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XYLOSANDRUS COMPACTUS AND LIPARTHURUM COLCHICUM (COLEOPTERA SCOLYTINAE) IN TUSCANY: A PRELIMINARY SCREENING OF ASSOCIATED FUNGI

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Benvenuti C., Strangi A., Iovinella I., Barzanti G. P., Simoni S., Vitale S., Luongo L., Francardi V., Roversi P.F. - *Xylosandrus compactus* and *Liparthrum colchicum* (Coleoptera Scolytinae) in Tuscany: a preliminary screening of associated fungi.

Xylosandrus compactus and *Liparthrum colchicum* are two Scolytinae recorded for the first time in Italy in 2011 and 2019, respectively. *X. compactus* is an “ambrosia beetle” causing damages to several plants typical of the Mediterranean maquis through its digging activity on twigs and branches of the host plants. *L. colchicum* is a “bark beetle”, monophagous on laurel shrubs and trees. During a survey performed in summer of 2020, in “Tenuta Salviati” located in the Migliarino Natural Park (Pisa, Tuscany), adults of *X. compactus* and *L. colchicum* were collected from the same branches and apical twigs of *Laurus nobilis* hedges. The study aimed at characterizing fungi isolated from the external surface of the two scolytids bodies, to evaluate similarities and even their role in fungal spreading in the environment.

KEY WORDS: ambrosia beetle; bark beetle; *Cladosporium* spp.; *Geosmithia* spp.; *Laurus nobilis*; phytopathogenic fungi.

INTRODUCTION

Xylosandrus compactus (Eichhoff) (black twig borer) is a scolytid beetle native to South Eastern Asia and accidentally introduced to several areas of Africa, North and South America and Europe (WOOD, 1982; WOOD and BRIGHT, 1992; WOOD, 2007). The adult females attack twigs and branches of the host plants digging breeding galleries where they lay eggs and spread ambrosia fungi (e.g. *Ambrosiella xylebori*, *A. macrospora*), which serve as food for adults and larvae. In the last decade, severe damages were reported in the EPPO region to cause the gradual decline and ultimate dieback of the attacked plants. More than 220 plant species, belonging to 62 different families, have been reported as suitable hosts for this pest in native or introduced areas; some of them (such as *Coffea arabica* L., *Camellia sinensis* L., *Mangifera indica* L., *Persea americana* Mill. and *Theobroma cacao* L.) have considerable commercial value (NGOAN *et al.*, 1976; WOOD, 1982; DIXON and WOODRUFF, 1983; MESHAM *et al.*, 1993; INTACHAT and KIRTON, 1997; DAEHLER and DUDLEY, 2002; MATSUMOTO, 2002). For this reason, since 2017, *X. compactus* has been included in the EPPO alert list (EPPO RS 2017/032) of alien and invasive species.

In Italy this pest was observed for the first time in 2011 (FITOLAB, 2011a, b) on *Laurus nobilis* L., *Quercus ilex* L., *Castanea sativa* Mill., *Fraxinus ornus* L. and *Celtis australis* L. in urban parks located near Naples (GARONNA *et al.*, 2012). Subsequently new discoveries

occurred in different Italian regions: Liguria and Tuscany in 2012, Lombardy in 2015, Lazio and Sicily in 2016, Emilia Romagna in 2018 and Veneto 2019 (FRANCARDI *et al.*, 2012; LONGO and TROPEA GARZIA, 2016; GUGLIUZZO *et al.*, 2019a; GUGLIUZZO *et al.*, 2019b; GUGLIUZZO *et al.*, 2019c; FACCOLI, 2021). The rapid spread of this insect, due to its adaptation to the Mediterranean climate, caused significant damages to several plant species typical of the Mediterranean maquis. In Tuscany, extensive damages were observed in several environments, as well as on laurel hedges in monumental public and private urban parks.

Moreover, it should be noted that: *X. compactus* is associated with a number of fungi (along the main nutritional symbionts) located not only on the mycangium but also in other parts of the beetle body (as showed by BATEMAN *et al.*, 2016); some of these fungi can be pathogenic such as *Fusarium solani*, *Geosmithia pallida* (Hypocreales: Bionectriaceae) and *Epicoccum nigrum* (Pleosporaceae: Hypocreaceae) (BATEMAN *et al.*, 2016; VANNINI *et al.*, 2017; MORALES-RODRÍGUEZ *et al.*, 2020). The combined effect due to both activities of colonizing insects and pathogenic fungi might represent a further stress factor in attacked plants and, consequently, a major concern from a phytosanitary perspective.

The surveys carried out on plants attacked by *X. compactus* allowed also the detection of another Scolytinae: *Liparthrum colchicum*. This species was recently recorded, for the first time in Italy, on *L. nobilis*

(FRANCARDI *et al.*, 2021). This bark beetle lives exclusively on laurel trees and it is currently considered a species native to Mediterranean countries (JORDAL *et al.*, 2004).

During the spring, *L. colchicum* generally attacks the distal part of laurel twigs; together with *X. compactus*, which is preferentially attracted to the basal parts of the same twigs, might contribute to the desiccation of the affected plants (FRANCARDI *et al.*, 2012).

Adults dig circular entrance holes, ranging from 0.5 to 1.0 mm in diameter, into the subcortical region of twigs. The main characteristics of maternal chamber and inner galleries have been described by WOOD (2007) for the genus *Liparthrum* spp. The tunnelling activity of larvae, inside the colonized twigs, is externally detectable by observing typical light yellow/orange circular streaks/rings. This insect might also play a role as a vector of phytopathogenic fungal species as suggested by KOLAŘÍK *et al.* (2007), that reported three species of the well-known phytopathogenic genus *Geosmithia* (Ascomycota, Hypocreales) associated with this bark beetle on laurel.

The aim of the present study was to characterize the fungal communities, including commensals, mutualistic and entomopathogenic fungi, carried by *X. compactus* and *L. colchicum* in “Tenuta Salviati” near Migliarino Natural Park – Pisa, a site where both species coexist and where the combined feeding activities resulted in a widespread damage on the same laurel hedges. The ecological and/or phytopathological significance of the distribution and association of the fungi with the insect species are discussed and evaluated to insight possible economic and ecological threats posed by the two insects.

MATERIALS AND METHODS

SAMPLING

Branches and apical twigs of *Laurus nobilis* with symptoms described by FRANCARDI *et al.* (2012); PENNACCHIO *et al.* (2012); FRANCARDI *et al.* (2017) were collected in laurel hedges in “Tenuta Salviati” near a natural park of Migliarino (Pisa) (43°46'07.00"N, 10°19'27.33"E). During summer of 2020, 100 short branches with leaves and twigs were sampled and each sample was approximately 20 cm in length with a diameter ranging from 0.5 to 1 cm. Single branches were placed separately into plastic bags for transportation and stored in Firenze CREA-DC laboratory at +4.0°C until analysis.

In the laboratory, all branches were cut lengthwise with a sterilised scalpel near the entrance holes of both adult insects and their galleries were carefully inspected under a stereo-microscope (Nikon SMZ 10A); all detected *X. compactus* and *L. colchicum* specimens were collected with a sterilized fine brush and maintained separately considering their respective galleries.

INSECT AND FUNGI IDENTIFICATION

a) Insect identification

The species identification of the insects was obtained through analysis of morphological characters and molecular procedures. Morphological identification was

performed by adopting the keys of PFEFFER (1995), WOOD (2007) and FRANCARDI *et al.* (2017). Both for *X. compactus* and *L. colchicum*, molecular identification was carried out for one single specimen using the protocol described by KIRAN *et al.* (2019); species attribution was assessed through homology search in GenBank database.

b) Fungal isolation and classification

In order to investigate the fungal taxa associated with *X. compactus* and *L. colchicum*, 30 adults of both species, previously identified, were individually set onto a PDA substrate (Potato Dextrose Agar, VWR) in a 90 mm Petri plate (1 adult/plate) and allowed to freely walk for 30 minutes, as reported by other authors (VANNINI *et al.*, 2017; GUGLIUZZO *et al.*, 2020). After this lapse of time, insects were discarded from the plates. All the plates, sealed with laboratory film, were placed at 24°C in the dark. After 72/96 hrs, plates were inspected for developing colonies by counting of Colony Forming Units (CFUs). From the resulting colonies, one transfer, representative of each colony type, was placed onto fresh PDA plates (60 mm) in order to obtain pure cultures growing at 24°C in the dark. Fungal colonies were sub cultured, examined for their microscopic characteristics and grouped according to their micro and morphological characters. The representatives of each morpho-type were selected and purified for molecular identification.

Aliquots of mycelium and conidia (about 10 mg maximum) representing the selected morpho-type were frozen at – 80°C and, subsequently, homogenized with a mix of 500 µm- and 2,0 mm-diameter glass beads (1:1 v/v) using a mechanical beater (Precellys 24, Precellys). Genomic DNA was extracted using Dneasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions; the final elution step was performed in 100 µl ddH₂O. The rDNA ITS region was amplified by conventional PCR using the primers ITS5 and ITS4 (WHITE *et al.*, 1990). Amplicons were sequenced in both directions at Bio-Fab Research Company (Rome, Italy) and assembled with Geneious 2021 software. Sequences were aligned with additional accessions obtained from GenBank using BLASTn program (<http://blast.ncbi.nlm.nih.gov>).

STATISTICAL ANALYSIS

Frequencies of association between *X. compactus*/ *L. colchicum* specimens and the identified fungal genera were assessed and analyzed by chi square test. Differences in fungal communities, within the two insect species, were assessed by different ecological parameters (Dominance, Shannon, Evenness, Simpson, Menhinick, Margalef, Equitability, Fisher and Berger-Parker indexes). Over all indexes, significance in diversity of fungi associated to the two insect species was evaluated by the Monte Carlo permutation test (BROWN and ROTHERY, 1993).

In order to evaluate the possibility of the two different insects to carry and spread the fungal community within the same habitat, a non-metric multidimensional scaling (NMDS) analysis was performed to represent, as closely as possible, the pairwise dissimilarity between the two insect species. Rarefaction curves were constructed to

estimate and compare the richness of the two fungal communities at the abundance level of the smallest sample (CHAO *et al.*, 2014).

The statistical analyses were computed using Past software (version 4.03) (HAMMER *et al.*, 2001).

RESULTS

The morphological and molecular analysis of all the specimens collected in Migliarino Natural Park allowed the identification of the two species as *Xylosandrus compactus* and *Liparthrum colchicum*. The sequences of COX I mitochondrial gene obtained from the two individuals respectively showed an identity of 100% with *Xylosandrus compactus* (Accession number MW961427) and 100% with *L. colchicum* (Accession number MT264965). Sequences of *X. compactus* and *L. colchicum* were submitted to GenBank with Accession numbers MW532748 and MT264966.

The screening focused on identification of the fungal isolates carried by the two scolytids resulted in 281 CFUs. More specifically, 204 CFUs from *X. compactus* were identified and grouped in 28 morpho-types, while 77 CFUs from *L. colchicum* were grouped in 14 morpho-types. Molecular analyses on morpho-types identified 11 and 9 different genera of fungi respectively. The most abundant fungal genera found on insect bodies were *Cladosporium* and *Geosmithia* (Fig. I). These genera represented the commonest fungi carried outside the body of *X. compactus* (83% and 76% specimens respectively) while the same genera were found on about 30% and 33% *L. colchicum* specimens respectively.

The number of fungal colonies originated from the two insects showed a greater association between *X. compactus* and fungi - all 30 *X. compactus* specimens tested were associated to two fungal genera at least - than that evaluated for *L. colchicum* (chi square test, $P=0.04$). By considering all the fungi registered, regardless of the genus, the number of CFUs originated by *X. compactus* was three times higher than that registered for *L. colchicum*.

However, considering the fungal communities associated with the two scolytids, we can observe non-significant differences between biodiversity indexes (Table 1). The only significant difference was found in the number of CFUs by Berger-Parker dominance index (Monte Carlo permutation test $P=0.04$): the higher value registered for *X. compactus* may be ascribable to the most abundant genera represented in these samples. The substantial uniformity between fungal communities associated with the two scolytids could be found also in the species richness. Assuming that individuals in the same environment are randomly distributed and sample size is sufficiently congruous, the individual rarefaction curves show that in a hypothetical population size of 75 CFUs, *L. colchicum* should have only 8 different genera, while *X. compactus* 9 genera (Fig. II). Considering the total number of CFUs and their frequencies, the NMDS analysis of the fungal communities showed that the two insect species are clearly separated in carrying ability for

a different quantity of fungal spores on the external surface of the body (Fig. III).

DISCUSSION

The association between bark and/or ambrosia beetles and fungi is well known and largely studied (e.g. BEAVER *et al.*, 1989; JANKOWIAK *et al.*, 2007; MORALES-RODRÍGUEZ *et al.*, 2020). The strength of association is deeply affected by the environment and species considered. Sampling places, isolation methods and species determination techniques are key factors to be considered when different associations between fungi and insects are compared. In this study, the fungal communities associated with the two scolytid species living on branches and apical twigs in a unique spot of laurel trees are compared.

As the unique environment and area of study, this could be explained with the different morphology of external structures where spores could interact.

Ambrosiella fungi have been reported to be associated with ambrosia beetles in several papers, but we did not obtain any CFU of this genus: this evidence could be explained as an effect of the methodology used to obtain the fungi colonies as suggested by BATEMAN *et al.* (2016). Moreover, only dispersing individuals have fully developed mycangia containing spores of the main fungal mutualists (LI *et al.*, 2019) and it could be possible that beetles collected from the galleries could not have reached the dispersal life stage. Moreover, *Ambrosiella* fungi are contained, and unexposed, mainly in the mycangi or tunnels, and their presence on the body surface of *X. compactus* could be limited. A similar explanation could be for the lack of *Fusarium*, previously found by INTACHAT and KIRTON (1997), on the surface of *X. compactus*. Indeed, in our experiment, the beetles were allowed to release spores on plate with a free walk, while Bateman and co-authors (2016) isolated fungi from the washing solution of the surface of insects. Although the choice of primers for the amplification of ITS locus could introduce a bias in the identification of some fungal genera, in this work we successfully amplified and sequenced this target sequence from all the colonies analysed.

The present study describes the fungi associated with the body surface of *X. compactus* and *L. colchicum* when these species coexist in laurel twigs collected in one common natural environment. Both scolytids seem to be able to carry potential phytopathogenic fungi, such as *Geosmithia* spp. and *Cladosporium* spp.; the presence of such species on *X. compactus* adults was significantly higher than in *L. colchicum*. *Cladosporium* is an extremely common fungal genus that can be isolated from a huge variety of substrates (from soil to plants, air, etc.) and its great abundance is not unexpected. Generally, it includes saprophytic species even if phytopathogenic, mycopathogenic and some weakly human parasitic species are also included (BENSCH *et al.*, 2012; 2015). Several species of *Cladosporium* have already been reported associated with *X. compactus* in

Fig. 1 - Percentage of *X. compactus* and *L. colchicum* specimens associated with the identified fungal genera. Scolytinae specimens were rated as associated with fungi when at least one CFUs could be detected on Petri plates.

Table 1 - Biodiversity indexes and Monte Carlo permutation test's probability (P).

Biodiversity indexes, significance (Permutation test)			
	on <i>Lyparthrum</i>	on <i>Xylosandrus</i>	P
Taxa S	9	11	0.8486
Individuals	77	204	----
Dominance	0.3105	0.2732	0.3613
Shannon H	1.487	1.637	0.4113
Evenness e ^H /S	0.4914	0.4673	0.8808
Simpson indx	0.6895	0.7268	0.3613
Menhinick	1.026	0.7702	0.4547
Margalef	1.842	1.88	1
Equitability J	0.6766	0.6827	0.9319
Fisher alpha	2.643	2.49	0.8586
Berger-Parker	0.4935	0.3676	0.0404

Italy and with other bark beetles (JANKOWIAK *et al.*, 2007; BATEMAN *et al.*, 2016; MORALES-RODRÍGUEZ *et al.*, 2020). However, this is the first time that the fungus was found associated with *L. colchicum*.

Geosmithia species are, instead, cosmopolitan but understudied fungi; most of them are associated with phloem-feeding bark beetles on various woody hosts (ČÍŽKOVÁ *et al.*, 2005; JANKOWIAK *et al.*, 2014; HÄNZI *et al.*, 2016; PEPORI *et al.*, 2015). This genus has been previously associated on the body of *L. colchicum* as previously described in KOLAŘÍK *et al.* (2004, 2007). The presence of *Geosmithia* spp. with *X. compactus* has been reported for the first time only recently (VANNINI *et al.*, 2017; GUGLIUZZO *et al.*, 2020; MORALES-RODRÍGUEZ *et al.*, 2020). Three fungal genera were found associated only with *X. compactus*: *Clonostachys*, *Aureobasidium* and *Acremonium*.

Fig. II - Individual rarefaction curves of *X. compactus* (continuous line) and *L. colchicum* (dashed line).

Fig. III - Non-metric multidimensional scaling (NMDS) analysis of the fungal communities carried by *X. compactus* (black dots) and *L. colchicum* (empty dots).

Filamentous fungi belonging to the genus *Clonostachys* are worldwide spread and colonize several habitats, particularly soil. Species of this genera are known as excellent parasite of numerous fungal plant pathogens, nematodes and insects. According to MORALES-RODRÍGUEZ *et al.* (2020), *Clonostachys* is reported on *L. nobilis* and *Q. ilex* associated with *X. compactus*.

Aureobasidium is a ubiquitous and generalist black, yeast-like fungus that can be found in different environments. This genus was reported as associated with ambrosia beetle such as *X. saxesenii* (MALACRINO *et al.*, 2017; RASSATI *et al.*, 2019).

Furthermore, the *Acremonium* genus includes more than 100 species, mostly saprophytic but also pathogenic for plants, insects and other fungi. This genus was found associated to *X. compactus* by MULENKO *et al.* (2008)

and PERDOMO *et al.* (2011) on *Laurus nobilis*. *Hyphopichia* is a ubiquitous and generalistic yeast-like fungus that can be found in different environments. *Alternaria* is an ubiquitous fungal genus that is frequently found in Italy on rotting fruit and vegetables as well as on plants, in which it plays a role as a phytopathogen. *Phaeosphaeria* is a major fungal pathogen affecting all major crop plant families and its association with *X. compactus* has been reported for the first time only recently (VANNINI *et al.*, 2017, GUGLIUZZO *et al.*, 2020, MORALES-RODRIGUEZ *et al.*, 2020), while *Acremonium* sp., *Alternaria* sp., *Aureobasidium* sp., *Cladosporium* sp. and *Clonostachys* sp. are genera well known to be associated to *X. compactus*. At the same time, two genera were found associated only with *L. colchicum* but not with *X. compactus*: *Penicillium* and *Cryptococcus*.

The genus *Penicillium* is ubiquitous, epiphytic in different crops and endophytic in the inner tissues of living plants. Species of the genus *Penicillium* play a key role in post-harvest conservation and spoilage of vegetal material (LOUW and KORSTEN, 2014).

The yeast genus *Cryptococcus* is a well-known mammalian pathogen but generally not of phytopathogenic interest, however some species, such as *C. laurentii*, could be used in biocontrol strategies (QIN and TIAN, 2005).

Considering the data obtained in this study and those reported in literature, *X. compactus* could be considered a vector for *Clonostachys*, *Aureobasidium* and *Acremonium*, while, so far, *L. colchicum* did not seem to be associated to these genera. On the other hand, *L. colchicum* could contribute to diffusion of *Cryptococcus* but it could not be considered an exclusive factor for the diffusion of *Penicillium* because this genus was previously found associated also with *X. compactus* on *L. nobilis* by several authors (PETERSON *et al.*, 2003; PETERSON *et al.*, 2005; PETERSON *et al.*, 2015 and CAO *et al.*, 2019). In addition, *Penicillium* spores are primarily wind-dispersed and could not strictly depend on beetle vectors for dissemination (GRINN-GOFRON, 2011). According to the emerged evidences, by considering overall the biodiversity indexes measured, not remarkable differences were highlighted between the composition of fungal communities carried by *X. compactus* and by *L. colchicum*. Only the Berger-Parker dominance index resulted to be statistically significant due to the higher frequency in *X. compactus* of the most common fungal genera detected.

A fundamental difference between the two beetles consists in the polyphagous behaviour of *X. compactus*, able to colonize different hosts, while the laurel is the specific host of *L. colchicum*. For this reason, the possible spread of *X. compactus* and its associated fungi can represent an added major risk for woody plants, cultivated for fruit production, ornamental and forestry purposes in EPPO region.

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