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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.

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'OrtoTrivelli 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 template 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′-GGTCAACAAATCATAAAGATATTGG-3′and HCO: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′(Simon et al., 1994). The 3′(Jerry-to-Pat) region of the COI gene was amplified using the primers Jerry: 5′- CAACATTTATTTTGATTTTTTGG-3′and Pat: 5′-TCCAATGCACTAATCTGCCATATTA-3′ (Simon et al., 1994). Samples were amplified in 25-μL reactions containing 15.375 μL HPLC water, 5 μL of 5× Gotaq buffer, 0.5 μL of 10 mm dNTPs, 0.125 μL of GoTaq® DNA Polymerase (Promega), 1 μL of each primer (10 mm); and 2 μl 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

includes, besides the Argentinean, other COI sequences available from genetic databases: ten of *Carpophilus* from different species, three from species in other genera of Carpophilinae (*Urophorus* Murray and *Epuraea* Erichson) and Nitidulinae (*Aethina* Erichson), plus four chosen as more distant outgroups in Erotylidae (*Languria* Latreille), Cucujidae (*Cucujus* Fabricius), Cryptophagidae (*Cryptophagus* Herbst), and Protocucujidae (*Ericmodes* Reitter), using the later to root the trees. The program PAUP (Swofford, 2002) was used to edit the original matrix and to export it to nexus and phylip formats, for analyses with TNT and RAxML respectively.

Parsimony analysis was conducted in TNT version 1.5 beta (Goloboff et al., 2008), with a heuristic search consisting in 1000 random addition sequences plus TBR as swapping algorithm, saving 10 trees per replication, under equal weights. Nodal support was evaluated with 100 bootstrap replications. Maximum Likelihood analysis was performed on RAxML version 8.2.8 (Stamatakis, 2014) on the CIPRES portal (Miller et al., 2010), using the -f a algorithm, which computes a rapid bootstrap analysis and search for best-scoring ML tree in one single run, with 100 bootstrap replications and GTRCAT model, without partitioning.

3. Results

3.1. Morphological identification and diagnosis

The taxonomic position and species identity of the nitidulid specimens found infesting walnut is *Carpophilus dimidiatus* (see Table 1 for a list of synonyms). The genus is in the Subfamily Carpophilinae. Beetles in this subfamily are characterized by having the elytra short and distally truncated, leaving exposed the pygidium (tergite VII), and usually also the next one or two precedent tergites (Fig. 1 A, C); the males have the tergite VIII button-like and visible ventrally, at apex of pygidium, in an excavation of ventrite 5 (Fig. 1 D). In addition, they share with subfamily Nitidulinae the meso- and metatibiae with double row of spines aligned longitudinally, a feature that distinguishes them from other Nitidulidae. Species in the genus *Carpophilus* have two abdominal tergites exposed dorsally beyond the elytra (Fig. 1A, C) (those in the closely related genus *Urophorus* have three tergites visible in dorsal view). Ventrites 2 and 3 are shorter than ventrites 1, 4, and 5 (Fig. 1B, D).

3.2. Morphological diagnosis of C. dimidiatus (Figs. 1–3)

General aspect (Fig. 1A–D). Length: 2–3 mm; body subparallel; color brown, dark to very dark in pronotum and head, often with paler areas on elytra, but these without sharp limits; vestiture of decumbent golden setae. Head (Fig. 2A–C): Antennal segment 3 longer (about 1.2 ×) than 2 (Fig. 2 A); labrum with lobes broadly rounded (Fig. 2 B); male mandibles symmetrical (Fig. 2 C). Prothorax: Pronotum (Fig. 1 A, C) with sides evenly arcuate, anterior and posterior angles rounded; disc convex, with punctures strongly impressed, separated by 1–2 diameters; pronotal carina (medial inpunctate strip) present, narrow. Prosternun and hypomerum densely and distinctly punctured (Fig. 2 D, F); prosternal process apically rounded and not expanded laterally (Fig. 2 D). Meso- and metathorax: mesoventrite punctate, without carinae (Fig. 2 D); mataventrite with axillary space of moderate size, present to a level of ¼ the length of mesepisternum (Fig. 2 E). Elytra with punctures strongly impressed, separated by 1–2 diameters (Fig. 1 A, C). Female genitalia: ovipositor as in Fig. 3 A. Male genitalia: sternite VIII as in Fig. 3 B; parameres as in Fig. 3 C, D.

Table 1
List of synonyms of *Carpophilus dimidiatus* (Fabricius).

Nitidula dimidiata Fabricius, 1792
Nitidula hemiptera Fabricius (non Linnaeus), 1792
Carpophilus pusillus Stephens, 1830
Carpophilus auripilosus Wollaston, 1854
Carpophilus tempestivus Jacqueline du Val, 1856
Nitidula contingensis Walker, 1858
Carpophilus puberulus Montrouzier, 1860
Carpophilus vittiger Murray, 1864
Eidocolastus dilutus Murray, 1864
Eidocolastus limbalis Murray, 1864
Eidocolastus nigritus Murray, 1864
Eidocolastus robustus Murray, 1864
Haptoncus testaceus Murray, 1864
Carpophilus biguttatus Gemminger and von Harold (non Motschulsky), 1868
Carpophilus lewisi Reitter, 1884

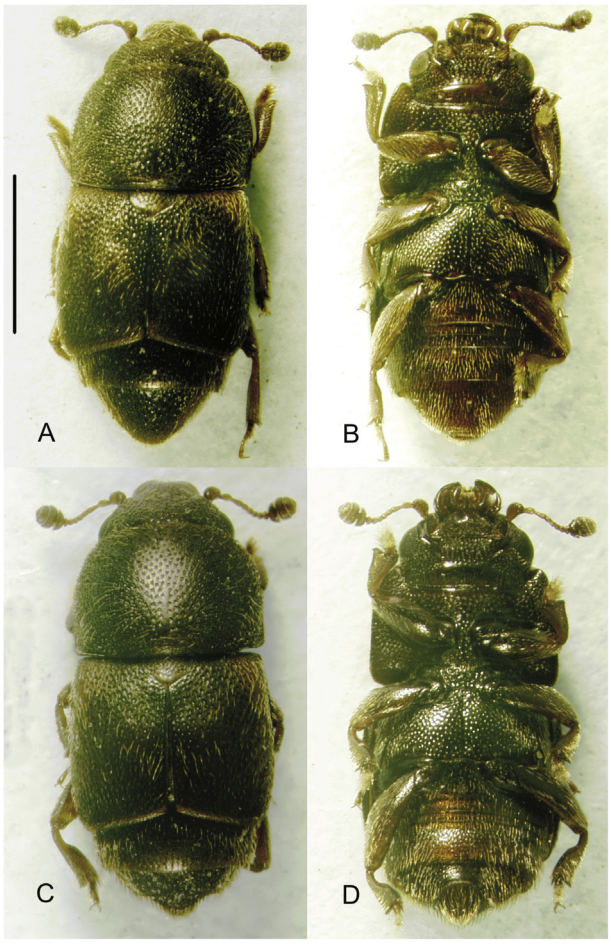

Fig. 1. A–D. *Carpophilus dimidiatus*, adult habitus: A, Female, dorsal; B, Female, ventral; C, male, dorsal; D, male, ventral. Scale bar 1 mm.

3.3. Molecular identification and diagnosis

We obtained the 5′region ("barcode) of COI of 658 bp length and the 3′region of COI of ~800 bp, both being useful for species identification. The sequence was deposited in GenBank under accession number MG679359. The phylogenetic tree resulting from maximum likelihood is shown in Fig. 4. Parsimony analysis resulted in two most parsimonious trees 962 steps long, the strict consensus of which presents most nodes in common with the ML tree, marked with asterisk in Fig. 4. Results from both MP and ML phylogenetic analyses show that the COI sequence from the specimen of Catamarca (Argentina), morphologically identified as *C. dimidiatus*, is most closely related, with high support values, to a COI sequence of

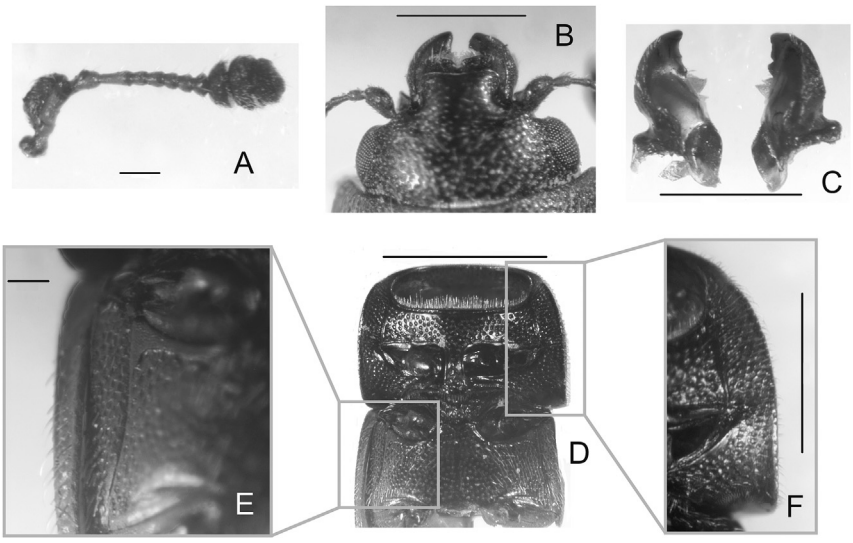

Fig. 2. A–F. *Carpophilus dimidiatus*, adult external morphology: A, antenna; B, head, dorsal; C, male mandibles, dorsal; D, thorax, ventral; E, portion of metathorax, showing detail of axillary space; F, portion of prothorax, showing detail of punctuation on prosternum and hypomeron. Scale bars for A and E, 0.10 mm; for B, C and F, 0.50 mm; for D, 1 mm.

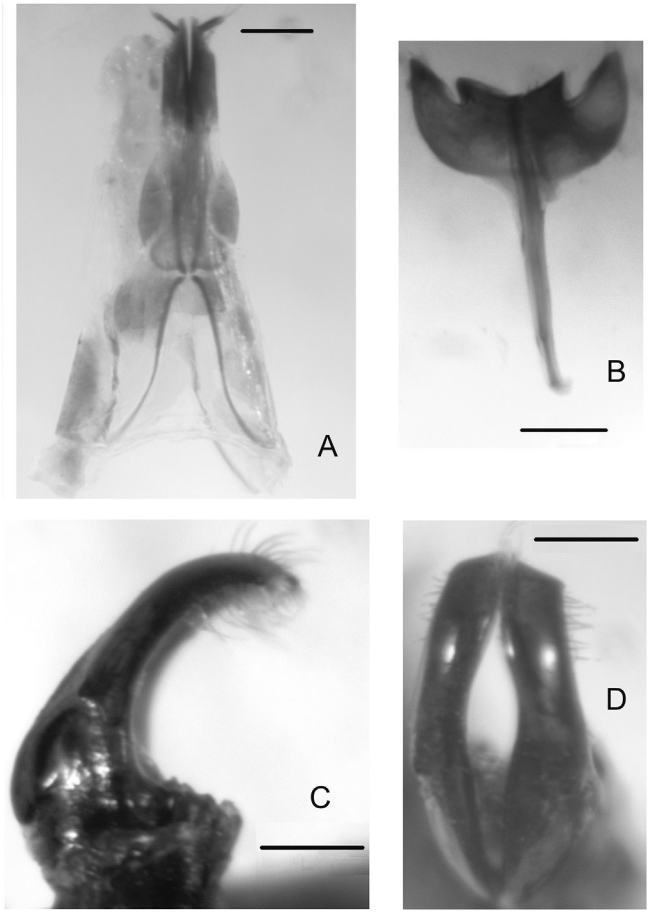

Fig. 3. A–D. *Carpophilus dimidiatus*, genitalia: A, Female, ovipositor; B, male, sternite VIII; C, parameres, lateral; D, parameres, dorsal. Scale bars 0.10 mm.

a specimen of this species, the only one so far available in public data bases when the current research was performed. Thus, molecular evidence supports the morphological identification performed. Also, the molecular tree shows that *C. dimidiatus* and *C. mutilatus* are clearly separated, distinct species (in agreement with results by Brown et al., 2012).

4. Discussion

Carpophilus dimidiatus is morphologically very similar to *C. mutilatus* and to a lesser extent to *C. hemipterus*, but sharing same ecological roles (Dobson, 1954; Audisio, 1993; Leschen and Marris, 2005; Brown, 2009; Jelínek et al., 2016). Distinctive characters of *C. mutilatus* are: the 3rd antennomere shorter than 2nd; male mandibles often strongly asymmetrical; the hypomeron unpunctate, lightly granulate; pronotum closely punctate (punctures separated by less than 1 diameter); the axillary space reaching to 1/3 the length of metepisternum; while *C. dimidiatus* has: the 3rd antennomere longer than 2nd; male mandibles symmetrical; the hypomeron distinctly punctate; pronotum moderately punctate (punctures separated by 1–2 diameters); the axillary space relatively small, reaching to 1/4 the length of metepisternum. Both species have distinct characters in male and female genitalia as illustrated in Fig. 3 (this paper) and in Dobson (1954) and Audisio (1993). However, as noted by Ewing and Cline (2004), *Carpophilus dimidiatus* and *C. mutilatus* were considered synonyms and then biological and host information regarding these species could be incorrectly described in the literature, particularly from 1913 until 1954. In the catalogue of Argentinean coleoptera by Bruch (1914) *C. mutilatus* is listed as a variety of *C. dimidiatus*. However, *C. dimidiatus* and *C. mutilatus* are distinct species, as shown by the morphological and molecular results in this paper.

Carpophilus hemipterus must also be distinguised from *C. dimidiatus* because they are similar in their general aspects, are widespread worldwide, and often associated with dried fruits in anthropogenic habitats. However, they can easily be distinguished because *C. hemipterus* has a distinct color pattern, with well-defined yellow patches on humeral and apical areas of elytra; the antennomeres 2 and 3 subequal in length; prosternal process expanded laterally behind coxae; mesoventrite with discal carinae; and metaventrite with axillary space very small, reaching to 1/5 the length of metepisternum.

In Argentina, the pest status of *C. dimidiatus* remains unrecognized by SINAVIMO (2017), while *C. hemipterus* is cited as pest of walnut in Northern Patagonia (Cichón et al., 2015). The occurrence

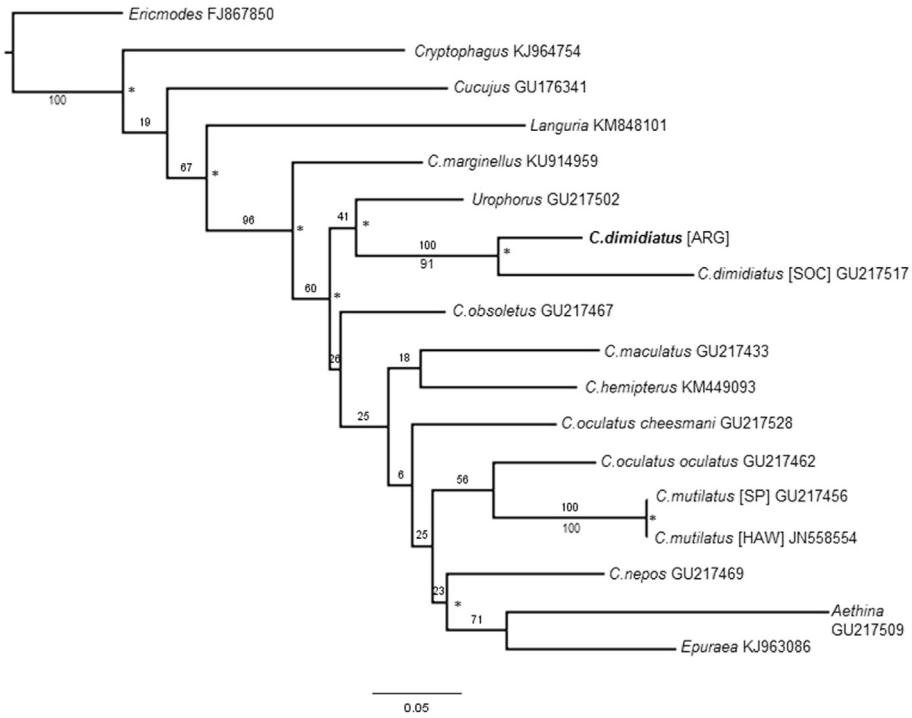

Fig. 4. Phylogenetic tree of COI sequences of *Carpophilus* species and allies, based on maximum likelihood, with nodes in common with parsimony tree marked with asterisk. Numbers above branches are ML bootstrap values; numbers below branches indicate MP bootstrap values over 50%. Letter codes in brackets, for *C. dimidiatus* and *C. mutilatus* terminals, indicate general geographic source of the specimens: ARG-Argentina, SOC-Society Islands, SP-South Pacific, HAW-Hawaii. Sequences from Gen Bank are identified with their accession numbers on the right.

herein reported of *C. dimidiatus* infesting walnut in Catamarca, Northwestern Argentina, is not unexpected, since *C. dimidiatus* is known to be less tolerant to cold weather than *C. hemipterus*. These species are known to develop where average annual temperature is not inferior to 14 °C and 10 °C, respectively (Audisio, 1993).

Among the insects reported to be associated with stored-products, beetles in the genus *Carpophilus* are particularly challenging, because of their high diversity and the abundance of economically important species that are morphologically too similar. In this paper, we identified *Carpophilus dimidiatus*, infesting walnut (*Juglans regia* L) in production areas in Northwestern Argentina, representing new host and distribution records for this nitidulid species from Catamarca Province. Morphological identification, performed on the basis of adult external and internal morphology, was then confirmed with DNA evidence from the COI gene. The 5′region ("barcode") of COI of 658 bp length and the 3′region of COI of ~800 bp, both are useful for *Carpophilus* species identification. Results of the phylogenetic analyses performed on the barcoding region of COI supported the identity of the specimen from Catamarca (Argentina) as *C. dimidiatus,* and furthermore, showed that *C. dimidiatus* and *C. mutilatus* are clearly separated, distinct species. The present study demonstrated the value of combining morphological and molecular evidence to ascertain species identity of stored product pests. Sustained morphological and molecular research on *Carpophilus* beetles is necessary, being the combined approach also crucial for resolving taxonomic issues in systematic revisions and reconstructing phylogenetic relationships.

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