

# Morphological and Molecular Identification of the Invasive *Xylosandrus crassiusculus* (Coleoptera: Curculionidae: Scolytinae) and Its South American Range Extending Into Argentina and Uruguay

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## Abstract

The occurrence of the exotic “granulate ambrosia beetle” *Xylosandrus crassiusculus* (Motschulsky) in Argentina and Uruguay is reported for the first time, documenting expansion in South America. Morphological characters are provided to allow distinction of this species from other similar ambrosia beetles. Molecular identification was also done on the basis of the 5' region of the mitochondrial cytochrome c oxidase I (COI) gene, obtained from a specimen from Argentina and compared with available “barcode” sequences in public databases. Phylogenetic analyses, via maximum likelihood and parsimony, were performed using COI sequences from 17 terminals, including 14 sequences of *X. crassiusculus* from different continents plus other *Xylosandrus* and *Cnestus* species as outgroups. Results from the sequence analyses confirm the identity of *X. crassiusculus*. The specimen from Argentina and the two sampled from United States showed no differences in their COI sequences. Further studies are needed, on the genetic variation through the native and introduced ranges of the granulate ambrosia beetle, to ascertain the source/s of origin of this biological invasion in the Americas. The present paper reports the expansion of this exotic species in South America into Argentina and Uruguay.

**Key words:** Xyleborini, ambrosia beetle, exotic species, DNA

The rate of establishment of exotic species worldwide is increasing mainly as a result of human activity (Brockerhoff et al. 2010, Ricciardi et al. 2011). In particular, the international transportation of wood packing and plant material may increase the risk of colonization of new areas by bark and ambrosia beetles, considered one of the most harmful threats both in plantations and native forests. Although most of these insects attack weakened or stressed trees, many species colonize and kill healthy, vigorous hosts during epidemic outbreaks (Sauvard 2004, Wood 2007).

Although the study of bark and ambrosia beetles is highly relevant to the forestry industry, research on these insects in South American countries is quite recent. Furthermore, surveillance networks of exotic wood-boring insects in the region are not entirely successful (Knížek 2007). Thus, species may be undetected for many

years before discovery (Liebhold et al. 1995). More than 40 species of scolytines have been recorded in Argentina, but only a few were observed in commercial plantations (Blackwelder 1947, Viana 1965, Bright 2004). In Uruguay, four exotic species of scolytines have been documented causing severe damage in pine trees and eucalyptus commercial plantations (Gómez et al. 2012, Gómez and Martínez 2013, Gómez et al. 2013). In Brazil, at least 80 species of scolytines have been detected, in relation with the increase in damage in eucalyptus and pine tree plantations during the past decade (Oliveira et al. 2008, Flechtmann and Cognato 2011). However, only a few ambrosia beetles are considered economically relevant (Flechtmann et al. 2001). The granulate ambrosia beetle, *Xylosandrus crassiusculus* (Motschulsky), most likely native to tropical and subtropical Asia, has become one of the most widespread

wood-boring beetles on the planet. Being highly polyphagous, haplodiploid, and an inbreeder, it has colonized most warm and humid regions of the world (Kirkendall and Faccoli 2010). Introduced and established long ago in Sub-Saharan Africa and Oceania (Wood and Bright 1992), it has also been introduced in Europe in recent years (Kirkendall and Faccoli 2010, Smith and Hulcr 2015, European and Mediterranean Plant Protection Organization [EPPO] 2015). The distribution records of *X. crassiusculus* in the New World has just been reviewed and updated by Flechtmann and Atkinson (2016). The species is currently well-established throughout eastern North America, where it was first detected in South Carolina in the 1970s (Atkinson et al. 2011). In Central America, it has been known to occur since the 1990s, in Costa Rica and Panamá (Kirkendall and Ødegaard 2007) and was recently detected in Guatemala (Flechtmann and Atkinson 2016). Only recently, *X. crassiusculus* has been detected in South America, with one record in French Guiana (in 2009) and several records, since 2012, from northern and southern Brazil (Flechtmann and Atkinson 2016). The present paper reports two new country records of *X. crassiusculus* from South America, in Argentina and Uruguay.

Commercial forestry in Argentina and Uruguay is primarily based on intensively managed monocultures of tree species. The increasing forested area in both countries, simultaneously with the growing of international trade, facilitates the establishment of exotic pests and diseases. Therefore, the occurrence of *X. crassiusculus* in Argentina and Uruguay is not unexpected.

Identification of bark and ambrosia beetles has traditionally been based on morphological characters, which can be not only challenging but also time-consuming due to the small body size and their superficially similar morphology (Wood 1986). Phytosanitary efforts to monitor or control exotic species will often be ineffective without correct taxonomic and biogeographic information (Knížek 2007, Kirkendall and Faccoli 2010). Thus, in the past 20 yr several studies have been employing molecular diagnostic tools based on DNA sequences, which have proven valuable to achieve accurate taxonomic identification and reveal possible sources of origin of the introduced pests (Buhroo and Lakatos 2011 and references therein, Jordal and Kambestad 2014, Cognato et al. 2015, Briski et al. 2016).

The current investigation uses morphology-based taxonomy and molecular diagnosis through DNA barcoding in order to confirm the identity of *X. crassiusculus*. In addition, we conducted DNA sequence analyses including COI sequences from Argentina and other regions, in order to place it in a phylogenetic and geographical context. The potential impact of this recently introduced species on the commercial forestry industry is discussed.

## Materials and Methods

### Specimen Collection for Morphological and Molecular Identification

The specimens examined in this study were obtained from recent surveys in Argentina and Uruguay, using both direct search and

ethanol-baited traps. Logs were brought to the laboratory and emerging live beetles were captured and stored in ethanol until identification. Of the 16 (female) specimens from Argentina examined, eight were preserved dry for morphological studies, and the others kept in 96% ethanol (one used for DNA extraction). Voucher specimens are deposited at the Instituto Nacional de Tecnología Agropecuaria, Instituto de Recursos Biológicos (Buenos Aires), and at the collection of the División Entomología del Museo de La Plata. The 10 (female) specimens from Uruguay were compared by S. M. Smith with specimens in the A. J. Cook Arthropod Research Collection, Michigan State University (East Lansing, MI), from SE Asia and United States, and found to be conspecific. Voucher specimens are deposited in the Forestry Entomological Collection of the Instituto Nacional de Investigación Agropecuaria, Tacuarembó, and Michigan State University.

### Morphological Examination

Diagnostic structures were examined and documented using standard stereomicroscopy and also scanning electron microscopy. Specimens, preserved in 96% ethanol, were air-dried, mounted on stubs, and coated with a gold-palladium (40–60%) alloy by a Thermo VG Scientific SC7620, and then observed using a Philips XL30 TMP New Look Scanning Electron Microscope (Philips, The Netherlands).

### Acquisition of Cytochrome Oxidase Subunit I (COI) Sequences

From material collected in Argentina, we obtained the 5' region of the mitochondrial cytochrome c oxidase I (COI) gene, in order to complement the species identification with molecular evidence. The DNA extraction and PCR amplification were done using facilities at the Instituto Argentino de Investigaciones de Zonas Áridas (IADIZA—CONICET Mendoza, Argentina). Total genomic DNA was extracted from 96% ethanol-preserved specimen by using the "salting out" protocol (adapted from Sunnuks and Hales 1996). The primers used in both amplification and sequencing were LCO (5'GGTCAACAAATCATAAAGATATTGG), and HCO (TAAACCTCAGGGTGACCAAAAAATCA; Simon et al. 1994, McKenna et al. 2009), covering ~600 bp fragment of the COI gene. Purified PCR products were sequenced by the INTA-Castelar internal sequencing service (Buenos Aires, Argentina). Sequences were edited with ProSeq software (Filatov 2002), and then checked and compared with others available in GenBank through BLAST tool ([www.blast.ncbi.nlm.nih.gov](http://www.blast.ncbi.nlm.nih.gov)). We obtained a 678-bp length fragment that covers for the standardized DNA "barcode" for animal species identification (Hebert et al. 2003). The barcode sequence was deposited in GenBank under accession number KX685266.

### Genetic Databases

We searched GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) and BOLD (<http://www.boldsystems.org/>) for sequences covering the "barcode" 5' region of the COI gene of *X. crassiusculus* from

**Table 1.** Geographical location of *X. crassiusculus* new records in two South American countries: Argentina and Uruguay

Country	Department	Host tree species	GPS coordinates	Year of detection
Argentina	Campana	<i>Populus deltoides</i> W. Bartram cv <i>deltoides</i>	34° 08'21.6" S, 58° 47'43.2" W	2013
Argentina	Campana	<i>Carya illinoensis</i> Wangenh	34° 07'50.5" S, 58° 49'22.4" W	2013
Uruguay	Paysandú	<i>Pinus taeda</i> L.	32° 18'19.40" S, 57° 33'35.34" W	2010
Uruguay	Rivera	<i>Pinus taeda</i> L.	31° 9'2.20" S, 55° 21'46.86" W	2013
Uruguay	San José	<i>Pinus taeda</i> L.	34° 32'54.58" S, 57° 0'26.41" W	2015

different countries and of three other *Xylosandrus* and *Cnestus* species chosen as outgroups (taking into account results by Dole et al. 2010).

### Molecular Analysis

We conducted Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses in order to place the COI sequence obtained from the Argentinian specimen in a phylogenetic and geographical context. The data matrix includes, besides the Argentinian, other COI sequences of *X. crassiusculus* from several regions, available from GenBank and BOLD databases: two from United States (North Carolina and Maryland), one from Madagascar, one from Thailand, and nine from French Polynesia from two different islands. Sequences from *X. morigerus* (Blandford), *Cnestus ater* (Eggers), and *Cnestus improcerus* (Sampson) were included as outgroups, using the former to root the trees. Sequences were aligned manually after reading frame localization, giving a final matrix with 17 taxa and 678 sites.

Maximum Likelihood analysis was performed on RAxML version 8.2.8 (Stamatakis 2014) on the CIPRES portal (Miller et al. 2010), using the *-f* algorithm, which computes a rapid bootstrap analysis and search for best-scoring ML tree in one single run, with 1,000 bootstrap replications and GTRCAT model, without



Fig. 1. Occurrence of *X. crassiusculus* in Central and South America. Flight intercept traps and multifunnel traps were employed in Uruguay and Argentina, respectively.

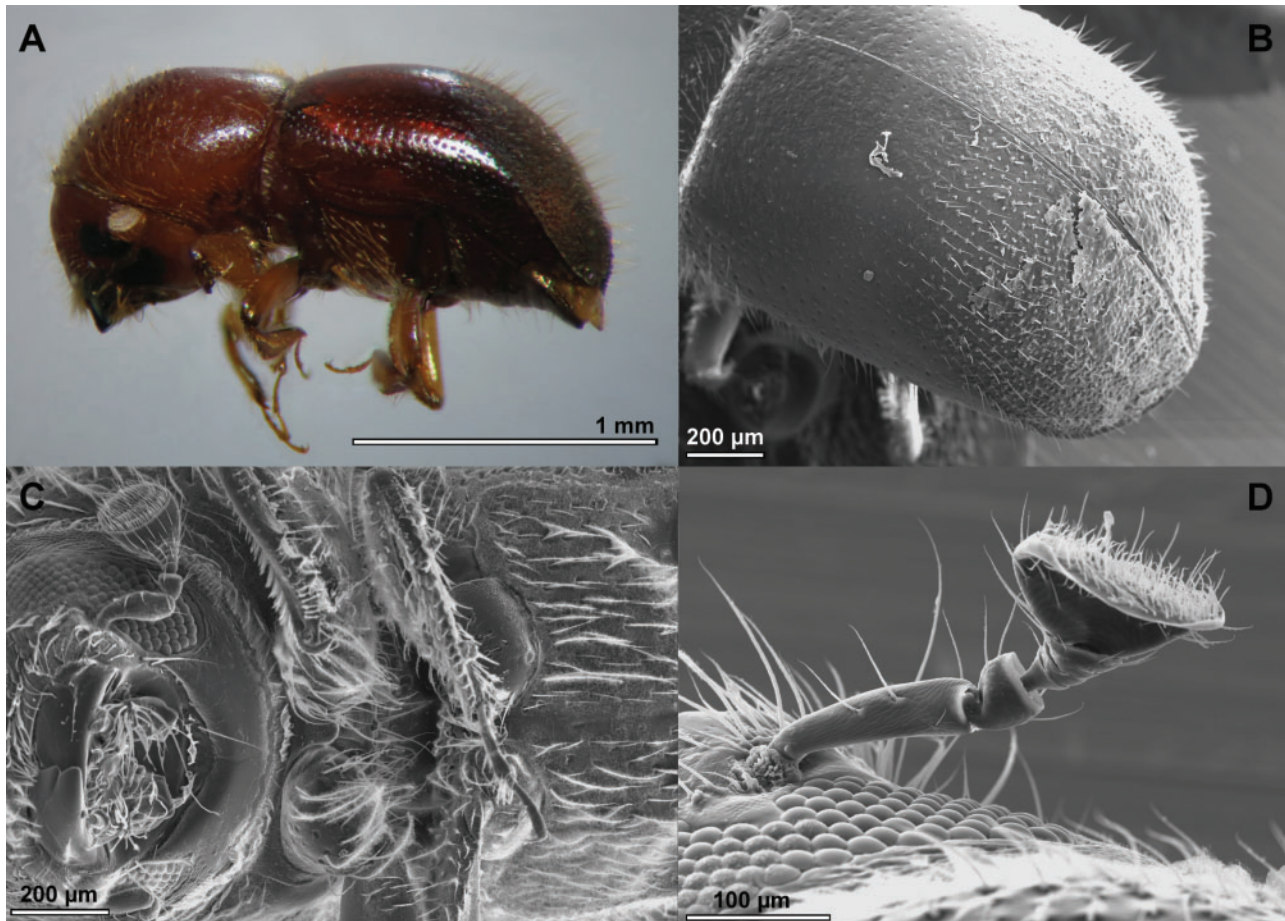
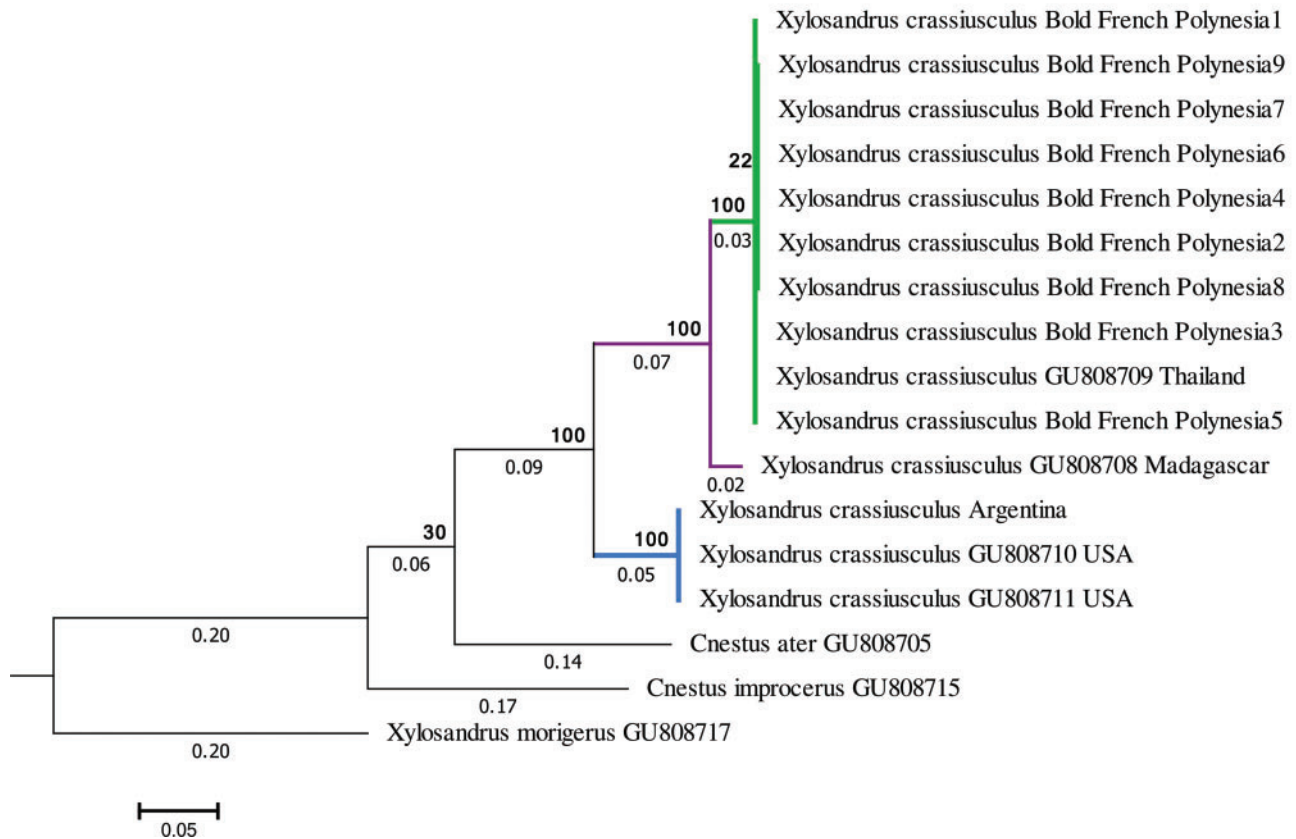


Fig. 2. *Xylosandrus crassiusculus*. (A) Habitus, female, lateral view. (B) Elytral declivity. (C) Noncontiguous procoxae and left antenna. (D) Detail of antenna.





**Fig. 3.** ML tree, numbers above and below branches indicate bootstrap values and branch lengths, respectively. Sequences from GenBank are identified with their accession numbers on the right. Other sequences were obtained from BOLD public database.

partitioning. MEGA version 6 (Tamura et al. 2013) was used in tree edition.

Parsimony analysis was conducted in TNT version 1.5 beta (Goloboff et al. 2008), with an heuristic search consisting of 1,000 random addition sequences plus TBR as swapping algorithm, saving 10 trees per replication, under equal weights. Nodal support was evaluated with 100 bootstrap replications.

## Results and Discussion

### New Country Distribution and Host-Plant Records

Table 1 presents the new data provided in this paper, including five new locality records of *X. crassiusculus* in two South American countries: Argentina and Uruguay. The map (Fig. 1) shows all known sites of occurrence of *X. crassiusculus* in Central and South America.

### Morphological Diagnosis

The genus *Xylosandrus* currently contains 40 species (Dole and Cognato 2010) from tropical and subtropical areas of the world. In their revision of the genus, Dole and Cognato (2010) provide a morphological diagnosis and delimitation of the genus with a key to its species, after transferring several species to other genera. However, monophyly of *Xylosandrus* is not recovered by phylogenetic analyses based on molecular and morphological data (Dole and Cognato 2010, Dole et al. 2010). In particular, the phylogenetic placement of *X. crassiusculus* as sister to *Cnestus* requires further study (Dole and Cognato 2010). Females of *Xylosandrus* species can

be distinguished from other New World Xyleborini by the stout body and the tuft of setae (though sometimes faint or abraded) at pronotal base (Fig. 2A); anterior margin of pronotum armed with serrations (six or more asperities of about equal size); truncate elytral declivity (Fig. 2B); separate, noncontiguous procoxae (Fig. 2C); lateral margin of the protibia bearing four to seven socketed teeth; and antenna with funicle five-segmented and with obliquely truncate club (Fig. 2C, D; Rabaglia et al. 2006, Wood 2007, Dole and Cognato 2010). The antennal club is peculiar because of its first segment being smooth, with its distal margin very acutely elevated into a continuous costa (forming a complete circle) extending from anterior face to apex (Fig. 2D). Males are rare, dwarfed, and flightless. *Xylosandrus crassiusculus*, the “granulate ambrosia beetle,” is distinguished from other *Xylosandrus* species in South America by the dull elytral declivity with dense small granules uniformly distributed from base to apex; and by the posterolateral margins of declivity sharply and distinctly carinate (Wood 2007). In addition, the body is reddish brown at maturity, the frons rugose, and the pronotum has a lateral costa. Females are larger (1.7–2.9 mm long) and more easily found than males (~1.5 mm).

### Molecular Analyses

The phylogenetic tree resulting from maximum likelihood is shown in Fig. 3. The specimen from Argentina cluster together with the two sequences from United States. These three samples are distinct from the remaining terminals included from Madagascar, Thailand, and Oceania. The tree topology obtained after Parsimony analysis (not shown) is fully congruent with the ML tree in Fig. 3, with the same nodes showing highest support values.

Morphological and phylogenetic analyses corroborate the identity of the Argentinian and Uruguayan specimens as *X. crassiusculus*. The absence of genetic difference in the COI sequences between the specimen from Argentina and the two available from United States is compatible with common source/s of the introduction. However, the information available does not allow inference whether the new records in Argentina (and Uruguay) represent new introduction/s or are expansions of the beetle from populations already established in the American continent. For that purpose, exhaustive surveys of the genetic variation through the native and introduced ranges of the species would be needed.

Like other *Xylosandrus* spp., *X. crassiusculus* is polyphagous and colonize any host of appropriate size and moisture content (Gorzlancyk et al. 2013, Smith and Hulcr 2015). It is capable of breeding in a wide variety of hosts, which include primarily deciduous species. Moreover, it prefers freshly dead wood and can use woody material which is drier than that required by most xyleborine ambrosia beetles. This species causes severe economic damage, killing small trees in nurseries and boring into stored hardwood lumber (Rabaglia et al. 2006, Gorzlanycyk et al. 2013, Smith and Hulcr 2015). The stressed condition of a host tree due to water damage, improper planting, drought stress, low temperature, and diseases plays an important role in the selection behavior of *X. crassiusculus* and other ambrosia beetles (Gorzlanycyk et al. 2013). In South America, and based on recent data from Brazil (Flechtmann and Atkinson 2016), we suggest that the geographical expansion of the species is still in progress. The establishment and spread of this ambrosia beetle would be favored by both international trade and special characteristics of the species deemed to facilitate colonization of new habitats, such as polyphagy, haplodiploid mating system, and regular inbreeding (Normark et al. 1999, Kirkendall and Faccoli 2010, Andersen et al. 2012, Biedermann et al. 2012).

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