



# First record of *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae, Scolytinae) in the Mediterranean scrubland in Southern Italy, and its co-presence with the co-generic species *X. compactus* (Eichhoff) and *X. crassiusculus* (Motschulsky)

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In the Circeo National Park (Italy), a survey assessed the occurrence of the ambrosia beetle *Xylosandrus germanus* in addition to the conspecifics *X. compactus* and *X. crassiusculus*. This is the first report in Europe of the co-presence of three *Xylosandrus* species, in an evergreen Mediterranean maquis dominated by holm oaks (*Quercus ilex* L.). Although no damage caused by *X. germanus* has been observed, the richness of plant species in the Circeo National Park may allow the establishment of this highly polyphagous ambrosia beetle, possibly endangering this native ecosystem. The dominance in the associated fungal community of *Ambrosiella grosmanniae* and *Fusarium* spp. reflects a stable symbiotic relationship of these taxa with *X. germanus*.

## Introduction

Ambrosia beetles (Coleoptera: Curculionidae, Scolytinae and Platypodinae) represent a particularly important group of alien species, capable of travelling undetected within many different woody materials and being difficult to find during phytosanitary inspections (Brockerhoff *et al.*, 2006). The damage they cause is related to both wood-boring activity and the introduction of associated fungi, which are of crucial importance for larval feeding development, as well as being pathogenic for the host plant.

Among ambrosia beetles, the species of the genus *Xylosandrus* belong to a highly damaging group of Asian tropical origin. In particular, *X. germanus* (Blandford), *X. crassiusculus* (Motschulsky) and *X. compactus* (Eichhoff) are spreading worldwide, invading also temperate areas of southern Europe, including Italy (Galko *et al.*, 2019). The highly polyphagous *X. crassiusculus*, attacking more than 200 plant hosts (Castrillo *et al.*, 2013), was reported for the first time in Europe since 2003 on Turkey oaks (*Quercus cerris* L.) growing in mixed woods in Tuscany. In a few years, the species spread in most of central and north Italy (Pennacchio *et al.*, 2003) also invading the Mediterranean maquis ecosystems (Francardi *et al.*, 2017, Gallego *et al.* 2017). It is currently not regulated by EPPO countries (EPPO, 2020). *X. compactus* was reported for the first time in 2011 in two urban parks in Naples and Portici (Campania, South Italy) on holm oaks (*Quercus ilex* L.) and evergreen Mediterranean shrubs (Garonna *et al.*, 2012). *X. compactus*

then spread quickly in other regions of the Italian mainland (Vannini *et al.*, 2017) and Sicily (Longo & Garzia, 2016), where it attacks twigs and small branches of a large number of Mediterranean host trees. *X. compactus* was added to the EPPO Alert List in 2017. In the EPPO region, it is a quarantine pest for Israel (EPPO, 2020). In the last 5 years, the coexistence of *X. compactus* and *X. crassiusculus* was reported in the Circeo National Park (Latium, central Italy), causing dieback of branches and twigs of many species of the Mediterranean maquis, such as *Ceratonia siliqua* L., *Quercus* spp. and *Arbutus unedo* L. (Francardi *et al.*, 2017). In particular, a serious outbreak of *X. compactus* was reported causing severe damage to the Mediterranean maquis in an area that extended for more than 13 hectares on the Circeo Promontory, within the stands of the Circeo National Park (Vannini *et al.*, 2017). Here, evergreen shrubs and trees suffered strong and quick desiccation of the small-diameter branches (up to 3 cm) caused by the wood-boring activities of the beetles and their associated fungal pathogens (Vannini *et al.*, 2017). A third species, *X. germanus*, was first reported in northern Italy in 1992 in stands dominated by chestnuts (*Castanea sativa* Miller; Bernabò, 2000; Dutto *et al.*, 2018), then in walnut plantations (*Juglans* spp.), in mixed broadleaf stands and in apple orchards (Stergulic *et al.*, 1999). *X. germanus* is currently not regulated in the EPPO region, but is a quarantine pest in the Comité de Sanidad Vegetal del Cono Sur (COSAVE) region (EPPO, 2018). Unlike the two previously described species, *X. germanus* has, to the authors' knowledge, never been recorded in Mediterranean maquis

ecosystems in Italy or in maquis ecosystems elsewhere. *X. germanus* is currently present in most of Europe (EPPO, 2018).

Within the framework of the monitoring activity carried out in Italy, France and Spain by the European LIFE project SAMFIX (SAving Mediterranean Forest from Invasions of *Xylosandrus* beetles and associated pathogenic fungi – LIFE17 NAT/IT/000609) the present paper reports the first detection of *X. germanus* in the Mediterranean maquis ecosystem in Europe, and the first report for Europe of the co-presence of the three *Xylosandrus* species in the same environment.

## Materials and methods

### Insect monitoring and collection

From 6 August until 6 November 2018 four black 9-funnel traps were set up in the Quarto Freddo site located in the Circeo Promontory of the Circeo National Park (Latium Region, central Italy) at about 160 m above sea level (41° 14' 17" N, 13° 04' 49" E). The area is characterized by the presence of mixed wood stands dominated by *Q. ilex* associated with a large number of tree and shrub species typical of the Mediterranean maquis. Traps were positioned about 100 m apart from each other and baited with a dispenser of 5 mL of pure ethanol and a dispenser of 1 mL of (–)-alpha-pinene. Both lures were put separately in polyethylene diffusion bags hung at the second funnel located above the trap collection cup. In the collection cups, an insecticide net was placed to kill collected specimens avoiding their escape. Lures and nets were renewed every 15 days, while trapped insects were collected and brought to the laboratory weekly. *Xylosandrus* species were determined based on morphological characteristics and following published keys (Dole & Cognato, 2010).

### Fungal isolation and identification

Among the adults of *X. germanus* collected from different traps and dates between 20 August and 3 September 2018, seven specimens were used to investigate the associated

fungal community through mass sequencing. Adult insects were repeatedly washed in sterile distilled H<sub>2</sub>O and dried on filter paper before DNA extraction. Total DNA from a bulk of the 7 adults was extracted using a DNeasy Power-Soil Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The ITS1 region was amplified with a dual indexing primer using the tagged primer pair ITS1F (50-xxxxCTYGGTCATTTAGAGGAAGTAA-30) and ITS2 (5-xxxxGCHRCGTTCTTCATCGDTGC-30), where xxx represents the barcoding key. The PCR reaction mixture comprised 12.5 mL of Maxima Hot Start PCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 1 mM of each primer in a total volume of 25 mL containing 24 mL of reaction mixture and 1 mL of template. The thermal cycle was an initial denaturation at 94°C for 10 min followed by 30 cycles of 95°C for 40 s, 60°C for 40 s and 72°C for 1 min, and a final elongation step of 72°C for 10 min. Amplicons were purified using MagJET NGS Cleanup (Thermo Fisher Scientific, Waltham, Massachusetts, USA), quantified with a Qubit Quantitation kit (Invitrogen, Carlsbad, California, USA) and pooled at equal concentrations for sequencing. Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq sequencer by Eurofins Genomics GmbH (Ebersberg, Germany). Data sets were analysed following the pipeline described by Morales-Rodriguez *et al.* (2019).

## Results

### Trapped species

A total of 283 *Xylosandrus* adults were collected in the four traps. Three species of *Xylosandrus* were identified: *X. compactus* and *X. crassiusculus*, previously reported at this site (Vannini *et al.*, 2017, Francardi *et al.*, 2017), and a third species, new for the area, identified as *X. germanus* (Fig. 1). *X. compactus* was the most common species trapped with a total of 266 individuals, followed by *X. germanus* with 15 specimens and 2 adults of *X. crassiusculus*. Results show the emergence peak of *X. compactus*, which occurred in middle September

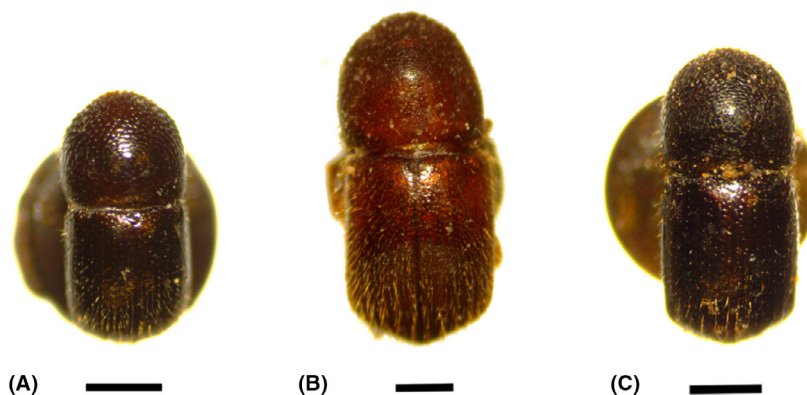
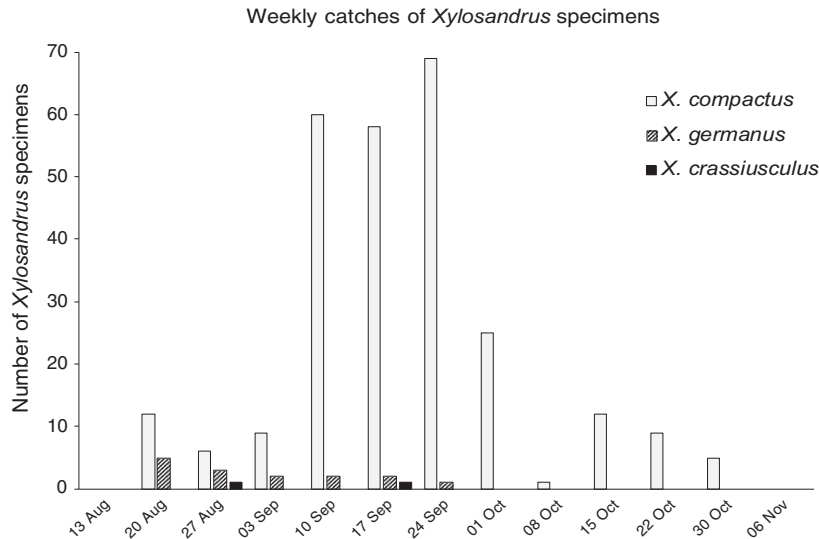


Fig. 1 (A) *Xylosandrus compactus*, (B) *Xylosandrus crassiusculus* and (C) *Xylosandrus germanus*. Scale bar 0.5 mm.



**Fig. 2** Number of specimens of *Xylosandrus* species trapped weekly in the Circeo National Park.

(Fig. 2). Captures of *X. germanus* and *X. crassiusculus* were too low for phenology and voltinism assessment.

#### Fungal community of *X. germanus*

From the insect bulk sample analysed, 289 331 reads were clustered to a 97% similarity level in 164 Operational Taxonomic Units (OTUs), among which *Ambrosiella grossmanniae* Meyers, McNew & Harr., *Geosmithia pallida* (G. Smith) M. Kolarík, Kubátová & Pazoutová, *Fusarium* spp., *Penicillium* spp. *Cladosporium* sp. and *Alternaria* sp. were the most representative taxa, accounting for 42% of the total reads.

#### Discussion

This is the first record of *X. germanus* presence in Mediterranean scrubland in Europe and Italy. Previous records in Europe confined this species to deciduous forests (e.g. *Juglans* spp., *Fagus sylvatica* L., *Castanea sativa*, *Quercus petraea* (Matt.) Liebl) and coniferous trees (CABI, 2020). In Italy, *X. germanus* was reported mainly in the northern part of the peninsula, preferentially associated with chestnut stands (Rassati *et al.*, 2016; Dutto *et al.*, 2018). Interestingly, this association was recently confirmed in spring 2018 in a chestnut coppice stand in Viterbo province, central Italy, suggesting the spread of this alien species southward across the Italian peninsula (Rassati *et al.*, 2020).

Furthermore, the survey activity carried out in the Circeo National Park reported, for the first time in Europe, the co-presence of the three *Xylosandrus* species, *X. germanus*, *X. crassiusculus* and *X. compactus*, in a heterogeneous stand of evergreen Mediterranean maquis dominated by holm oaks.

Even if *X. compactus* has a higher population in comparison to the other two species, the simultaneous presence of these three introduced alien pests endangers this delicate

ecosystem, which is already showing signs of the change in the forest structure. Moreover, the large number of plant species occurring in the Circeo National Park potentially susceptible to be colonized by these three highly polyphagous ambrosia beetles further increases the biological risk for these native ecosystems to be altered by the massive presence of *Xylosandrus* species and their associated fungi. In addition, the three *Xylosandrus* species exploit distinct ecological niches by colonizing different branch diameters of the host plant, exposing whole forest stands to further infestation risk. *X. compactus* differs from other species as it usually colonizes branches smaller than 2–3 cm, even though atypical behaviours are sometime reported (Gugliuzzo *et al.*, 2019), while *X. germanus* and *X. crassiusculus* bore galleries in stems larger than 2 cm.

*X. germanus* is a very polyphagous species, attacking more than 200 species of trees, including living trees, dying trees or trees which have recently died (Hoffmann, 1941; Weber & McPherson, 1983; Ranger *et al.*, 2015). Adult females, 2–2.3 mm long, penetrate the wood through entry holes 1 mm in diameter. Mother galleries widen in brood chambers where eggs are laid and larvae develop until the emergence of adults of the new generation (Agnello *et al.*, 2017). *X. germanus* completes two generations per year, three in the warmer southern regions of the United States (Weber & McPherson, 1983). Moreover, it presents haploid-diploid parthenogenesis with females and males produced from fertilized and unfertilized eggs, respectively. Female progeny mate with siblings inside the galleries before emergence and dispersal. However, even if very rare, mating among non-siblings is known (Keller *et al.*, 2011). The sex ratio is about 10 females per male, but it can vary depending on the host plant as well as brood size (Peer & Taborsky, 2004).

Colonization symptoms are not easy to detect. However, 3–4 cm long cylinders of woody frass are expelled by

adults from the entry holes during the gallery boring in the wood; sometimes, sap pours from the entry holes, staining and wetting the bark.

As for other ambrosia beetles, larval development and adult maturation depend on inoculation and growth on the inner walls of the brood chambers of symbiotic fungi used as food and carried in special tegumental structures, the mycangia. In the present study, the fungal community associated with *X. germanus* was investigated. The finding of *Ambrosiella grossmanniae* is consistent with the records in the literature that identify in this species the main symbiont associated with *X. germanus* (Mayers *et al.*, 2015). The OTU of *A. grossmanniae* accounted for 7% of the total reads, reflecting the importance of this species for the larval development of the insect. *Geosmithia pallida* accounted for 14% of the total reads. *G. pallida* is a species native from Europe accidentally introduced as an alien pathogen of live oaks in the United States (Lynch *et al.*, 2014). This fungus shows affinities with a wide range of wood-boring insects, such as bark beetles and sawyer beetles (Kolarik *et al.*, 2004). Moreover, it was already reported in association with *X. compactus* in the Circeo National Park by Vanini *et al.* (2017). Associations among *X. germanus* and *Fusarium* species were already known, often in presence of host dieback and stem cankers (Kessler, 1974; Weber & McPherson, 1984, 1985; Agnello *et al.*, 2017).

Further studies are already underway (1) to determine the effective damage caused by *X. germanus* in the Circeo National Park; (2) to update the list of the potential host plants; and (3) to better understand both the relationships between the three *Xylosandrus* species and their associations with fungi in order to assess the risk of biological invasions in sclerophyllous stands of southern Italy.

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## Premier signalement de *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae, Scolytinae) dans la guarrigue du sud de l'Italie, en présence des espèces du même genre *X. compactus* (Eichhoff) et *X. crassiusculus* (Motschulsky)

Une prospection a mis en évidence la présence du petit scolyte noir du Japon, *Xylosandrus germanus*, dans le parc national du Circé (Italie), ainsi que celle de ses congénères,

les scolytes *X. compactus* et *X. crassiusculus*. Il s'agit du premier signalement en Europe de la présence conjointe de ces trois espèces de *Xylosandrus*, et ce au sein d'un maquis méditerranéen à feuilles persistantes dominé par des chênes verts (*Quercus ilex* L.). Bien qu'aucun dégât causé par *X. germanus* n'ait été observé, la richesse des espèces végétales du parc national du Circé pourrait permettre l'établissement de ce scolyte très polyphage, présentant un danger éventuel pour cet écosystème indigène. La prévalence dominante d'*Ambrosiella grossmanniae* et de *Fusarium* spp. au sein de la communauté fongique associée reflète une relation symbiotique stable de ces taxons avec *X. germanus*.

## Первое сообщение о *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae, Scolytinae) в средиземноморском маквисе на юге Италии, и о его совместном присутствии с близкородственными видами *X. compactus* (Eichhoff) и *X. crassiusculus* (Motschulsky)

В Национальном парке Цирцео (Италия) было проведено обследование, в ходе которого было оценено присутствие амброзиевого жука *Xylosandrus germanus* в дополнение к принадлежащим к тому же роду *X. compactus* и *X. crassiusculus*. Это первое сообщение для Европы о совместном присутствии трёх видов рода *Xylosandrus* в вечнозелёном средиземноморском маквисе с преобладанием каменного дуба (*Quercus ilex* L.). Несмотря на то, что не было обнаружено никакого ущерба, нанесённого *X. germanus*, разнообразие видов растений в Национальном парке Цирцео может способствовать акклиматизации этого крайне многоядного амброзиевого жука, что может представлять угрозу для этой аборигенной экосистемы. Доминирование *Ambrosiella grossmanniae* и *Fusarium* spp. в ассоциированных грибных сообществах отражает стабильную симбиотическую связь этих таксонов с *X. germanus*.

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