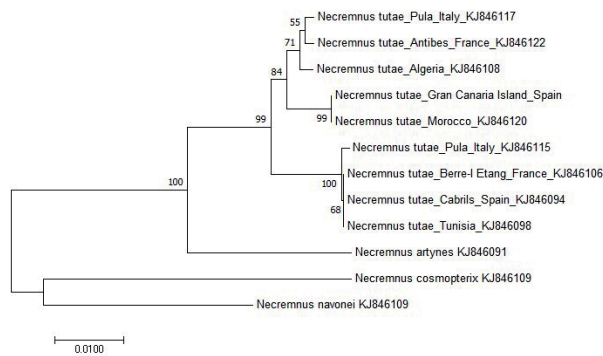


Fig. 1. Neighbor-joining tree of the artynes group COI sequences, using Kimura-2-parameter distance. Bootstrap support values are based on 1,000 replications.

Fig. 1. Árbol Neighbor-joining de las secuencias COI del grupo artynes, empleando el método Kimura-2-parameter distance. Los valores de bootstrap se basan en 1.000 repeticiones.

geria. The second cluster was composed of the COI-3P sequence of *N. artynes*. The sequences of *N. cosmopterix* and *N. navonei* were branched separately as an outgroup.

A total of four DNA barcodes were obtained in this study. All sequences were identical, as well as 650 base pairs (bp) long (0% ambiguities) with no evidence of stop codons and Nuclear Mitochondrial DNA sequences (NUMTs). The comparison of the sequence of *N. tutae* with referenced DNA barcodes stored in BOLD Systems confirmed that our sequence was the first complete DNA barcode record ever deposited in a database.

Morphology identification

Necremnus tutae belongs to a complex of closely related species called the artynes group. This species group is composed of *N. tutae*, *N. cosmopterix*, *N. artynes* and *N. navonei*. Males of *N. tutae* (Fig. 2A) are very similar to those of *N. artynes*, but they differ mainly by the relative position of the sensory pores on their scape (Fig. 2B). In *N. tutae* the sensorial area is broad, and the sensorial pores are large and placed in one irregular row such that the interspaces are smaller than pore size, and usually some pores are arranged in a second irregular row (Fig. 2C). In males of *N. artynes*, the sensorial area is narrow, and the sensorial pores are



Fig. 2. *Necremnus tutae*. A Male lateral view B Antennae lateral view C Sensory pores on the scape D Gaster dorsal view. Scale bars in micrometres.

Fig. 2. *Necremnus tutae*. A. Vista lateral de un macho B Vista lateral de la antena C Poros sensoriales del escapo D Vista dorsal del gaster. Barras de escala en micrómetros.

small so that they are separated by interspaces greater than a pore width. Females of *N. tutae* are also very similar to those of *N. artynes* and *N. navonei*, though they differ in the shape of the gaster (Fig. 2D). *N. navonei* and *N. artynes* have a slightly more elongated gaster than that of *N. tutae* (GEBIOLA *et al.*, 2015).

Materiel examined

SPAIN: 5♂♂ y 2♀♀; Canary Islands, Gran Canaria, Telde; 27° 56' 52" N; 15° 24' 04" W; 270 m; 07.vi.2019; Claudio Lang-Lenton Bonny leg.; on *Tuta absoluta*.

DISCUSSION

Rapid and accurate identification of biological control agents is a fundamental requirement for effective pest management procedures. In this study, we identified through molecular and morphological analyses the parasitoid *N. tutae* found on the island of Gran Canaria (Canary Islands). We also produced the first COI reference DNA Barcode sequence in order to make it publicly available in international databases. Many studies have proven that DNA barcoding is useful for selecting parasitoid species for

biological control (COCK *et al.*, 2019; KENIS *et al.*, 2019), but also, to assess host-parasitoid associations (SANTOS *et al.*, 2011; GARIEPY *et al.*, 2014). Thus, this sequence can be used in the future to identify *N. tutae*, consequently facilitating its monitoring, detection, and successful use for future pest management of the tomato leaf miner.

This study also provides the first report of *N. tutae* parasitizing *T. absoluta* on the island of Gran Canaria. The arrival of this species on Gran Canaria may have occurred through an accidental introduction, probably together with its host; similar to what happened with other parasitoid species in New Zealand (BERRY & WALKER, 2004; MANSFIELD *et al.*, 2011) and Italy (CALECA *et al.*, 2011). Its presence on the other islands is still unknown. However, the long distance dispersal capacity of several Eulophid parasitoid species, after release or accidental introduction, has been previously observed (ELZINGA *et al.*, 2007; GICHINI *et al.*, 2008; CALECA *et al.*, 2011). The case of *Tamarixia dryi* (Waterston, 1922) (Hymenoptera: Eulophidae) is a prime example of this ease of dispersal; when it was introduced on the island of Tenerife in 2018 within the African citrus psyllid *Trioxa erythrae* (Del Guercio, 1918) (Hemiptera: Trioxidae) biological control program. Soon after, *T. dryi* was found on all the other islands of this archipelago (HERNÁNDEZ-SUÁREZ *et al.*, 2020). All this suggests a heightened possibility of finding *N. tutae* in other tomato production areas of the Canary Islands. Therefore, future monitoring is needed to determine the prevalence and distribution of this parasitoid.

The occurrence of *N. tutae* could improve the biological control of the tomato leaf miner in tomato producing greenhouses in the Canary Islands. Many studies have suggested that *N. tutae* has great potential in the biological control of *T. absoluta*. CRISOL-MARTÍNEZ & VAN DER BLOM (2019) reported biological control by spontaneously appearing *N. tutae*; with high rates of parasitism, up to 70%, and mortality via host-feeding of almost 90% in some contexts. The use of insectary plants as alternative food sources has been reported to promote the attraction and establishment of this parasitoid in greenhouse

tomato crops (BALZAN & WÄCKERS, 2013; ARNÓ *et al.*, 2018; GIORGINI *et al.*, 2019). Since *N. tutae* has only been recorded with *T. absoluta*, future studies are needed to understand its ecological relationships with other native insect fauna in the Canary Islands, such as with native Lepidopteran species. Consequently, this study could represent the first step for including the parasitoid *N. tutae* in the Integrated Pest Management of *T. absoluta* in the tomato crops of the Canary Islands.

ACKNOWLEDGEMENTS

We are grateful to Claudio Lang-Lenton Bonny for collecting material of parasitoid specimens in *Tuta absoluta* in Gran Canaria. Saskia Bastin is recipient of a 2019-2023 PhD grant from the Agencia Canaria de Investigación Innovación y Sociedad de la Información (ACIISI), Consejería de Economía, Industria, Comercio y Conocimiento of the Gobierno de Canarias and the European Social Fund. This research was carried out with financial support from the research project CU-ARENTAGRI (MAC2/1.1a/231).

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