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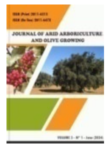

Beetles infesting pistachio (*Pistacia vera* L.) in Tunisia and their auxiliary fauna

Karama HADJ TAIEB[1,2,*], Houda GHARSALLAH[1], Ines KSENTINI[1], Andreas LECLERQUE[3,4], Haifa BEN GHARSA[3,4], Mohamed Ali TRIKI[1], Mohieddine KSANTINI[1]

[1]Laboratoire des Ressources Génétiques de l'Olivier : Caractérisation, Valorisation et Protection Phytosanitaire, Institut de l'Olivier, IRESA, Université de Sfax, 3000 Sfax, Tunisie

[2]Institut Supérieur Agronomique de Chott Meriem, Université de Sousse, 4042 Sousse, Tunisie

[3]Institute for Microbiology and Biochemistry, University of Geisenheim, D-65366 Geisenheim, Germany

[4]Laboratory of Insect-Associated Microorganisms and Microbial Control, Department of Biology, Technische Universität Darmstadt, 64287 Darmstadt, Germany

*Corresponding author: karama.hadjtaieb@gmail.com

ORCID : 0000-0001-9762-4482

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Abstract

Numerous pests, like beetles, which have expanded widely due to climate change, harm pistachio crops. Using morphological and barcoding methods, we looked into the biodiversity of pistachio beetles and their parasitoids. Seven different species of insects were found in pistachio branches. Two molecular markers, the mitochondrial cytochrome oxidase I (COI) and the region (D2-D3) of the nuclear ribosomal RNA operon's 28S rRNA gene, were used to identify these insects. Five species a similarity ≥97% were identified. For the remaining two species, sequence identities did not exceed 93% and their identification was only made at the genus level. Our findings indicated that the four following species of beetles develop on pistachio trees: *Chaetoptelius vestitus* (Mulsant & Rey, 1861) (Coleoptera, Curculionidae), *Carphoborus perrisi* (Chapuis, 1869) (Coleoptera, Curculionidae), *Phoracanthas emipunctata* (Fabricius, 1775) (Coleoptera, Cerambycidae) and *Sinoxylon* sp. (Coleoptera, Bostrichidae). Furthermore, three species were identified as parasitizing these beetles: *Doryctes leucogaster* (Nees, 1834) (Hymenoptera, Braconidae), *Cheiropachus quadrum* (Fabricius, 1787) (Hymenoptera, Pteromalidae) and *Ecphylus* sp. (Hymenoptera, Braconidae).

Keywords: Pistachio, Identification, Pests, Auxiliary fauna, Coleoptera, Hymenoptera

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1. Introduction

Pistachio world production is substantially increasing due to its medicinal and nutritional properties as well as the tree adaptation to drought conditions and salinity (Bullo et al., 2015; Dreher, 2012; Esmaeilpour et al., 2015). Despite the ability of the pistachio tree to be cultivated under adverse agricultural conditions, warming associated with climate change increases its vulnerability to the attack of xylophagous insects especially bark beetles (Hart et al., 2014). In fact, the term «bark beetle» is both a taxonomic and ecological designation. In the taxonomic sense, bark beetle species formerly formed the family of Scolytidae. Currently, the phylogenetic classification inserts them as a subfamily of Scolytinae, of the family of Curculionidae (Thompson, 1992). In the ecological sense, bark beetles are species of Scolytinae whose larvae and adults live in and consume phloem of trees and woody plants (Hulcr et al., 2015). These insects could be associated with phytopathogenic fungi aggravating their damage (Hulcr et al., 2011). Pistachio cultivations are damaged by many beetles such as *Chaetoptelius vestitus* (Mulsant & Rey, 1861) (Coleoptera, Curculionidae), *Carphoborus perrisi* (Chapuis, 1869) (Coleoptera, Curculionidae) and *Sinoxylons exdentatum* (Olivier, 1790) (Coleoptera, Bostrichoidea) (Chebouti-Meziou et al., 2009; Mourikis et al., 1998; Rassati, 2018). In Tunisia, which ranks 7^{th} worldwide producer of pistachio (FAOSTAT, 2019), pistachio cultivations were reportedly attacked by the pistachio bark beetle *C. vestitus* (Jerraya, 2003). This species is univoltine, developing one generation per year and having two distinct phases for feeding and reproduction (Balachowsky, 1949). Females lay eggs in tree-weakened branches, the hatched larvae develop on the tree bark, whereas the emerged adults feed on both buds and young twigs of damaged and healthy pistachio trees. This leads to the weakening of the tree and a decrease in its productivity (Ghrissi, 2019). The subcortical development of this pest and other wood-boring beetles complicates their management. (Marannino et al., 2008). Chemical control requires repeated applications to coincide with adults flight period. This approach is generally unsatisfactory due to its negative impact on non-target fauna, humans, and environmental ecological balance (Iyaniwura et al., 1993). The complexity of beetles' chemical control and its adverse environmental effect encourage the research for alternative management strategies, including biological control. Dry twig traps can attract pests for breeding, and destroying these traps can provide a sustainable management method (Sönmez and Mamay, 2022).

Biological control involves identifying natural enemies, like parasitoids, from the same region as the pests. These organisms can provide sustained pest control due to their capacity for natural reproduction (Rathee et al., 2018). The latter can provide long-lasting pest control due to their ability to multiply naturally in their populations (Rathee et al., 2018). There are some promising studies, conducted on rearing and releasing beneficial insects against agricultural pests that provide successful results (Mamay and Mutlu, 2019; Özgen et al., 2022). In this context, we aimed to investigate beetles infesting pistachio branches as well as their parasitoids in two pistachio orchards in

Tunisia. Through field prospections, beetles and parasitoids were collected and identified by analysis of COI and the region (D2-D3) of the 28S rRNA gene of the nuclear ribosomal RNA operon.

2. Materials and methods

2.1. Insects

Beetles and their parasitoids were collected from pistachio trees (cv. Mateur) in two pistachio orchards in Tunisia: Taous (34°55’17” N, 10°36’47” W), situated in Sfax governorate, and Ksar Ghris (34°55’55.0” N, 9°55’14.4” W), situated in Sidi Bouzid governorate. Both orchards are managed using rain-fed methods, in conventional mode, and include pistachio trees of the “Mateur” variety, with a spacing of 14m/14m. For this purpose, trap branches, 50–60 cm in length and 20–30 mm in diameter were cut in autumn, from pistachio trees (cv. Mateur) of the experimental fields. They were used to trap insects during 6 weeks. There-after, trap branches containing insects in reproduction stage were transferred to the laboratory and were put in wooden cages for insect rearing at 25°C and under natural light. Daily monitoring of the trap branches allowed us to collect the different emerging adults. These adults were stored at -20°C for molecular identification.

2.2. Molecular markers

2.2.1. Cytochrome oxidase I (COI)

A portion of DNA corresponding to the end of the 5' region of the mitochondrial gene coding for cytochrome oxidase I (COI) (658 base pairs), was chosen as a universal molecular marker. COI is a transmembrane protein involved in the Krebs cycle. It is highly conserved across species that employ oxidative phosphorylation in their metabolism. The COI region contains 11 primer positions, allowing different parts of the gene to be amplified, including LCO1490/HC02198 which allows the gene to be amplified over its entire length (Gibson et al., 2014) (Figure 1).

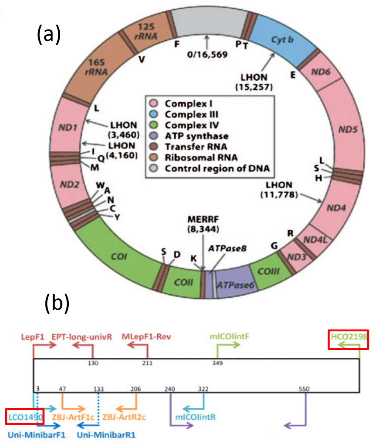


Figure 1. (a) Organization of insect barcode regions of mitochondrial DNA (Jalali *et al*., 2015); (b) map of primers’ positions on the COI (Brandon-Mong *et al*., 2015).

2.2.2. 28S rRNAgene

The nuclear 28S rRNA gene includes a mixture of conserved and divergent regions. The divergent regions of the 28S gene are called “D” regions and are numbered in the 5'→3' direction of the rRNA (Redmond et al., 2011) (Figure 2). Segments D2 and D3 are generally chosen in phylogenetic studies because of their suitability for primer design and there are many informative sites there. As a result, the D2-D3 region is increasingly used in phylogenetic analyses (Sonnenberg et al., 2007).

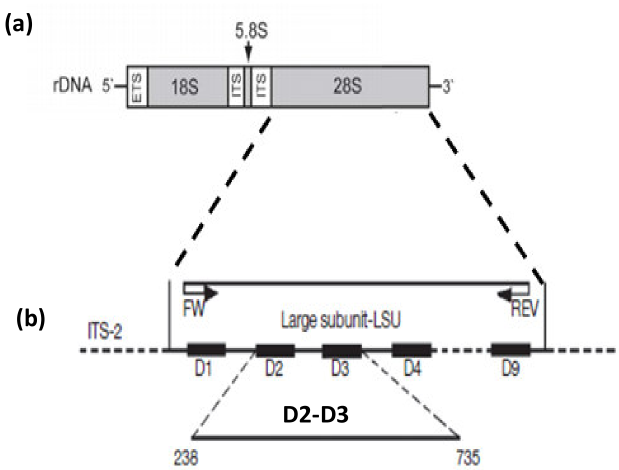


Figure 2. (a) Organisation of the nuclear ribosomal RNA: genes: 18S, 5.8S and 28S, Spacers separate these genes, namely the external transcribed spacer (ETS) and the internal transcribed spacers (ITS 1 and ITS 2) (Muirand Schlötterer, 1999); (b) Organization of 28S barcode region (Gou et al., 2013)

2.3. Molecular identification of insects

The DNA of seven insects was extracted with the Qiagen Blood and Tissue Mini Kit (Qiagen® GmbH, Hilden, Germany), following the manufacturer's protocol. The mitochondrial cytochrome oxidase I was amplified using the primer pair LCO 1490/ HCO 2198. The D2-D3 region of the 28S rRNA gene of the nuclear ribosomal RNA was amplified with two PCR primers D2F1/ D3R2 and 3665/4048 (Table 1).

Table 1: PCR primers, amplicon size (bp) and annealing temperatures used for the amplification of gene sequences.

Molecular marker	Primer	Primer sequence	Amplicon size (bp)	Annealing Temp. (°C)	Reference
COI	LCO 1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	630-650	50	Hebert *et al.* (2003)
	HCO 2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'			
28S	D2F1	5'-ACTGTTGGCGACGATGTTCT-3'	500-570	50 or 55	Jordal*et al.* (2008)
	D3R2	5'-TCTTCGCCCCTATACCC-3'	500-570		
	3665	5'-AGACAGAGTTCAAGAGTACGTG-3'	600-750		
	4048	5'-TTGCTCCGTGTTTCAAGACGGG-3'	600-750		

PCR was performed in a 50 µL volume reaction mixture with 49 µL master mix containing 42.25 µL of H_2O, 5 µL of Taq PCR Buffer (10X), 1 µL of dNTPs mix (200µM), 0.25 µL of each primer (0.4 µM), 0.25 µL of Taq Polymerase (1.25 units/ 50 µl PCR) (New England Biolabs®, Germany) and 1 µL DNA template. PCR amplification was carried out using a Master cycler device (Eppendor f®, Germany) under the following conditions: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 50-55°C for 45 seconds and elongation at 68ºC for 2 minutes, with a final extension at 68ºC for 5 minutes. Each set of reactions included negative (no DNA template) and positive (insect DNA) controls. To successfully amplify the 28S molecular marker, PCR was repeated at the annealing step under 55°C temperature for both primer pairs D2F1/D3R2 and 3665/4048, as their cited annealing temperatures are 50 or 55 (Jordal et al., 2008). Moreover, the PCR was repeated with three dilutions of DNA (1:2; 1:4; 1:10) for the three used primer pairs (LCO1490/HCO2198, D2F1/D3R2 and 3665/4048) to amplify the target fragments. PCR products obtained were controlled by agarose gel electrophoresis on 1% agarose Tris-acetate-EDTA gel, stained with Roti®-GelStain(Carl Roth® GmbH, Karlsruhe, Germany). PCR product sizes were estimated by comparison with an NEB 2-Log DNA Ladder size standard (New England). PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen® GmbH, Hilden, Germany) following the manufacturer's instructions and were sequenced in both directions by StarSEQ® GmbH (Mainz, Germany) using the primers listed above. The program Molecular Evolutionary Genetic Analysis software, ver. 7.0 (MEGA7.0; https://www.megasoftware.net/) was used to assemble obtained sequences from raw sequence data. The resulting consensus sequences were aligned and compared with those available in the Gen Bank database from the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST), using the BLAST® server (Altschul et al., 1997; Zhang and Madden, 1997).

3. Results and Discussion

3.1. Pistachio Beetles and their auxiliary fauna

This study is built on our research in pistachio orchards, where we discovered that this crop has the potential to valorize area with low rainfall and poor soils (Figure 4). However, dry conditions and excessive warm increased pistachio trees stress and consequently, their vulnerability to beetle attacks (Figure 3).

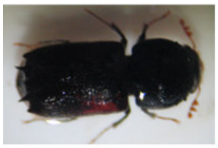
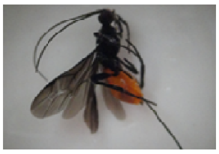
Figure 3. Fructification of pistachio trees under conditions causing the drying out of olive trees (Site: Menzel bouzayen, Sidi Bouzid) (Ksantini, 2012)

Field investigations showed that pistachio branches were attacked by different beetles (Figure 4). Four beetle species and three species of auxiliary fauna were found in both studied sites. These insects were identified based on their morphological characteristics, referring to previous descriptions (Table 2).

Figure 4. Beetles infesting *Pistacia vera*: Attacked branches presented roughened and cracked bark; heavily infested branches showed growth stopping.

Table 2. Morphological identification of beetles infesting pistachio branches and their auxiliary fauna

Code	Identification (Reference)	
A	*Chaetoptelius vestitus* (Coleoptera, Curculionidae) Balachowsky (1949) and Fragoulis (2006)	
b	*Carphoborus perrisi* (Coleoptera, Curculionidae) Yanik and Yücel (2001)	

c	*Phoracantha* sp. (Coleoptera, Cerambycidae) Mesbah et al. (2010)	
d	*Sinoxylon* sp. (Coleoptera, Bostrichidae). Liu and Beaver (2018) ; Zhang et al. (2022) ; Lesne (1906)	
e	*Doryctes* sp. (Hymenoptera: Braconidae) Farahani et al. (2014)	
f	*Cheiropachus quadrum* (Hymenoptera, Pteromalidae) Zeiri et al., (2013)	
g	*Ecphylus* sp. (Hymenoptera, Braconidae) Gebiola et al. (2015)	

3.2. *Chaetoptelius vestitus* development

The predominance of the pistachio bark beetle *C. vestitus* (Coleoptera, Curculionidae) was noted in both study sites. In fact, during the autumn and winter seasons, the presence of insects was noted by the abundance of adult penetration and emergence holes in the branches, as a consequence of the insect reproduction. During the spring and summer, period of insect feeding, attacks were observed on young twigs. Following these attacks, the young twigs become desiccated and budless resulting in a decrease of the infested pistachio tree productivity. Thus, the insect exacerbates the tree's deterioration during its reproductive stage (Figure 5). The adult of *C. vestitus* was present in pistachio orchards throughout the year. In fact, adults colonized the dry twig traps from October to February, in both study sites. This period corresponds to the reproduction of the insect. Between March and September, a period matching with the feeding phase of the insect, the adult was present in the young twigs.

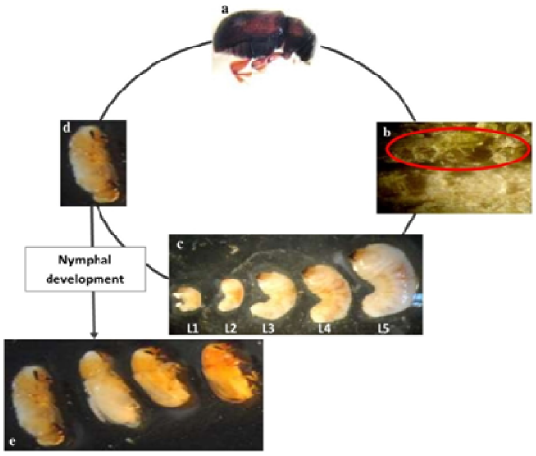


Figure 5. Life cycle of *Chaetoptelius vestitus*. **(a)** Adult; (**b)** Eggs; **(c)** Larvae; (**d,e)** Nymph

3.3. Identification of insects

Seven insect species emerging from the rearing of beetles in pistachio trap branches were subject to molecular identification. DNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific), with values ranging from 50 to 200 ng/µL for the extracted DNA. A 1:10 dilution was then performed, yielding a final working concentration of 5 to 20 ng/µL, which was used as the template in PCR reactions. The PCR carried out using DNA dilutions 1:10 at the annealing temperature of 50°C allowed the best amplification for the three used primer pairs (LCO1490/HCO2198; D2F1/D3R2 and 3665/4048). Consequently, we retained PCR reactions performed in these conditions. Twenty-one amplicons have been recovered. The optimized PCR allowed the amplification of 16 out of 21 fragments. Among them, 13 sequences were highly interpretable (Table 3).

Table 3. Amplification and sequencing of both used molecular markers (with a volume of 1 µl of DNA diluted 1/10 at a hybridization temperature of 50°C)

Molecular Marker	COI		28S			
Primers	LCO-HCO		D2-D3		3665-4048	
Samples	Amplification	Sequencing	Amplification	Sequencing	Amplification	Sequencing
A						
B						
C						
D						
E						
F						
G						

Presence of amplicon and sequence of good-quality
Presence of amplicon and sequence of poor quality
Absence of amplicon and sequence

The alignment of the obtained sequences with those of the GenBank database allowed the identification of five out of the seven species with an identity value>= 97%. For remaining species, identification was assigned to the genus level since their identity values were between 84 and 93%. DNA barcoding analysis confirmed the molecular identity of *C. vestitus*, *C. perrisi*and *C. quadrum* and allowed the identification of *P.semipunctata* (Coleoptera, Cerambycidae), *D. leucogaster*, *Sinoxylon* sp. and *Ecphylus* sp. (Table 4).

Table 4. Molecular identification of pistachio beetles and their parasitoids obtained from sequencing of COI and 28S rRNA gene

Code	Gene and primers	Closest match	Identity (%)	[1]Size
a	28S (D2F1-D3R2)	*Chaetoptelius vestitus*	100	200
	COI		97.31	483
b	28S (D2F1-D3R2)	*Carphoborus perrisi*	100	319
	28S (3665-4048)		99.22	383
	COI		98.90	368
c	28S (D2F1-D3R2)	*Phoracanthas emipunctata*	99.35	310
d	28S (3665-4048)	*Sinoxylon* sp.	93	400
	COI		84.35	377
e	28S (3665-4048)	*Doryctes leucogaster*	99.50	402
f	28S (D2F1-D3R2)	*Cheiropachus quadrum*	100	290
	28S (3665-4048)		99	410
g	28S (3665-4048)	*Ecphylus* sp.	93.45	351
	COI		91.82	550

COI, cytochrome oxidase I
[1]Size the length of base pairs compared

3.4. Discussion

This study aims the identification of beetles of pistachio branches and their parasitoids in Tunisia. For this purpose, two molecular markers (COI and 28S), considered as some of the most widely used markers for the phylogenetic identification of insects (Hulcr et al., 2015) were used. To overcome PCR inhibition, DNA was diluated. This technique is known to optimize PCR (Piterina et al., 2010).We opted for the molecular identification even though that the classification of insects is based on their morphology. Indeed, morphological identification could be subjective. This is due to the experience and opinion of the taxonomist, as well as their assessment of distinguishing morphological characteristics (Chant and McMurtry, 1994). *C. vestitus* was reported in Tunisia since 2003 (Jerraya, 2003) and has recently been the subject of several research works (Ghrissi et al., 2019; Hadj Taieb et al., 2019, 2020). Over the cited damages, we have reported in a previous study (Hadj Taieb et al., 2019) the association of *C. vestitus* with phytopathogenic fungi, including trees dieback agents. In this study, we recorded the presence of another bark beetle (C. perrisi) on pistachio branches. Several pistachio-producing countries, including Iran (Mehrnejad, 2001), Turkey (Yanik and Yücel 2001) and Italy (Rassati, 2018) have reported cases of this pest attacking weakened trees and causes the browning of their leaves and fruits. Climate change may have contributed to its expansion in Tunisia. In fact, because of the positive effects of global warming on their life cycle, bark beetle populations have been growing recently (Bentz and Jonsson, 2015). Indeed, Tunisia is home to some of the world's warmest pistachio orchards (Elloumi et al., 2013). Concerning P. semipunctata, it was accidentally introduced in Tunisia in 1962. Although larval development of the insect can exclusively take place in Eucalyptus species and those closely related (Mifsud, 2002), adults could attack several host trees, including pistachio (Hanks et al., 1995). The identification of *Sinoxylon*sp. In this study was restricted to the genus level, which includes 52 species with a wide range of hosts (Filho et al., 2006). Adults and larvae of these species damage weaker trees by deeply penetrating their stems and twigs (Nair 2007). In this study, three species of parasitoids (*D.leucogaster*, *C. quadrum* and *Ecphylus* sp.) were identified. These species are known as beetle parasitoids (Belokobylskij, 2009; Farahani et al., 2014; Khanday et al., 2019). In light of the potential biological control of beetles attacking pistachio trees, the parasitism potential of the identified parasitoids will be further examined.

Conclusion

The study highlights the impact of climate change on pistachio crops, specifically by increasing the prevalence of beetle pests. Through detailed morphological and genetic analysis using two molecular markers, we identified seven species of insects present in pistachio branches, including four beetle species and three parasitoid species that could potentially control these pests. While five of these species were accurately identified, two could only be classified at the genus level. This research emphasizes the need to understand pest and parasitoid biodiversity to improve pest management strategies for pistachio crops, particularly as climate change continues influencing pest populations.

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