



Morphological and molecular identification of *Carpophilus dimidiatus* (Coleoptera: Nitidulidae) associated with stored walnut in Northwestern Argentina

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ABSTRACT

The beetle genus *Carpophilus* (Coleoptera: Nitidulidae: Carpophilinae) includes important stored-product pest species, many of which are widespread worldwide and often difficult to identify using external morphological features. The present research work provides the identification of *Carpophilus dimidiatus* (Fabricius), infesting walnut in production areas in Catamarca Province, Northwestern Argentina, these being new host and distribution records for the province. It was based on both morphological and molecular information. In order to ensure accuracy and facilitate further studies requiring recognition of taxonomically challenging species of *Carpophilus*, a revised morphological diagnosis of *C. dimidiatus* is provided and illustrated with photos of habitus of adult male and female and of relevant external and genital characters, including comparative notes to allow distinction of this species from other similar nitidulid beetles. For molecular identification of *C. dimidiatus*, sequencing of the 5' and the 3' regions of the mitochondrial cytochrome c oxidase I (COI) gene was performed. Fragments of 658 bp and ~800 bp, respectively, are made available as additional diagnostic tools. Phylogenetic analyses were also done on the barcode fragment of COI of several *Carpophilus* species and outgroup taxa, available in genetic databases, with results confirming the species identity.

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1. Introduction

The sap beetle family Nitidulidae Latreille embraces more than 4500 described species worldwide, with diverse feeding habits, but mostly saprophagous and mycetophagous on decaying fruits and fermented plant material (Jelínek et al., 2010). Most of the nitidulid species are not of agronomic concern because they feed on damaged or overdone fruits and vegetables with high moisture content (Prado, 1987). However, a lesser group of nitidulid species are regarded as economically important pests of stored products worldwide, in particular several species of the cosmopolitan genus

Carpophilus Stephens, which affect stored grain and byproducts, dry fruits, oilseeds, cacao, and many other commodities (Dobson, 1954; Dell'Orto-Trivelli and Arias Velázquez, 1985; Prado, 1987; Audisio, 1993; Artigas, 1994; Leschen and Marris, 2005; Brown et al., 2012; Jelínek et al., 2016).

The genus *Carpophilus* includes approximately 200 species distributed mainly in tropical and temperate regions of the World (Dobson, 1954; Gillogly, 1962; Prado, 1987; Leschen and Marris, 2005; Brown et al., 2012). Some species often become pests of relevant economic importance, particularly sixteen of these are known to be associated with stored products (Dobson, 1954; Leschen and Marris, 2005). Many of them were distributed and introduced by commercial trading among regions and countries, like *C. hemipterus* (L.), *C. obsoletus* Erichson, *C. ligneous* Murray and

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C. dimidiatus (Fabricius), all reported as highly destructive pests on non-processed dried fruit (Dobson, 1954). Some species, like *C. mutilatus* Erichson, *C. hemipterus* and *C. dimidiatus*, are relevant in food industry when developing in accumulations of fruits because of the indirect damage they can cause as vectors of fruit diseases that seriously spoil the stored product (Leschen and Marris, 2005; Barth et al., 2009).

Argentina is one of the largest walnut producers in South America. By 2014, walnut orchards in the country covered an area of 16,500 ha, producing annually about 17,000 tons, showing a strong increasing tendency (Doreste, 2013; Cólica, 2015). The Argentine walnut producing provinces are Catamarca (in the Northwest), followed by Mendoza, La Rioja and San Juan (in the Central-west) and Río Negro (in Northern Patagonia). However, the main walnut producing area in Argentina is located in the North of the country, in the central part of the Catamarca province (departments of Andalgalá, Belén, Ambato, Santa María, Pomán, Capayán) at an altitude of 800 to 2000 m, where the maximum yield per Ha is currently about 2.5 MT (Fernández Górgolas, 2012).

In Catamarca, at post-harvest, walnuts are negatively affected by insect pest species and fruit-damaging microorganisms during storage and transport, causing between 35% and 74% of losses (Fernández Górgolas and Aybar, 2012). The damage caused by insect pests on fruit kernels are basically: loss of weight, lessen of germination capacity and loss of commercial value due to kernel injury. Considerable losses are caused as well by product spoilage through undesirable mold growth, as well as the presence of mycotoxins due to development of toxigenic fungi (García Gutiérrez et al., 2009; Luttfullah and Hussain, 2011). In central areas of Catamarca province, post-harvest walnut damage was caused by a complex of beetle species including *Carpophilus* sp. (Nitidulidae), *Blapstinus punctulatus* (Solier) (Tenebrionidae) and *Oryzaephilus surinamensis* (L.) (Silvanidae) (Fernández Górgolas and Aybar, 2012). Moreover, the nitidulids often found in association with other stored-product beetles, are by far the most abundant and recurrent species infesting walnuts in Catamarca. Larvae and adults of these beetles cause extensive damage and product loss by their feeding on or boring into seeds and due to product contamination with frass, cadavers, cast skins and fecal droppings, as well as increased moisture leading to the growth of mold and bacteria (Artigas, 1994).

Accurate species identification is important to ensure proper selection of effective control methods and management tools of stored-product beetle pests. However, this is not always an easy task, particularly regarding some *Carpophilus* species which are morphologically very similar and difficult to identify even for expert taxonomists (Audisio, 1993; Leschen and Marris, 2005; Brown et al., 2012). Recent application of molecular methods has proved helpful in resolving phylogenetic and taxonomic problems in *Carpophilus*. In addition, these techniques also show the usefulness of the COI barcode region to discriminate species in this genus (Brown et al., 2012). Alternative molecular techniques, based on species-specific primers, are being proposed for identification of common nitidulid pests (Bai et al., 2017). It is expected that increased availability of DNA sequences of species of *Carpophilus* in genetic databases would facilitate the identification, even by non-specialists, of economically important species. Molecular sequences are also advantageous because they can be used to identify the species at any one stage of their life cycle. Diagnosis of insect species based on adult morphology is, and will continue to be, a fundamental step to ensure accuracy and verifiable findings, as all research studies should be based on correctly identified voucher specimens. The aim of the current research work is to provide the identification of a *Carpophilus* species found in large numbers infesting walnut in Catamarca province, the main production area in Argentina, throughout both morphological and molecular information.

2. Materials and methods

2.1. Insects

During 2015, walnut samples, of around one kilogram each, were collected from the field as well as from storage facilities in two different production areas in Catamarca province, department of Andalgalá, located at El Potrero and Chaquiago. Samples were taken to the laboratory and inspected in search of insect pest species. Beetle specimens found (adult and immature stages) were placed in glass vials and preserved in 96% ethanol for further morphological and molecular identification.

2.2. Morphological identification

Taxonomic identification was done using keys and descriptive comparative works on *Carpophilus* published by nitidulid experts, mainly: Dobson (1954), Prado (1987); Audisio (1993), Leschen and Marris (2005); Jelínek et al. (2010). Adult male and female specimens were examined under stereomicroscope to observe external diagnostic characters. Also, two males and two females were dissected to examine genitalia. Photos of habitus and of diagnostic structures were taken with a digital camera associated with the microscope. Studied and identified specimens are deposited in the Entomology collection of the Museo de Ciencias Naturales de La Plata (MLP, La Plata, Buenos Aires, Argentina).

2.3. Molecular identification

The acquisition of Cytochrome Oxidase Subunit I (COI) sequences was done in order to verify the species identification with molecular evidence. The 5' region of COI of 658 bp length, corresponding to the standardized DNA "barcode" for invertebrate species identification, as well as the 3' region of COI of ~800 bp, which is also widely used for that purpose, were obtained.

Total genomic DNA was extracted from 96% ethanol preserved adult voucher specimen by using the DNeasy Blood and Tissue Kit (QIAGEN, MD, U.S.A.) from head and thorax. The extracted DNA was stored at -20 °C. Amplification and sequencing of mtDNA (COI): The 5' (barcoding) region of the COI gene was amplified using the primers LCO: 5'-GGTCAACAATCATAAAGATATTGG-3' and HCO: 5'-TAAACTTCAGGGTGACCAAAAATCA-3' (Simon et al., 1994). The 3' (Jerry-to-Pat) region of the COI gene was amplified using the primers Jerry: 5'-CAACATTTATTTGATTTTTGG-3' and Pat: 5'-TCCAATGCACTAATCTGCCATATTA-3' (Simon et al., 1994). Samples were amplified in 25- μ L reactions containing 15.375 μ L HPLC water, 5 μ L of 5 \times Gotaq buffer, 0.5 μ L of 10 mM dNTPs, 0.125 μ L of GoTaq[®] DNA Polymerase (Promega), 1 μ L of each primer (10 mM); and 2 μ L of the DNA extraction. PCR conditions were: initial denaturation for 2 min at 94 °C, followed by 40 cycles at 94 °C for 15 s, 45 °C for 30 s, 72 °C for 75 s, and a final extension at 72 °C for 7 min (Brown et al., 2012). The PCR products were purified and bi-directionally sequenced by Macrogen Inc. (Seul, South Korea). Electropherograms were edited using ChromasPro v.1.5 and BioEdit v7.0.9.0 (Hall, 1999) software.

Molecular comparison and analyses are described as follows. The 5' and 3' COI fragments obtained were checked and compared with others available in GenBank through BLAST tool (www.blast.ncbi.nlm.nih.gov). Phylogenetic analyses were performed on the 5' (barcoding) region of the COI gene as sequences for this locus were available for several *Carpophilus* species in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) and BOLD (<http://www.boldsystems.org/>) databases. Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were conducted on a data matrix of 18 terminal taxa and 658 characters. The data matrix

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