DOI: 10.1111/epp.12956

ORIGINAL ARTICLE

From a cause of rapid fig tree dieback to a new threat to mango production: the invasive bark beetle *Cryphalus dilutus* Eichhoff (Coleoptera: Curculionidae, Scolytinae) and its associated fungi found on mango trees in Europe

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Abstract
Bark and ambrosia beetles (Coleoptera: Curculionidae) are receiving increasing scientific interest because of the economic losses that they can cause in invaded areas. Nonetheless, there are a number of emerging pest species, belonging to different tribes, for which invasions have likely been overlooked in the past due to their inconspicuousness, taxonomic confusion and/or misidentification. In the summer of 2022, severe infestations by a bark beetle species belonging to the tribe Cryphalini were observed on mango (*Mangifera indica* L.) in Eastern Sicily (Italy). No information about bark beetle attacks on mango trees in Europe was available previously. However, the occurrence of a cryphaline bark beetle species causing rapid common fig tree (*Ficus carica* L.) dieback was reported for the same investigated area in 2014 and 2015. We aimed to identify the bark beetle species attacking mango and common figs, and to describe the fungal pathogens vectored by this beetle species. Our results revealed that the bark beetle species attacking mango and fig trees in the studied area is *Cryphalus dilutus* Eichhoff and that different fungal pathogens, i.e., species of Botryosphaeriaceae, *Ceratocystis ficicola* and *Neocosmospora* spp., are vectored by this beetle species.

KEYWORDS
biological invasions, *Ficus carica*, invasive pest, *Mangifera indica*, Scolytinae

Cause de dépérissement rapide des figuiers et nouvelle menace pour la production de mangues : le scolyte envahissant *Cryphalus dilutus* Eichhoff (Coleoptera : Curculionidae, Scolytinae) et les champignons associés découverts sur manguiers en Europe
Les scolytes (Coleoptera : Curculionidae) suscitent de plus en plus l'intérêt scientifique en raison des pertes économiques qu'ils engendrent dans les zones envahies. Pour autant, il existe un certain nombre d'espèces émergentes de ces organismes nuisibles, appartenant à différentes tribus, dont les invasions ont probablement été négligées par le passé en raison de leur caractère discret, de confusion taxonomique et/ou d'une erreur d'identification. Au cours de l'été 2022, de fortes infestations d'une espèce de scolyte appartenant à la tribu Cryphalini ont été observées sur manguier (*Mangifera*

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indica L.) en Sicile orientale (Italie). Aucune information sur les attaques de scolytes sur manguiers en Europe n'était disponible jusqu'alors. Cependant, la présence d'une espèce de scolyte cryphale provoquant un dépérissement rapide du figuier commun (*Ficus carica* L.) a été signalée dans la même zone lors d'une étude en 2014 et 2015. Nous avons cherché à identifier l'espèce de scolyte attaquant les manguiers et les figuiers communs, et à décrire les champignons pathogènes transmis par cette espèce de coléoptère. Nos résultats ont révélé que le scolyte attaquant les manguiers et les figuiers dans la zone étudiée était *Cryphalus dilutus* Eichhoff et que différents champignons pathogènes, à savoir Botryosphaeriaceae spp., *Ceratocystis ficicola* et *Neocosmospora* spp., sont transmis par cette espèce de coléoptère.

От выяснения причины быстрого усыхания деревьев инжира к пониманию новой угрозы для производства манго: инвазивный короед *Cryphalus dilutus* Eichhoff (Coleoptera: Curculionidae, Scolytinae) и сопутствующие грибы, обнаруженные на деревьях манго в Европе

Короеды и амброзиевые жуки (Coleoptera: Curculionidae) вызывают все больший научный интерес из-за экономического ущерба, который они могут наносить в зонах инвазии. Тем не менее, существует целый ряд видов, относящихся к разным трибам этих жуков, на инвазии которых в прошлом, вероятно, не обращали внимания из-за их малозаметности, таксономической путаницы и/или неправильной идентификации. Летом 2022 г. на востоке Сицилии (Италия) на манго (*Mangifera indica* L.) было отмечено сильное заселение короедом, относящимся к трибе Cryphalini. До этого информация о заселении короедами деревьев манго в Европе отсутствовала. Однако в 2014 и 2015 гг. в той же исследуемой местности сообщалось о присутствии вида короеда-крифалина, вызывающего быстрое усыхание деревьев инжира (*Ficus carica* L.). Была поставлена задача определить вид короеда, поражающего манго и инжир, и описать грибные патогены, переносимые этим видом жуков. Наши результаты показали, что в исследуемой зоне нападает на манго и инжир короед *Cryphalus dilutus* Eichhoff; он же является вектором различных грибных патогенов, таких как виды сем. Botryosphaeriaceae, *Ceratocystis ficicola* и *Neocosmospora* spp.

1 | INTRODUCTION

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are usually considered to be an integral part of natural ecosystems and can play a valuable ecological role as rejuvenation agents and biodiversity promoters in natural forests (Kulakowski, 2016; Morris et al., 2017; Netherer & Hammerbacher, 2022; Raffa et al., 2015). Some species are also known for the massive economic and ecological damage that they can cause during outbreaks in their native range and especially after introduction in new areas. The number of invasion events caused by bark beetles and associated microorganisms in new environments has increased rapidly in recent decades (Biedermann et al., 2019; Faccoli et al., 2020; Li et al., 2022; Marincowitz et al., 2020; Schebeck et al., 2023; Tanin et al., 2021) as a consequence of climate change and the intensification of international trade (Lantschner et al., 2020; Loehle et al., 2023; Vilardo et al., 2022; Ward et al., 2023), reaching alarming levels. The situation may be even more serious than currently known due to ongoing cryptic invasions (Mas et al., 2023; Morais & Reichard, 2018).

In addition to the bark beetle taxa that have been studied the most, including that of many well-known pests, there are a number of species for which invasions have likely been overlooked in recent years due to their inconspicuousness, taxonomic confusion and/or misidentification (Johnson et al., 2017, 2020; Kirkendall et al., 2015; Li et al., 2021). Bark beetle species in the genus *Cryphalus* (formerly *Hypocryphalus*, Johnson et al., 2020) have recently received increasing interest from the scientific community because of their massive attacks, followed by a rapid decline, in cultivated trees of high economic value (Faccoli et al., 2016; Johnson et al., 2017; Masood et al., 2010; Zheng et al., 2019). The common fig (*Ficus carica* L.), and mango (*Mangifera indica* L.) are among the main fruit crops affected by some of these invasive bark beetles and the pathogenic fungi they spread (Al Adawi et al., 2013a, 2013b; Galdino et al., 2016, 2017; Pereira et al., 2021).

In Europe, the bark beetle *Cryphalus dilutus* Eichhoff, initially identified as *Hypocryphalus scabricollis*, was reported for the first time in South-Eastern Sicily (Italy) in 2014 and 2015, where it caused serious damage to common fig trees together with another non-native Scolytinae beetle, the ambrosia beetle *Xyleborus bispinatus* Eichhoff (Faccoli et al., 2016). Populations of *C. dilutus* and *X. bispinatus* are currently considered as established in

Italy and fig plants infested by these pests showed the co-occurrence of some pathogenic fungi, including *Fusarium* spp. (Di Silvestro et al., 2021). However, the current distributional range and the identity of fungal pathogens which can be vectored by these beetle species in Southern Italy remained unclear.

During the summer of 2022, a severe bark beetle infestation of mango trees was observed in a mango plantation in Eastern Sicily (Italy). This was caused by a species belonging to the tribe Cryphalini. A cryphaline bark beetle species infesting mango had previously been reported in different tropical regions and identified as *Cryphalus mangiferae* Stebbing, formerly *Hypocryphalus mangiferae* (Stebbing, 1914) (Al Adawi et al., 2013b; Galdino et al., 2016, 2017; Masood et al., 2010) so this could have been considered as a potential causal agent. However, Johnson et al. (2017) hypothesized that *C. mangiferae* is a species specialized to *Mangifera* spp. and preferentially colonizing dead or highly stressed mango trees without causing severe damage. Previous records concerning bark beetle species infesting mango trees and vectoring different plant pathogens in Oman, Pakistan, Bangladesh and Mexico also referred to *C. mangiferae* (Galdino et al., 2016, 2017). However, Johnson et al. (2017) found no evidence that *C. mangiferae* can act as a vector of serious plant pathogens, including the lethal mango fungal pathogen *Ceratocystis manginecans*. Furthermore, Johnson et al. (2017, 2020) hypothesized that the bark beetle species infesting mango trees in the above-mentioned regions and acting as a vector of serious mango pathogens is actually *C. dilutus*.

Our study aimed to identify the invasive bark beetle species infesting cultivated mango trees in Southern Italy (Sicily), as well as its associated fungal pathogens. Furthermore, we aimed to clarify the identity of the bark beetle species and associated fungi occurring on common fig trees in the same environment.

2 | MATERIALS AND METHODS

2.1 | Wood and beetle samplings

Wood samples were taken in May 2021 and in August 2022 on fig and in November 2022 on mango plants showing attacks of wood-boring insects associated with wilt symptoms. Samples were taken in a fig orchard located in Noto (Siracusa, Italy) and some ancient fig trees of a private residence in Aci Castello (Catania, Italy), as well as in a mango orchard located in Giarre (Catania, Italy) (Figure 1a,b).

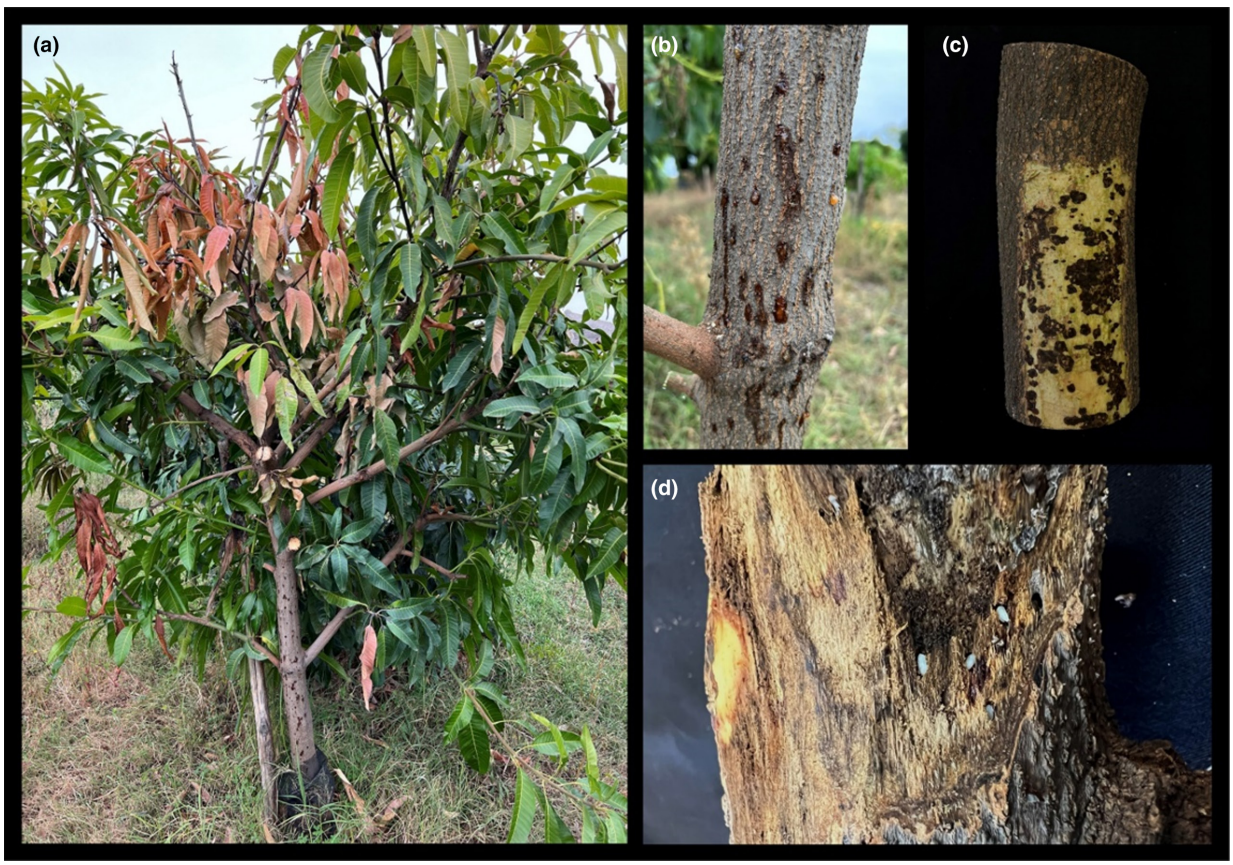

FIGURE 1 (a) Wilting of mango tree Kensington Pride grafted on Gomera 3 infested by the bark beetle *Cryphalus dilutes*. (b) Gum exudates from entry holes of *C. dilutus* on mango trunk in the field (c) underlying woody necrotic tissues associated with galleries on a mango trunk section. (d) Larvae and pupae of *C. dilutus* in galleries of a mango branch section showing wood necrosis and discoloration. Photographs taken in November 2022 by G. Polizzi.

Regarding the fig orchard, almost all the monitored trees (about 400) were found to be infested by the beetle, while only a few mango trees showed signs of infestation at the time of sampling in the mango orchard. A portion of sampled branches and trunks of five common fig and two mango trees, showing signs of bark beetle attacks and/or wood necrosis, were cut into 30 cm sections, placed inside plastic boxes and brought to the laboratory. Once in the laboratory, a group of trunk and branch sections was immediately dissected to isolate fungal species associated with the infested wood and to collect beetle larvae for species identification (Figure 1c,d). The remaining boxes were kept at 24 ± 2°C and 60 ± 10% relative humidity for 8 weeks and checked every 2–3 days for beetle emergence. Adult beetles spontaneously emerging from the sampled trunk and branch sections were collected and placed individually in sterile vials before being processed for species identification and/or fungal isolation.

2.2 | Beetle identification

2.2.1 | Morphological identification of the beetles

The morphological identification of the *Cryphalus* sp. individuals collected from infested branch and trunk sections of common fig and mango was based on the description of the most common *Cryphalus* pest species infesting these two tree species by Johnson et al. (2017). Moreover, all collected adult males (>30 for mango and >200 from common fig) were observed at 100× magnification to ascertain the possible presence of the mesofemoral spine on the proximal face of mesoferum, nearer to the apex, as the unique morphological trait specific for *C. dilutus* if compared with *C. mangiferae* and other similar *Cryphalus* spp. (Johnson et al., 2017).

2.2.2 | Molecular Identification of the beetles

We conducted molecular identification of the collected beetles by sequencing the amplified cytochrome oxidase I mtDNA (COI) and 28S rDNA (28S) as follows. The larval and adult individuals (10 from fig and five from mango for both larvae and adults) were separately crushed with a sterile pestle in a 2-mL Eppendorf tube and subjected to DNA extraction using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek, Inc.). In the DNA extraction, we also included a DNA positive control and a negative control without DNA. Universal primer pairs (Table 1) were used to amplify the COI and 28S targeted regions. PCR was carried out following the protocol according to Ricupero et al. (2021). Briefly, the reactions were performed in 20-μL volumes with 8.5 μL of FailSafeTM PCR 2X PreMix F (Lucigen Corporation), 0.5 μL of each primer 10 μM, 1.5 U of Taq DNA Polymerase 5 U (Invitrogen, Thermo Fisher Scientific) and 2 μL of DNA template. The cycling conditions were as follows: 96°C for 5 min, 35 cycles at 96°C for 45 s, 45°C for 1 min, 72°C for 1 min, followed by a final cycle at 72°C for 10 min. Reactions and cycling conditions were carried out in an Applied Biosystems™ MiniAmp™ Plus Thermal Cycler.

PCR product amplification success was checked by electrophoresis using 1% agarose gel. When DNA bands of the expected size were visualized in the gel, the remaining PCR products were shipped to a BMR Genomics (Padova, Italy) Sanger sequencing service that purified and sequenced the PCR products. The obtained coding regions were manually checked for errors, trimmed for low quality and aligned to reference sequences from the National Center for Biotechnology Information (NCBI) GenBank® through Basic Local Alignment Search Tool (BLAST) sequence analysis tool for species identification. Representative sequences of *C. dilutus* for COI and 28S were deposited in GenBank with the accession numbers OR352489 and OR352496.

2.3 | Fungal isolation from beetle individuals

The assessment of the fungal community associated with the bark beetle on mango was conducted using eight beetle individuals among those spontaneously emerged from infested woody samples. Concerning common fig, a total of 15 and 14 beetle individuals among those emerged from infested material belonging from the Noto and Aci Castello locations, respectively, were also examined. Fungal species were isolated by first grinding individual bark beetles,

TABLE 1 Primer pairs used in this study to identify the collected beetles.

Primer name	Sequence (5′–3′)	References
COI-LepF1	ATTCAACCAATCATAAAGATATTGG	Hebert et al. (2004)
COI-LepR1	TAAACTTCTGGATGTCCAAAAA	
28S_S3690F	GAGAGTTMAASAGTACGTGAAAC	Sequeira et al. (2000)
28S_A4285R	CCTGACTTCGTCCTGACCAGGC	

spontaneously emerging from the sampled wood, in a sterile phosphate-buffered saline (PBS) solution (Gugliuzzo et al., 2023). The obtained mixture was then diluted 1:100 and 200 μL of this dilution was spread on potato dextrose agar (PDA) plates. There were three plates for each tested bark beetle specimen. Plates were incubated in the dark at 25°C until the appearance of fungal colonies. After 5 days of incubation, fungal colonies were inspected and transferred on fresh streptomycin sulphate (PDAS) to make pure cultures. Macro and microscopic observations of the fungal colonies were conducted using an Olympus SZX-ILLB2-200 stereoscope (Olympus) and a Leica 020-518.500 DM/LS microscope (Leica Microsystems Holding GmbH).

2.4 | Fungal isolation from wood samples

Fungal isolations from infested wood samples were conducted as follows: small sections (from 0.2 to 0.3 cm^2) of symptomatic tissues (internal wood necrosis) close to beetle galleries were surface-sterilized for 1 min in 1.5% sodium hypochlorite, rinsed in sterile deionized water, dried on sterile absorbent paper under a laminar hood and placed on PDA (Lickson) amended with 100 mg/L of PDAS (Sigma-Aldrich) to prevent bacterial growth, and then incubated at 25°C for 3–5 days until fungal colonies were large enough to be examined. Subsequently, colonies of interest were subcultured in fresh PDAS to make pure cultures for macro- and microscopic observations. Colony characteristics, such as texture, density, obverse and reverse colour, margin and zonation, were observed usually after 7 days of incubation. Representative colonies were deposited in the fungal collection of the Department of Agriculture, Food and Environment of the University of Catania (Italy). For some fungal specimens, sporulation was induced by transferring the isolates onto Technical Agar (AT, 1.2% Agar; Biolife) with sterilized pine needles and incubating at room temperature for 14 days. Observations of fruiting bodies were made with an Olympus SZX-ILLB2-200 stereoscope (Olympus), whereas spore observations were conducted using an Olympus-BX61 fluorescence microscope (Olympus) coupled to an Olympus DP70 digital camera.

3 | RESULTS

3.1 | Morphological identification of the beetles

All individuals emerging from branch and trunk sections of both common fig and mango trees were morphologically identified as *C. dilutus* (formerly *Hypocryphalus dilutus*). No other bark beetles from the genus *Cryphalus* or other genera emerged from the infested and isolated woody material.

3.2 | Molecular identification of the beetles

The sampled individuals shared the same haplotype on COI and 28S sequence data. We confirmed the morphological identification of *C. dilutus* through both COI and 28S fragment amplification from genomic DNA, followed by direct sequencing and BLAST searches. The resulting sequences were aligned to reference sequences from NCBI compared with publicly available data on GenBank and yielded an identity score of 100% and E-value = 0.0 with *C. dilutus* (listed as *Hypocryphalus dilutus*) isolates obtained from infested common fig trees in Italy (accession number MG051153.1; Johnson et al., 2017).

3.3 | Identification of fungi associated with symptomatic samples and dispersing beetles

Fungal colonies derived from symptomatic woody samples of common fig resembled three major fungal groups. In particular, species of Botryosphaeriaceae, *Ceratocystis ficicola* (Crous et al., 2023) and *Neocosmospora* spp. were consistently yielded from fig samples. Only species of Botryosphaeriaceae and *Neocosmospora* spp. were isolated from mango. Botryosphaeriaceae were characterized, as reported by Slippers and Wingfield (2007), by a 'fluffy' mycelium, either white to creamy, greenish brown or grey to grey-black. Conidia harvested from pycnidia grown on pine needles were hyaline, nonseptate and ellipsoid, having a typical fusicoccum-like shape. *Ceratocystis ficicola* showed wiry greenish grey mycelium with black, long-necked perithecia, and *Neocosmospora* spp. had flat, felty to floccose, aerial mycelium, white becoming sulphur yellow, producing typical micro (hyaline, obovoid, short clavate to cylindrical) and macro (hyaline, falcate, wedge-shaped) conidia, whereas from bark beetles collected from common fig trees only colonies belonging to *Ceratocystis ficicola* and *Neocosmospora* spp. were identified. Colonies of species of Botryosphaeriaceae and *Neocosmospora* spp. were consistently detected from both infested woody samples and insects emerging from mango tree sections.

4 | DISCUSSION

To the best of our knowledge, we report, for the first time in Europe, attacks by the invasive bark beetle *C. dilutus* associated with wood infections by some fungal pathogens in cultivated mango trees in Sicily (Italy). *Cryphalus dilutus* is currently considered an

emerging and serious pest in some areas of the world due to its polyphagy and known ability to vector lethal mango fungal pathogens (Johnson et al., 2017, 2020). Indeed, unlike most *Cryphalus* species, which are host specific, *C. dilutus* is able to feed on plants of at least two different plant families, i.e., Anacardiaceae (including mango) and Moraceae (including common fig) (Johnson et al., 2017; Wood & Bright, 1992). Moreover, this bark beetle is considered the main vector of mango wilt pathogens, including *C. fimbriata* (Hassan & Nazami, 2017).

In this study, different fungal species were found in association with *C. dilutus* individuals or woody necrotic tissues close to beetle galleries. In particular, three groups of fungi, i.e., species of Botryosphaeriaceae, *Ceratocystis ficicola* (Crous et al., 2023), and *Neocosmospora* spp., were isolated from infested common fig woody samples. Regarding the Mediterranean area, species of Botryosphaeriaceae were reported attacking cultivated fig plants, causing canker and shoot blight (Aiello et al., 2020; Çeliker & Michailides, 2012; Güney et al., 2022). *Ceratocystis ficicola* is responsible for one the most destructive disease of fig named fig wilt disease, recently reported in Greece, causing severe wilt and canopy defoliation (Tsopelas et al., 2021). In addition, two species of *Neocosmospora*, namely *N. caricae* and *N. metavorans*, were identified to cause stem and trunk cankers in Iran (Bolboli et al., 2022). Two groups of fungi were identified from both symptomatic mango woody samples and from beetle individuals emerged from mango, namely species of Botryosphaeriaceae and *Neocosmospora* spp.

The occurrence of Botryosphaeriaceae in the present study in association with mango trees infested by *C. dilutus* should be taken into consideration since the species *Lasiodiplodia theobromae* is reported as being involved in mango sudden decline (MSD; Al Adawi et al., 2006). MSD, also called mango wilt, and probably the same disease described in Brazil as 'secas' (Ploetz, 2003; Viegas, 1960), is a complex disease caused by more than one organism, i.e., *Ceratocystis manginecans* (previously frequently reported as *C. fimbriata*, and described as a new species within *C. fimbriata* s.l.; Van Wyk et al., 2007), *L. theobromae* and the bark beetles acting as vectors (Al Adawi et al., 2006). The role of the fungal species involved in the disease is not completely clear yet, although the literature seems to refer mostly to *Ceratocystis* as the main causal agent (Al Adawi et al., 2013a; Fateh et al., 2006; Galdino et al., 2016). Commonly, *L. theobromae* is able to cause complete tree death within weeks or a few months after the infection (Saeed et al., 2017). Interestingly, in the same area surveyed in the present study, previous investigations of mango woody canker and shoot blight conducted in 2022 revealed the presence of Botryosphaeriaceae, including *Botryosphaeria dothidea*, *Lasiodiplodia theobromae* and *Neofusicoccum parvum* (Aiello et al., 2022). Although previous investigation in the same territory did not reveal the presence of *Ceratocystis* spp. as involved in canker and dieback disease, *Lasiodiplodia theobromae* was found co-infecting mango along with other Botryosphaeriaceae as well as Diaporthaceae species. As *L. theobromae* is considered worldwide one of the most serious pathogens of mango (canker and fruit rot pathogen), its presence and dangerousness must not be underestimated, especially in light of its potential association with *C. dilutus*.

The preliminary results obtained in this study are on the association of relevant phytopathogenic fungal species with *C. dilutus* on mango and common fig in Sicily (Italy). Further investigations will aim to clarify the taxonomic identity of the different associated fungi. It is undoubtedly clear, even from this first study, that some isolated fungal species represent a serious risk for both investigated crops, i.e., mango and common fig. This is also supported by our observations, revealing the common occurrence of phytopathogenic fungi on the bark beetle body, after adult emergence from galleries, which suggests that they could be easily vectored within the tissues of other host plants in the vicinity by *C. dilutus* individuals. Moreover, with the simultaneous occurrence of several fungal pathogens in both common fig and mango trees in association with increasing populations of *C. dilutus* across the Mediterranean area, the likelihood of beetles vectoring fungal disease may increase in the near future. However, the relative importance of beetle transmission of the isolated pathogenic fungi in comparison to other means of spread or insect vectors in the investigated area remains to be determined.

Overall, we provide new knowledge on the identity of the bark beetle species and associated fungi occurring on mango and common fig trees in Southern Italy. Moreover, our results suggest that future management strategies toward this pest should be targeted to both dispersing beetle individuals before they bore under the bark of susceptible plants and to the fungal pathogens they can vector to healthy trees.

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How to cite this article: Gugliuzzo, A., Gusella, G., Leonardi, G.R., Costanzo, M.B., Ricupero, M., Rassati, D. et al. (2023) From a cause of rapid fig tree dieback to a new threat to mango production: the invasive bark beetle *Cryphalus dilutus* Eichhoff (Coleoptera: Curculionidae, Scolytinae) and its associated fungi found on mango trees in Europe. *EPPO Bulletin*, 53, 663–670. Available from: https://doi.org/10.1111/epp.12956