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Maria Inês Barros Matos

Mites and fungi associated with *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae): first report in northern Portugal

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Olga Ameixa, Investigadora Auxiliar (doutorada nível 1), do Departamento de Biologia da Universidade de Aveiro, da Doutora Maria João Santos, professora Associada do Departamento de Biologia da Universidade do Porto e Doutor Camilo Ayra-Pardo do CIIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental) da Universidade do Porto.

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À minha avó Celeste.

o júri

presidente

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palavras-chave

Rhynchophorus ferrugineus; palmeira; ácaros; fungos; inseto invasor; forésia; patogénio; interações hospedeiro-patogénio.

resumo

O escaravelho vermelho das palmeiras *Rhynchophorus ferrugineus* (RPW) é uma espécie invasora nativa do sudoeste asiático. É considerado uma peste significativa das palmeiras da família Arecaceae, principalmente da palmeira das canárias *Phoenix canariensis*. O seu efeito é considerável, tendo impactos económicos na produção agrícola e em meios urbanos por despesas de manutenção da peste e de plantas afetadas. Foram descritas várias espécies de ácaros, maioritariamente como foréticas, e fungos, saprófitos oportunistas, associadas a este escaravelho. Esta diversidade de ácaros ainda não foi registada em Portugal, bem como a natureza da interação destes com o hospedeiro. O objetivo deste trabalho é fazer um reconhecimento de populações deste escaravelho presentes na região norte de Portugal, identificar os ácaros e fungos associados, de forma a explorar a natureza das suas interações com o escaravelho, e finalmente discutir o seu potencial como agentes de biocontrolo.

Colocamos armadilhas nos distritos de Aveiro, Porto, Viana do Castelo e Braga entre os meses de julho 2021 e janeiro 2022. Os escaravelhos foram dissecados e as partes do corpo inspecionadas para observação de ácaros ou fungos. Foram anotados o comprimento do corpo, peso, sexo e foi fotografado o padrão torácico. Extrações de DNA de ácaros e fungos foram feitas para identificação molecular, para complementar a identificação morfológica.

A atividade de escaravelhos adultos nesta região foi praticamente constante em todo o período amostrado. Foram identificadas 2 espécies, 4 géneros e uma ordem de ácaros, todos já previamente associadas ao RPW. Verificou-se um pico de amostragem de escaravelhos e de número de ácaros nos meses mais quentes. A diversidade de espécies de ácaros, o número de ácaros por hospedeiro e a abundância de ácaros foram elevadas, indicando uma boa adaptação destas espécies aos habitats amostrados. A zona dos élitros do escaravelho foi a mais ocupada, apontando para a possibilidade de afetar a capacidade de voo do hospedeiro, sugerindo uma relação de parasitismo, em especial quando o número de ácaros for elevado.

Foram identificadas cinco espécies de fungos em que uma, *Scopulariopsis* sp., é pela primeira vez associada ao RPW. Os fungos identificados apresentam potencial para serem patogénicos para humanos e plantas agrícolas, o que sugere uma necessidade de controlo do RPW que se estende além da necessidade de preservação das palmeiras.

Este trabalho é a primeira documentação de espécies de ácaros associadas ao RPW em Portugal, é o primeiro que explora as relações interespecíficas entre ácaros e escaravelho nestas populações, e revela uma presença significativa desta peste, e uma necessidade acrescida do seu controlo. Os ácaros identificados podem ter potencial como agentes de biocontrolo, capacidade essa que deverá ser explorada e confirmada em trabalhos futuros.

keywords

Rhynchophorus ferrugineus; palm-tree; mite; fungi; invasive insect; phoresis; pathogen; host-pathogen interactions.

abstract

The red palm weevil *Rhynchophorus ferrugineus* (RPW) is an invasive species native to southeast Asia. This weevil is a pest of the Arecaceae palm tree family and is mainly considered the pest of *Phoenix canariensis*. It has a significant economic and ecological impact on agricultural production and urban environments due to the costs of pest and damaged plant management. Various mite species have been described, mainly as being phoretic, and fungi, mainly as opportunistic saprophytes, in association with the RPW. Mite diversity of these populations has not yet been described in Portugal, as well as the nature of their interaction with the host. The aim of this work is to describe populations of RPW in northern Portugal, to identify associated mites and fungi in order to explore the nature of their interactions with the RPW and to, finally, discuss their potential as biocontrol agents.

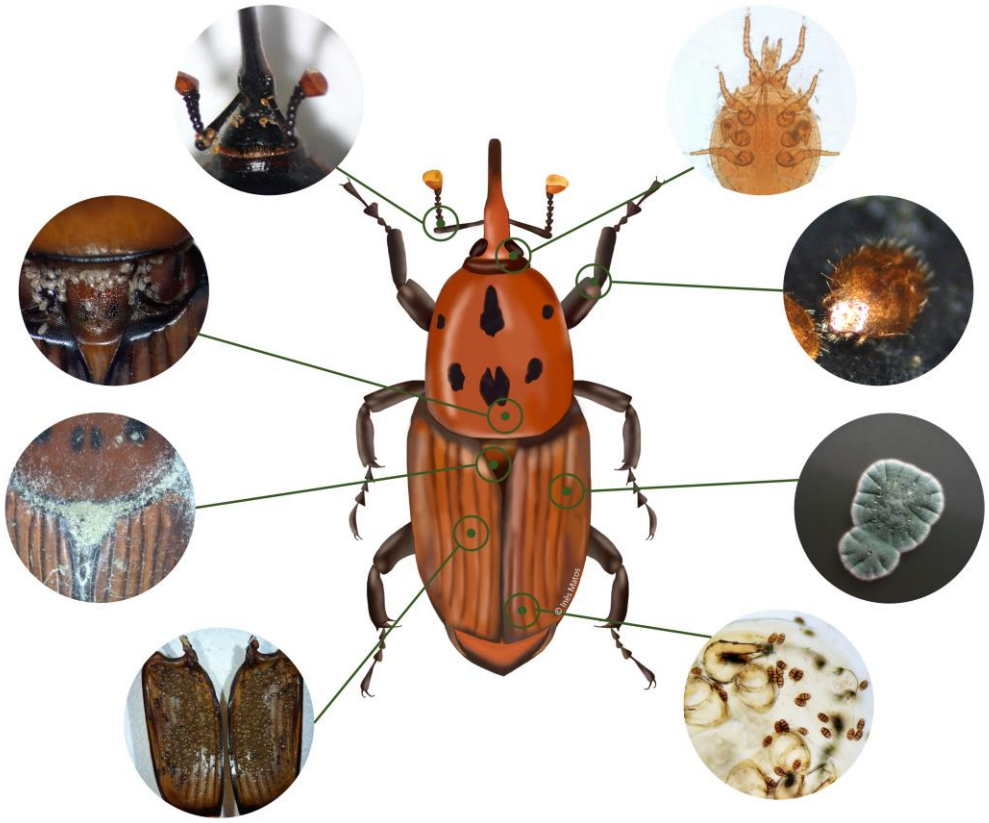
We placed traps in the districts of Aveiro, Porto, Viana do Castelo and Braga from July 2021 to January 2022. Weevils were dissected and each body part inspected for mites or fungi. Body length, body weight and sex were noted, and thoracic patterns photographed. DNA extractions of mite and fungal samples were made in order to complement morphological identification.

Activity of adult RPW in this region was essentially constant during the sampling period. We identified 2 species, 4 genera and one order of mites, all previously described in association with the RPW. The number of weevils collected in traps and the mite load per weevil was higher during the warmer months. Mite species diversity, mite load per host and mite species abundance were high, indicating a good adaptation of these species to studied habitats. The weevil's elytral area was associated with the highest mite load, suggesting the possibility of it hindering flight capacity, indicating a parasitic interaction with the host, in particular with high mite load.

Five species of fungi were identified, where one, *Scopulariopsis* sp., is for the first time described in the RPW. Identified fungi have pathogenic potential for humans and agricultural crops, indicating the need for RPW control that goes beyond the need for palm tree preservation.

This work provides the first description of mites associated with the RPW in Portugal, revealing a significant presence of this pest, and is the first exploring the interspecific relationships between mites and the RPW in these populations. It reveals a further need for its control. Identified mites could have potential as biocontrol agents, a capacity that should be explored and confirmed in further studies.

graphical abstract



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RPW	*Rhynchophorus ferrugineus* Olivier 1790
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
BLAST	Basic Local Alignment Search Tool
PDA	Potato dextrose agar
PCR	Polymerase chain reaction
UAE	United Arab Emirates
TBE	Tris-Borate-Acetate (TBE)
bp	Base pairs
ITS	Internal transcribed spacer
COI	Cytochrome c oxidase subunit I
18S	18S ribosomal RNA
EPPO	European and Mediterranean Plant Protection Organization
DGAV	Direção Geral de Alimentação e Veterinária
USA	United States of America

Communications and future papers based on this dissertation

In the context of this dissertation two oral presentations and four posters have been submitted and presented, two papers have been drafted and are currently being prepared to be submitted soon and 20 DNA sequences of identified mites and fungi have been submitted and published in the GenBank database.

One oral presentation and one poster, the presentation awarded with *distintion* for best oral communication in Biological Sciences (Matos *et al*., 2022) (diploma in **Annex A2**), were done in May 2022 at the "Young Researchers Meeting of U. Porto, IJUP 2022".

- **Inês Matos**, Diogo Silva, João Oliveira, Nuno Pereira, Cláudia Gonçalves, Rita Alves, Luís Rangel, Maria João Santos, Camilo Ayra-Pardo. Phoretic and parasitic organisms associated with *Rhynchophorus ferrugineus* Olivier 1790 in Northern Portugal. Oral Communication. Young Researchers Meeting of U. Porto, IJUP 2022. Porto (**Annex A1**);
- Diogo Silva, **Inês Matos**, Maria João Santos, Camilo Ayra-Pardo. World distribution of mites associated with the Red Palm Weevil (*Rhynchophorus ferrugineus Olivier,* 1790). Poster Communication. Young Researchers Meeting of U. Porto, IJUP 2022. Porto (**Annex A3**);

Three posters have been accepted and will be presented in august 2022 at "ICOPA 2022: 15th International Congress of Parasitology in Copenhagen":

- **Inês Matos**, Diogo Silva, João Oliveira, Nuno Pereira, Claúdia Gonçalves, Rita Alves, Luis Rangel, Maria João Santos, Camilo Ayra-Pardo. Site distribution analysis of mites from *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionoidea): first report in Portugal. Poster Communication. 15th International Congress of Parasitology (ICOPA 22), Copenhagen, Denmark (**Annex A4**);
- **Inês Matos**, Diogo Silva, Luis Rangel, Maria João Santos, Camilo Ayra-Pardo. Identification of pathogenic fungi associated with the Red Palm Weevil-mite complex in Portugal. Poster Communication. 15th International Congress of Parasitology (ICOPA 22), Copenhagen, Denmark (**Annex A5**);
- Diogo Silva, **Inês Matos**, Maria João Santos, Camilo Ayra-Pardo. World distribution of mites associated with *Rhynchophorus ferrugineus* Olivier, 1790 (Red Palm Weevil).

Poster Communication. 15th International Congress of Parasitology (ICOPA 22), Copenhagen, Denmark (**Annex A6**).

One oral presentation has been accepted and will be presented in September 2022 at "17th Yes Meeting Scientific Competition":

- João Oliveira, **Inês Matos**, Diogo Silva, Luis Rangel, Maria João Santos, Camilo Ayra-Pardo. Distribution of mites on *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionidae) in Northern Portugal and its consequences on public health. 17th Yes Meeting Scientific Competition, Porto, Portugal (**Annex A7**).

Paper titled "Site distribution analysis of mites from *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionoidea): first report in Portugal." will be submitted soon to *International Journal of Acarology*.

Paper titled "Fungi associated with *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionoidea) in Northern Portugal" will be submitted soon to *Mycologia.*

20 DNA sequences of fungi and mites have been published in the GenBank database with accession codes (**Annex B2**).

1. Introduction

1.1. Red palm weevil (*Rhynchophorus ferrugineus*)

1.1.1. Hosts

Rhynchophorus ferrugineus Olivier 1790 (Coleoptera: Curculionidae), also known as the Red Palm Weevil (RPW) has been reported to compromise 26 species of palms across 16 genera worldwide. It has been described on almost all the Arecaceae family (Dembilio & Jaques, 2015). It was first known as a pest of the coconut tree *Cocos nucifera* L. in Asia and of the date palm tree *Phoenix dactylifera* L. in the Middle East (Fiaboe *et al.*, 2012). It has also been reported on the giant bird of paradise *Strelitzia nicolai* Regel and K.Koch (Fiorello *et al.*, 2015), sugarcane *Saccharum officinarum* L. (Prabhu & Patil, 2009) and century plant *Agave americana* L. although the two latter were only described in an experimental setting and not in field observations. Although it was initially reported as a *C. nucifera* pest, it is now mainly documented as a pest of *Phoenix canariensis* Chabaud.

1.1.2. Life cycle

The RPW has a life cycle comprised of 4 stages: egg, larva, pupa, and adult (**Figure 1**). After flying, feeding and mating, females lay small (2.6 mm wide) white eggs in carved holes on the host plant's steam and petiole at any time during the day or night (Dembilio & Jaques, 2015). After deposition, egg-laying chambers are closed with a protective secretion (Murphy & Briscoe, 1999). A single female lays an average of 4 eggs per day, and it can lay up to 24 eggs per day (Ince *et al.*, 2011). Hatching occurs after three to several days depending on temperature and humidity conditions (Murphy & Briscoe, 1999). Larvae have a legless 50 mm long body with 13 segments and a brown head and may take 24 to 128 days to develop into pupae depending on temperature, humidity and diet (Salama *et al.*, 2009). Larvae cause the most significant damage to the host plant as they feed on soft tissues, boring through the apical meristems and then, in more mature stages, in the peripheral area of the trunk (Dembilio & Jaques, 2015). Pre-pupal stage lasts for 3 days and then converts into pupae inside the cocoon (Murphy & Briscoe, 1999). The pupae are initially cream white and develop into brown over a period of 11 to 45 days before emerging as adults. Adults are black and rusty red with a long-curved nostrum that allows for excavation. They have a pair of wings that allows them to fly, covering distances of up to 5 000 m, and quickly disperse

to neighbouring host plants (Ávalos *et al.*, 2014). Male weevils, in contrast to females, have hairs on the legs and on the tip of the nostrum (Dembilio & Jaques, 2015). The total life cycle takes 4 to 5 months to be completed (Ince *et al.*, 2011).

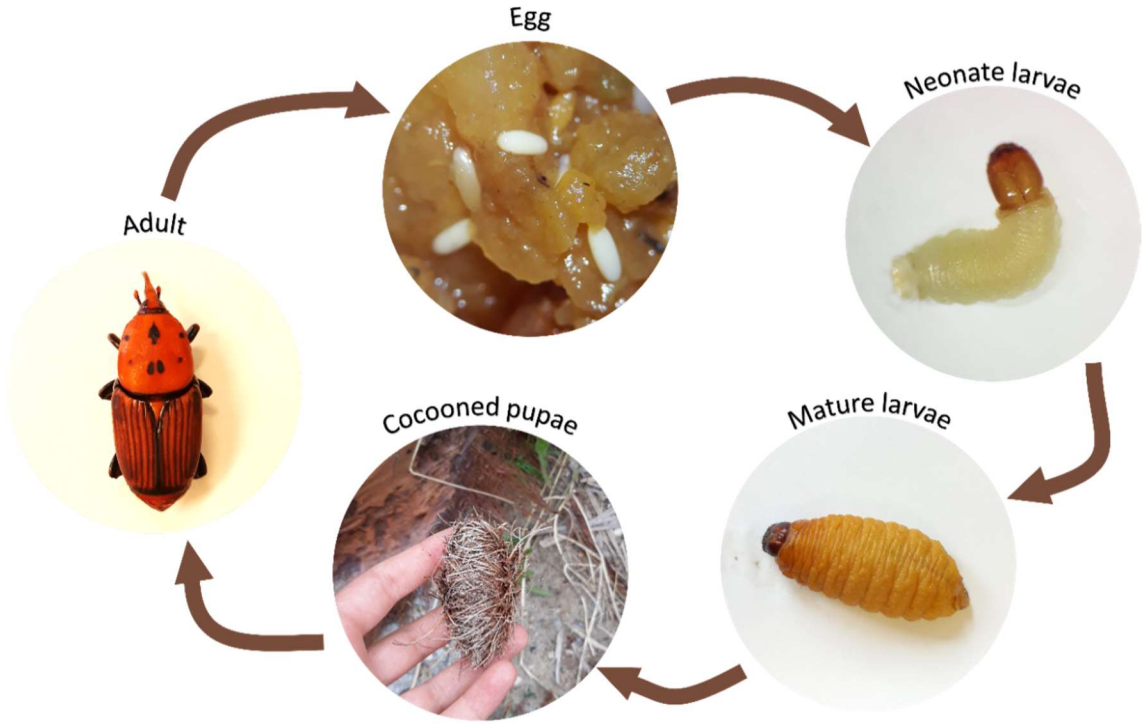

Figure 1. Life cycle of *Rhynchophorus ferrugineus*.

1.1.3. History of introduction

Despite its flight ability, the RPW, originating in Southern Asia, was spread anthropogenically through the trade of infested palm trees and offshoots. It reached Saudi Arabia, United Arab Emirates, Oman and Egypt by 1992 having been first detected in Europe, in Spain, by 1994 (Ferry & Gomez, 2002), and in Portugal, in the region of Algarve, by 2007 (Boavida & Franca, 2008). It has been found in all Mediterranean countries and in the Maghreb region with the exception of Algeria (Ferry, 2019), and is now present on all continents, except Antarctica (Dembilio & Jaques, 2015). The import of palms is mainly done in order to collect seedlings for date production and for ornamental reasons (Ferry, 2019).

The impact of this pest in the middle east and northern African countries is of significant economic impact considering that many countries in these regions profit from date production (Ferry, 2019). The damage is otherwise ornamental. However, ornamental damage also leads to economic impact considering the cost of pest management and potential future expenses with hosts switching to economically relevant hosts (Ferry, 2019).

1.2. Canary Island date palm (*Phoenix canariensis*)

Phoenix canariensis is a palm tree species indigenous to the Canary Islands where it can be found in fertile volcanic soils. Its extensive root system allows it to reach subterranean water at long distances. This trait combined with its ability to resist temporary swamping, allows it to live in various types of soils (Morici, 1998).

The palm tree only has visible symptoms of infection by RPW once considerable damage has been done. Symptoms and signs of infection include yellowing, withering, and thinning of leaves, crunching noise from inside the palm tree and slowing down of new leaf growth (EPPO, 2007). Once the symptoms are easily detectible, the damage is most likely to have been too extensive to be reversible.

Many specimens of *P. canariensis* have been affected by the RPW in Portugal, namely in the region of Porto and Aveiro (**Figure 2**) (Fernandes, 2016).

Figure 2. Damaged *Phoenix canariensis* palm trees in Porto, in front of the University headquarters (A) and in Avenida do Brasil (B), and in Aveiro in Rua da Pega in the vicinity of the University campus (C). Image C retrieved from Google maps.

1.3. Control methods

Due to the difficulty in timely detection of the damage caused by *Rhynchophorus* species during the early stages of infestation (particularly because of the cryptic habits of the larvae), integrated pest management is mainly considered with an attempt to combine public awareness, precautionary measures and efficiency of control methods (Mazza *et al.*, 2014). Since chemical applications elicit serious concerns related to environmental pollution and insect resistance, and affect human health, eco-friendly biological control has now attracted high interests (Lahlali *et al.*, 2022). Biological control (or biocontrol) is a broad term including a variety of management actions based on the use of natural enemies of the invader.

1.3.1. Natural enemies

An array of natural enemies of RPW have been described, including viruses, bacteria, fungi, yeasts, nematodes, mites, insects and vertebrates (Mazza *et al.*, 2014).

There is a vast array of viruses that have been observed to be entomopathogenic. However, only the cytoplasmatic polyhedrosis virus (family Reoviridae) has been found in association with the RPW in India and Egypt (El-Minshawya *et al.*, 2005; Gopinadhan *et al.*, 1990). This virus has been found to lead to deformed adults and reduced populations when there is infection at the larval stage (Gopinadhan *et al.*, 1990).

Bacteria from the families Bacillaceae, Pseudomonadaceae, Enterobacteriaceae, Streptococcaceae and Micrococaceae have been documented to reveal entomopathogenic potential (Tanada & Kaya, 2012). However, only the genera *Bacillus* and *Serratia* have been registered for the control of insects (Tanada & Kaya, 2012). Both these genera have been found in association with adult and larvae of RPW (Alfazairy, 2004; Alfazairy *et al.*, 2003; Dangar & Banerjee, 1993). *Bacillus thuringiensis*, *B. sphaericus*, *B. megaterium*, *B. laterosporus* and *Pseudomonas aeruginosa* have been observed to increase RPW larval mortality either through force-feeding or direct inoculation (Alfazairy, 2004; Banerjee & Dangar, 1995; Salama *et al.*, 2004). However, *B. thuringiensis*, *B. sphaericus* and *B. megaterium* revealed no pathogenicity to RPW eggs (Francesca *et al.*, 2009), which indicates that larvae are a more promising target for biocontrol with bacteria.

Fungi can infect the insect host by direct contact with fungi or by vertical (through progeny) or horizontal (through direct contact with infected hosts) transmission. *Beauvaria*

bassiana Vuill and *Metarhizium anisopliae* (Metschn.) Sorokīn are two of the most widely studied entomopathogenic fungi. *B. bassiana* has been isolated from the RPW (El-Sufty *et al.*, 2009) and has revealed promising potential as a biocontrol agent given that it not only has increased mortality of directly infected adults, but also of their progeny (Dembilio *et al.*, 2010b; Llácer *et al.*, 2013) and is spontaneously disseminated by adult weevils (Besse *et al.*, 2011; El-Sufty *et al.*, 2011). *Metarhizium anisopliae* has also shown to be effective against both RPW larvae and adults (Francardi *et al.*, 2012). Besides the latter mentioned, strains of *Aspergillus* sp. P. Micheli, *Fusarium* sp. Link, *Penicillium* sp. Link, *Trichothecium* sp. Link, *Thrichoderma* sp. Link, *Alternaria* sp. Nees among other agents have been documented in association with the RPW, amounting to a diversity of over 40 fungal species (Ramos *et al.*, 2015; Tarasco *et al.*, 2008).

Nematodes have revealed entomopathogenic activity in particular in the Heterorhabditidae and Steinernematidae families that are not phylogenetically close but share similar life histories (Mazza *et al.*, 2014). These families carry pathogenic bacteria that are released into the host's hemocoel after infection. *Steinernema* sp. Travassos nematodes have revealed promising potential as biocontrol agents against the RPW (Dembilio *et al.*, 2010a; El Sadawy *et al.*, 2020; Nurashikin-Khairuddin *et al.*, 2022) and are currently the more widespread control method in Portugal in combination with chemical insecticides (DGAV, 2013).

Mites are employed as a method of control, with varied species being sold commercially for, for example, pest control in agricultural crops (Mazza *et al.*, 2014; Orr & Suh, 1999). A vast diversity of mites have been found in association with the RPW in various countries, namely India, Malaysia, Indonesia, Papua New Guinea, Thailand, the Philippines, China, Egypt, Saudi Arabia, United Arab Emirates (UAE), Turkey, Brazil, Ecuador, Malta, Italy, and Spain (Abolafia & Ruiz-Cuenca, 2020; Dilipkumar *et al.*, 2015). Some parasitic mites have been associated with the RPW namely *Rhynchopolipus swiftae* Husband and Oconnor and *R. rhynchophori* Ewing, the latter being found to be effective in decreasing RPW populations at various development stages due to the fact that it predates on the host's body fluid (Abdullah, 2009). The mite life cycle comprises egg, larva, protonymph, deutonymph, tritonymph, and adult (Baker & Wharton, 1952). Most mites associated with the RPW are in the deutonymphal life form that is mainly described as phoretic, i.e., attaching to the host with the sole purpose of transportation (Mazza *et al.*, 2014).

Predating and parasitoid insects have a long history of use for pest management. However, recent increase in more stringent regulation over importation, collection and distribution of species and the awareness of the vastness of non-target species has limited the use of these as the primary control method (De Clercq *et al.*, 2011). *Chelisosches morio* Fabricius has been reported as a predator of RPW in coconut plantations in India (Abraham & Kurian, 1973), *Euborellia annulipes* Lucas of RPW eggs in *in vitro* conditions (Mazza *et al.*, 2014), and *Sarcophaga fuscicauda* Bottcher as an attacker of RPW adults in South India (Iyer, 1940). However, no large-scale studies on the impact of the introduction of these species as a biocontrol agent have been done.

1.3.2. Control methods in Portugal

The control of this pest in Portugal focuses either on chemical or biocontrol in case the damage has not yet been considerable. Chemical control consists of the application of phytosanitary chemical solutions (abamectin, imidaclopride or tiametoxame). Biocontrol is based on the application of commercial solutions of entomopathogenic nematodes (*Steinernema* sp. or *Heterorhabditis bacteriophora* Poinar) in Chitosan, a chemical saccharide that activates defence mechanisms in the plant and increases lignification (Llácer *et al.*, 2009), formulation (DGAV, 2013). If the plant is significantly damaged, the complete removal and destruction via fire of the plant are recommended (DGAV, 2013).

However, in spite of established protocols and management efforts across the country, the RPW still has a significant impact. Therefore, novel and more effective control methods are warranted.

1.4. Objectives

Although the presence of the RPW has been described in Portugal (Boavida & Franca, 2008; Ramos *et al.*, 2015), no studies have been made on populations of this weevil in northern Portugal nor on mites associated with the RPW in this region. The nature of the relationship of these mites with their host is still uncertain, however the identification of mite species is the first necessary step to further comprehend these interactions. Fungal species associated with the RPW have only been studied in the Lisbon region of Portugal (Ramos *et al.*, 2015), however no studies of fungal diversity in other regions have been made yet.

This insect still results in considerable damage to palm trees in Portugal and other countries. Even though the only currently documented damage done by the RPW in Portugal is to ornamental palm trees, a variety of other hosts have been described and, therefore, effective control is of utmost importance considering prospect economic and agricultural impacts.

To broaden existing knowledge of the RPW and associated organisms, the following main objectives are proposed:

- To document the presence and seasonal activity of RPW populations in northern Portugal;
- To identify and quantify mite species associated with the RPW in northern Portugal and to study their relationships, through host data and mite's ecological parameters prevalence, abundance and mite load);
- To test the applicability of molecular tools for identification of mites associated with the RPW;
- To identify fungal species associated with the RPW in northern Portugal;
- To discuss the potential of using identified mites and/or fungi as biological control agents of RPW.

2. Materials and Methods

2.1. Weevil sampling

To capture adult individuals, Picusan Traps (supplied by Koppert, the Netherlands) were placed in mainland Portugal from July 2021 through January 2022 in the vicinity of visibly infected Canary Island date palm trees (*P. canariensis*) (**Figure 3**). RPW-specific aggregation pheromones (Ao Midori Biocontrol 2021) were placed in the inner lid of the containers to attract the weevils in the vicinity and Canarian palm tree fibres were placed on the bottom surface to serve as food source for the captured insects until collection. Pheromone baits were replaced every three months as recommended by the supplier. Traps were placed at the ground level and at more than 5 meters distance from palm trees in order to attract more weevils (Hallett et al., 1999). Collection areas were selected according to personal observations or available information from owners of infected palm tree. A total of 9 traps were placed in the northern region of Portugal, specifically 4 in the district of Porto: Botanical Garden (41.153008, -8.642730), Marquês (41.161283, -8.604427), Esmoriz (40.955908, -8.650886) and Foz (41.147716, -8.670845); 3 in the district of Aveiro: 2 at the University of Aveiro's Campus (40.635053, -8.659500; 40.635133, -8.659395) and 1 in Praia da Barra (40.632630, -8.748307); 1 in the district of Braga in Famalicão (41.361287, -8.539348); and 1 in the district of Viana do Castelo in Moledo do Minho (41.847698, -8.864375) (**Figure 3**).

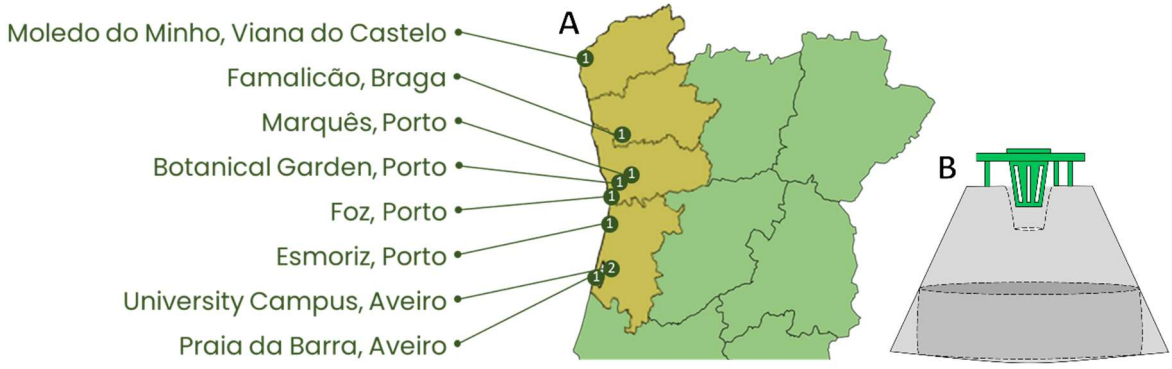

Figure 3. Map of northwestern portuguese localities (A) in which Picusan traps containing aggregation pheromone for *Rhynchophorus ferrugineus* were installed. The number displayed in each location indicates the number of traps set per locality. Illustration of a trap used for *R. ferrugineus* sampling (B).

Traps were checked weekly, and captured weevils were transported to the laboratory in plastic containers containing apple slices (**Figure 4**). Specimens were then kept under low light conditions and fed with apple slices (Shahina *et al.*, 2009). They were either dissected within a week or they were stored in the fridge at 4 ºC and fed apple slices until dissection (n = 174) (non-frozen weevils). Weevils sampled from 28th of September onwards (n = 64) were fed apple slices until 15th of December, after which they were frozen at - 18 ºC (frozen weevils) due to high amount of available weevils to dissect and due to the difficulties in access to the laboratory as a result of COVID restrictions.

Figure 4. Pheromone trap placed in the vicinity of a visibly infected palm tree (*Phoenix canariensis*) at the University of Aveiro's Campus (40.635053, -8.659500) (A). A palm tree damaged by *Rhynchophorus ferrugineus* close to Costa Nova in Aveiro (40.632630, -8.748307) (B). Plastic containers with apple slices and palm tree fibers used for transportation of *R. ferrugineus* from traps to the laboratory (C).

The weevils' sex was identified based on sexual dimorphism (EPPO, 2007) with main focus on the presence (males) or absence (females) of hair-like setae on the rostrum (**Figure 5**).

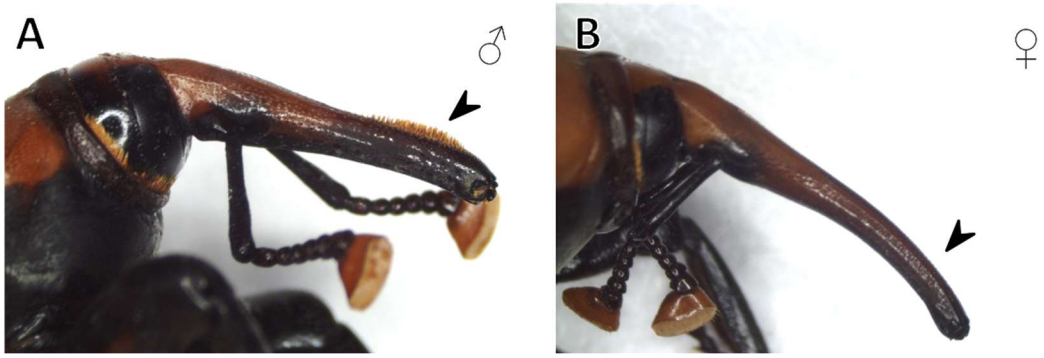

Figure 5. Sexual dimorphism in presence of hair-like setae on rostrum of *Rhynchophorus ferrugineus* male (presence, A) and female (absence, B) weevils.

2.2. Mites

2.2.1. Mite sampling and identification

Weevils were frozen for 30 minutes in order to immobilize them and desensitise them prior to dissection as described by Dilipkumar *et al.* (2015). Weevils were weighted, and measured in their length (with and without their snout), respective sex was noted, and their thoracic patterns photographed. Under a stereomicroscope, body surface (head, antenna, legs, and thorax) was inspected; the area under the elytra, membranous wings and abdomen was observed after dissection. Mites were counted on each body part and specimens from each observed species were mounted on microscopic slides in lactic acid 90% medium (Helle & Sabelis, 1985) for further identification with Zeiss Axiophot microscope. Photos of mites mounted in semi-permanent slides, in lactic acid 90%, were taken with LAS X (Leica X) software.

Mites were identified, to the lower taxonomic level possible, based on original descriptions and recent revisions of morphological traits (Abo-Shnaf & Allam, 2019; Fain, 1974; Farahani *et al.*, 2016; Griffiths, 1970; Kinn, 1984; Kontschan *et al.*, 2014a; Krantz & Walter, 2009; Lindquist, 1975; McGraw & Farrier, 1969; Porcelli *et al.*, 2009; Wisniewski *et al.*, 1992; Womersley, 1954). Ten specimens of each identified species were measured, with Zeiss Axiophot microscope and LAS X (Leica X) software, for morphometric comparison with other described species from the existing literature.

2.2.2. Ecological parameters

Ecological parameters were determined such as Prevalence (%) (number of infected weevils / number of analysed weevils x 100), Mean Abundance (mean number of mites per RPW analysed, considering only one mite species), Mite load (total number of mites per RPW, considering all mite species), Diversity (number of species of mites present in each RPW) (Bush *et al.*, 1997). Parameters were calculated for each locality (district), for each host sex, for each sampling month, and for each body part (site).

2.2.3. Molecular analysis of mites

There is currently no existing literature that explores molecular protocols for identification of mites associated with the RPW. Therefore, we wanted to test multiple

extraction protocols and amplification primers in order to access which more successfully yielded DNA sequences that could be used for identification.

Our initial approach was to work with only *Centrouropoda* sp. mites and to test two different extraction methods (Chelex 10% and Thermal shock), three sets of primers for three different regions (ITS, COI and 18S) with different number of mites (3 samples with one mite, one sample with 5 mites, 4 samples in total for each extraction method and each set of primers).

Once the more efficient extraction method and set of primers were determined, we proceeded to do extractions with samples of *Centrouropoda* sp., *Nenteria extremica*, *Acarus* sp., *Dendrolaelaps* sp., *Uroobovella* sp. and Mesostigmata. *Curculanoetus rhynchophorus* was not isolated for extraction and amplification considering that this mite could not be isolated from the RPW at the time of molecular analysis given that it is not very prevalent.

2.2.3.1. DNA extraction

Considering that the first extractions with one and five *Centrouropoda* sp. mites were successful, we proceeded with genomic DNA extractions from samples with one and three mite specimens for each type of mite observed (Konakandla *et al.*, 2006). Mites were collected directly from the weevil and immediately placed into extraction solutions or stored in an ethanol 70% solution (Alasaad *et al.*, 2009). With the goal of determining the more effective method for mite DNA extraction, two protocols were tested: one using thermal shock based on Soglia *et al.* (2009) and one using a chelating resin solution (Chelex 10%) adapted from Walsh *et al.* (1991) and tested for a single mite sample by Konakandla *et al.* (2006).

2.2.3.1.1. Thermal shock extraction

Each mite, or group of mites, was placed in a 1.5 ml Eppendorf tube containing 180 µl of elution buffer AE from DNA extraction kit DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). Samples were heated to 100 ºC in a dry bath incubator and vortexed every 10 + 5 + 5 minutes. Tubes were then centrifuged at 12,000 xg for 6 min at room temperature and pellet was discarded. Samples were stored in the freezer at - 18 ºC.

2.2.3.1.2. Chelating resin Chelex 10% extraction

Chelex 100 is a metal-chelating resin, with iminodiacetate ions that act as chelating groups. The alkaline pH of the solution (pH 10 – 11) in combination with high temperatures results in membrane rupture and DNA denaturation but not in DNA degradation due to the metal ions that provide DNA stability (Singer-Sam, 1989).

Each mite was placed in a 1.5 ml Eppendorf tube containing 200 µl of 10% Chelex (Chelex 100 Molecular Biology Grade Resin, Bio-Rad, CA, USA) diluted in Milli-Q filtered water. A 10% Chelex solution was prepared in a 20 mL Falcon tube using 1 ml Chelex 100 and 9 ml of sterile distilled water. Before pipetting, solution was vortexed to ensure homogenous resin distribution and avoid varying Chelex concentrations. Samples were heated, vortexed, centrifuged and stored in the above-mentioned thermal shock protocol.

2.2.3.2. PCR amplification

PCR amplification solution was prepared as described in the master mix product protocol (*NZYTaq II 2x Green Master Mix Protocol*, 2022). Total 25.0 µl volume containing: 12.5 µl PCR master mix (NZYTaq II 2x Green Master Mix, NZYtech, Lisbon, Portugal), 9.5 µl Milli-Q filtered water, 0.5 µl (10 µM) of forward, 0.5 µl (10 µM) of reverse primer and 2 µl of DNA. All PCR amplifications were done in XT^{96} thermocycler (VWR, Pennsylvania, USA).

2.2.3.3. Amplification primers

Different primers were used in order to test amplification efficiency of distinct primers with the extracted samples.

The internal transcribed spacer (ITS) has been proven efficient in mite DNA barcoding (Yang *et al.*, 2011). The nuclear ribosomal ITS2 marker, was amplified using ITS2-F1 (5’ - ATA TGC TTA AAT TCA GCG GG - 3’) and ITS2-R1 primers, (5’ - GGG TCG ATG AAG AAC GCA GC - 3’) (Navajas *et al.*, 1998) which are anchored in the highly conserved 5.8S and 28S regions. Thermocycler protocol for ITS2 amplification was: initial denaturation cycle at 95 ºC for 3 min, followed by 30 cycles of 94 ºC for 1 min, 52 ºC for 1:30 min, 72 ºC for 2 min, and a final extension at 72 ºC for 5 min.

Cytochrome c oxidase subunit I (COI) is one of the more documented mite genomic regions in current databases and has been found to be a good barcoding region for mites

(Knee *et al.*, 2012b). Primers LCO1490 (light cytochrome oxidase) (5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3') and HCO2198 (heavy chain oxidase) (5' - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3') have been found to be effective for mite samples (Folmer, 1994; Knee *et al.*, 2012b). Thermocycler protocol for COI amplification was: initial denaturation cycle at 95 ºC for 3 min, followed by 40 cycles of 94 ºC for 45 s, 45 ºC for 45 s, 72 ºC for 1 min, and a final extension at 72 ºC for 7 min.

The 18S ribosomal RNA (18S) region is widely used as a barcoding sequence for phylogenetic studies considering that it is highly conserved and allows for clade identification to higher levels (Bhadury & Austen, 2010; Meyer *et al.*, 2010). It has been used for mites, namely single mites, for identification to the family level (Xue *et al.*, 2017; Zhao *et al.*, 2012). The 18S region was amplified with two sets of primers 18e/18R (5' – CTG GTT GAT CCT GCC AGT – 3' / 5' – CTA CGG AAA CCT TGT TAC G – 3') (Hillis & Dixon, 1991; Whipps *et al.*, 2003) and WormA/WormB (5' – GCG AAT GGC TCA TTA AAT CAG – 3' / 5' – CTT GTT ACG ACT TTT ACT TCC – 3') (Littlewood & Olson, 2014). These primers were compared to existing sequences from GenBank database to confirm complementarity with mite 18S region DNA. Thermocycler protocol for 18S amplification was: initial denaturation cycle at 95 ºC for 3 min, followed by 35 cycles of 94 ºC for 1 min, 54 ºC for 1 min, 72 ºC for 2 min, and a final extension at 72 ºC for 7 min.

2.2.3.4. PCR products

PCR products were visualised in a 1% agarose (NZYtech) in Tris-Borate-Acetate (TBE) (NZYtech) buffer gel electrophoresis stained with GelRed Nucleic Acid Gel Stain (Biotium, California, USA). For DNA sequencing, the samples were sent to Stab Vida (stabvida.com). Obtained sequences were aligned in Molecular Evolutionary Genetics Analysis software (Mega 11.0.11, 2022) and compared to related sequences from National Center for Biotechnology Information's (NCBI) GenBank sequence database using Basic Local Alignment Search Tool (BLAST).

2.2.3.5. Phylogenetic analysis

Multiple sequence alignments and phylogenetic reconstructions were performed using the NGPhylogeny.fr platform (ngphylogeny.fr; Lemoine *et al.* (2019)). Sequences from this study and sequences retrieved from GenBank were submitted and aligned using MAFFT (Rozewicki *et al.*, 2019); ambiguously aligned regions and highly variable characters were

removed using BMGE (Block Mapping and Gathering with Entropy; Criscuolo and Gribaldo (2010)). Bootstrap confidence levels were determined with 1000 replicates. The graphical representation and editing of the phylogenetic tree was done with iTOL version 6.5.4 (Letunic & Bork, 2021) (itol.embl.de). Outgroup sequences from the same genera, family and a different order were included in the phylogenetic tree for contextualization of samples into clades.

All sequences resulting from this study were submitted to GenBank (in Annex B2, page 91).

2.3. Fungi

2.3.1. Sampling

Fungal samples were collected from weevils or mite specimens with visible mycelial growth or spores (**Figure 6**). Several species were collected from different localities, and at different times of the year.

Fungal species were identified based on morphology and by DNA sequencing. For morphological identification, photos of colony growth on potato dextrose agar (PDA) (Sigma-Aldrich, USA) were taken in the first 7 days of growth in a climatic chamber at 25 ºC after which a small sample of fungal growth was removed with a pipette tip and placed on 90% lactic acid medium and methylene blue stain and mounted on microscopic slides for observation of micromorphological traits.

For identification based on DNA sequencing, a small portion of the visible spores or mycelium was removed with a sterilised inoculation loop and plated on the centre of 9 cm Petri dishes with PDA. Plates were then left to grow in a climatic chamber at 25 ºC for 7 days and re-inoculated onto fresh PDA plates in order to obtain single-species colonies. All inoculation work was done under a laminar flow.

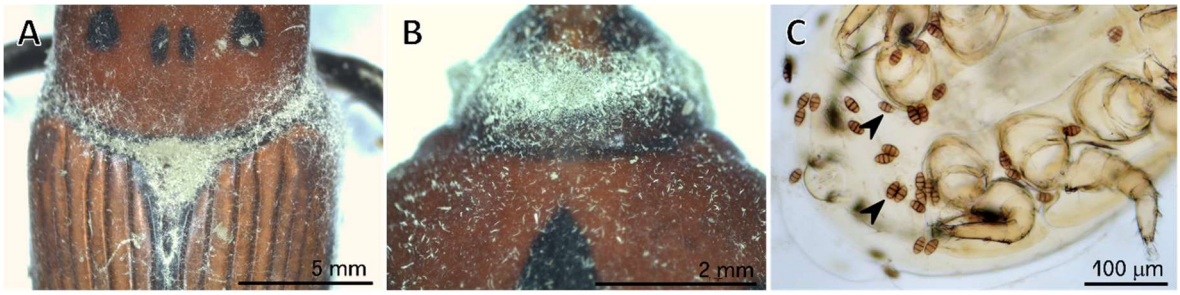

Figure 6. Visible fungal growth on *Rhynchophorus ferrugineus* on the intersection between abdomen and thorax (A) and between head and thorax (B). Multiple fungal spores (black arrows) on a mite (C).

2.3.2. Molecular analysis

2.3.2.1. DNA extraction

Fungal gDNA was extracted from mycelium grown on PDA plates for which had been selectively grown by serial plating. A small sample of mycelium was removed with a sterilised inoculation hoop and placed in a 1.5 ml Eppendorf tube and extraction was performed according to the above-described protocol for mite DNA extraction with Chelex 10% (Ferencova *et al.*, 2017).

2.3.2.2. PCR amplification

PCR amplification solution was prepared as described in the master mix product protocol (*NZYTaq II 2x Green Master Mix Protocol*, 2022), identically to above described procedure for mite DNA amplification.

2.3.2.3. Amplification primers

Two different sets of primers were initially used in order to test amplification efficiency of distinct primers with the extracted samples.

The internal transcribed spacer (ITS) and the small subunit 18S rRNA (18S), constitute two of the most extensively used sequences for fungi DNA barcoding (Raja *et al.*, 2017; Schoch *et al.*, 2012). The evolution of 18S is slower than ITS, thus has less variation among taxa (Mitchell & Zuccaro, 2006). Therefore, the 18S is usually used for identification at higher taxonomic level and the ITS for lower taxonomy (species level) (Raja *et al.*, 2017).

We initially amplified both ITS and 18S regions of extracted fungal DNA. ITS was amplified using ITS1F (5' - CTT GGT CAT TTA GAG GAA GTA A – 3') and ITS4 (5' - TCC TCC GCT TAT TGA TAT GC – 3') primers which yield a ~ 450 – 800 bp sequence (White *et al.*, 1990). 18S was amplified using NS1 (5' - GTA GTC ATA TGC TTG TCT C – 3') and NS4 (5' - CTT CCG TCA ATT CCT TTA AG – 3') primers which yield a ~1200 bp sequence (White *et al.*, 1990). Thermocycler protocol for both ITS and 18S amplification was: initial denaturation cycle at 95 ºC for 5 min, followed by 35 cycles of 94 ºC for 30 s, 52 ºC for 30 s, 72 ºC for 1 min, and a final extension at 72 ºC for 8 min as described in Raja *et al.* (2017).

Primers for the ITS and 18S region were used for amplification of all samples.

2.3.2.4. PCR products

PCR products were visualised in a 1% agarose (NZYtech) in Tris-Borate-Acetate (TBE) (NZYtech) buffer gel electrophoresis stained with GelRed Nucleic Acid Gel Stain (Biotium). For DNA sequencing, the samples were sent to Stab Vida (stabvida.com, Caparica, Portugal). Obtained sequences were compared to related sequences from National Center for Biotechnology Information's (NCBI) GenBank sequence database using Basic Local Alignment Search Tool (BLAST) algorithm.

2.3.2.5. Phylogenetic analysis

Multiple sequence alignments and phylogenetic reconstructions were performed using the NGPhylogeny.fr platform (ngphylogeny.fr; Lemoine *et al.* (2019)). Sequences from this study and sequences retrieved from GenBank were submitted and aligned using MAFFT (Rozewicki *et al.*, 2019); ambiguously aligned regions and highly variable characters were removed using BMGE (Block Mapping and Gathering with Entropy; Criscuolo and Gribaldo (2010)). Bootstrap confidence levels were determined with 1000 replicates. The graphical representation and editing of the phylogenetic tree was done with iTOL version 6.5.4 ((Letunic and Bork (2021); itol.embl.de). Outgroup sequences from the same genera were included in the phytogenic tree for contextualization of samples into clades.

All sequences resulting from this study were submitted to GenBank (in Annex B2, page 91).

2.4. Statistical analysis

Host and mite features, RPW weight and length, mite load of each body part, and mite species abundance, were tested for a normal distribution with a Kolmogorov-Smirnov test. All values were found to be non-normally distributed ($Z > 0.080$, $p < 0.001$) and therefore analysis proceeded with non-parametric tests.

Four different non-parametric tests were used: Wilcoxon-Mann-Whitney, for comparison of two independent samples, Kruskal-Wallis, for more than two independent samples, Wilcoxon signed-rank test, for two paired samples, and Friedman's ANOVA for more than two paired samples. These non-parametric tests use ranking procedures to compare samples, where each sample is ranked considering all values and then the mean rank of each group is compared to the overall rank. Tests used for each comparison are listed in the following paragraphs in the order that they will be explored in the results and discussion sections.

Host features were analysed for their differences between male and female weevils in terms of body weight and total body length, with Wilcoxon-Mann-Whitney test. Body weight and body length of frozen and non-frozen samples were compared between the two groups with Wilcoxon-Mann-Whitney test. Body weight and length differences between districts were tested with Kruskal-Wallis test for general comparison followed by Wilcoxon-Mann-Whitney for pairwise comparison.

Mite species diversity was analysed for its differences between male and female weevils with Wilcoxon-Mann-Whitney test. Differences in this parameter between districts and between different sampling months were tested with Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney test for pairwise comparisons whenever differences were significant.

Mite load was analysed for its differences between male and female weevils with Wilcoxon-Mann-Whitney test, and differences between districts and different sampling months with Kruskal-Wallis test. Differences in mite load per body part between male and female weevils were tested with Wilcoxon-Mann-Whitney test, and differences in mite load between all body parts with Friedman's ANOVA test and then pairwise with Wilcoxon signed-rank test whenever differences were significant.

Mite abundance was analysed for its differences between all species with Friedman's ANOVA test. Differences in this parameter between sex for each species were tested with Wilcoxon-Mann-Whitney test, between districts and between months with Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney test for pairwise comparison, and between body parts with Friedman's ANOVA test and Wilcoxon signed ranks test for pairwise comparison.

Correlation between average total mite load per weevil and weevils body weight and total length was tested with Pearson correlation. Correlation of mite load and mite abundance with species diversity were also tested with Pearson correlation.

Significance level was $\alpha = 0.05$ for all statistical tests. All analysis were done with SPSS version 27.0 (SPSS Inc., Chicago, IL, 2020) software.

3. Results

The results from this dissertation will be presented by following an approach from broader to smaller populations, from macro to micro variations. Hence, we will first present the sampling and distribution of weevils: by first considering the ratio of females and males, their spatial and seasonal distribution, their variation in weight and length and, finally, the observed thoracic patterns. We will then present the identified mite species and the associated data that led to their identification (morphology) and the results of the molecular analysis. Followed by the mite's species diversity, prevalence, load, and abundance of mites. Each of the mentioned parameters will be presented first by value across all samples, then in males and females, then across different districts, different sampling months and different body parts (these last two parameters only when relevant). Finally, the obtained data from the fungal samples considering the species found, their association with specific mite species and the obtained phylogenetic data will be presented.

Selected non-parametric statistical tests are based on the comparison of mean ranks. The average for each parameter, considering that the mean rank value does not represent a real characteristic of each considered group, will be presented. The order of the mean ranks matches the order of averages, from greater to smaller, unless stated otherwise.

3.1. Weevils

A total of 238 weevils were collected from July 2021 until January 2022, in the northern districts of Continental Portugal.

3.1.1. Sex ratio

Weevils collected in the pheromone traps were mainly females (1 male: 1.9 females). A total of 155 females (65%) and 83 males (35%) were collected. The sex ratio varied between 1 male: 1.5 females in Viana do Castelo to 1 male: 2.2 females in Aveiro (**Table 1**).

In the frozen samples (n = 64) there were 41 females (64%) and 23 males (36%) (1 male: 1.8 females) and in the non-frozen sample (n = 174) there were 114 (66%) females and 60 (34%) males (1 male: 1.9 females). We therefore conclude that other differences found between storing methods are not a direct result of the sex ratio in samples since they were identical.

Table 1. Number of sampled females, males, sex ratio and total number of *Rhynchophorus ferrugineus* for each sampled district.

Locality (district)	Females (*n* (%))	Males (*n* (%))	Ratio females:males	Total *n*
Aveiro	76 (68.5%)	35 (31.5%)	2.2 : 1	111
Porto	30 (68.2%)	14 (31.8%)	2.1 : 1	44
Braga	31 (58.5%)	22 (41.5%)	1.4 : 1	53
Viana do Castelo	18 (60.0%)	12 (40.0%)	1.5 : 1	30
	155 (100.0%)	83 (100.0%)	1.9 : 1	238

The sex ratio of sampled weevils was similar throughout different months. The proportion of males and females varied between 1 male: 1.6 females in August and 1 male: 2.5 females in October and November (**Table 2**).

Table 2. Number of sampled females, males, sex ratio and total number of *Rhynchophorus ferrugineus* for each sampled month.

Month (2021/22)	Females (*n* (%))	Males (*n* (%))	Ratio females:males	Total *n*
July	23 (65.7%)	12 (34.3%)	1.9 : 1	35
August	35 (61.4%)	22 (38.6%)	1.6 : 1	57
September	68 (64.8%)	37 (35.2%)	1.8 : 1	105
October	20 (71.4%)	8 (28.6%)	2.5 : 1	28
November	5 (71.4%)	2 (28.6%)	2.5 : 1	7
December	0 (0.0%)	0 (0.0%)	-	0
January	4 (66.7%)	2 (33.3%)	2.0 : 1	6
	155 (100.0%)	83 (100.0%)	1.9 : 1	238

3.1.2. District distribution and seasonal variation

The number of weevils collected in pheromone traps was higher in Aveiro district (Aveiro *n* =111 (46.6%)), followed by Braga (*n* = 53 (22.3%)), Porto (*n* = 44 (18.5%)), and Viana do Castelo (*n* = 30 (12.6%)). Their number was also higher in the warmer months of August and September 2022 (July *n* = 35 (14.7%), August *n* = 57 (23.9%), September *n* = 105 (44.1%), than in cooler months as October *n* = 28 (11.8%), November = 7 (2.9%),

December n = 0 (0%), January n = 6 (2.5%)). Therefore, active RPW adults were recorded in almost all months of sampling, with the exception of December (**Figure 7**).

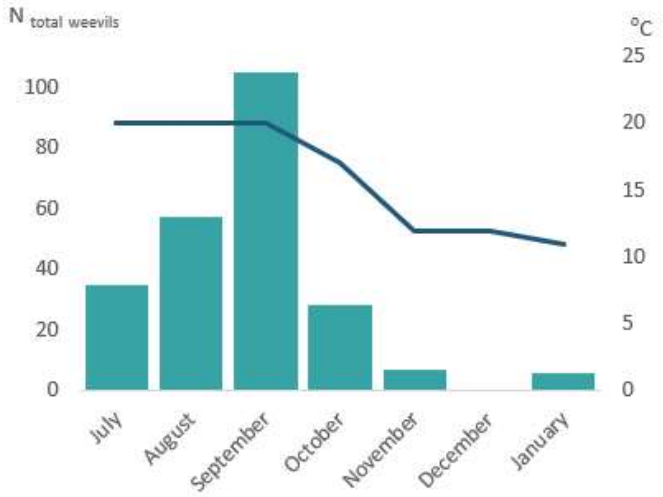

Figure 7. Number of collected *Rhynchophorus ferrugineus* (considering all traps) throughout sampling months from July 2021 to January 2022 (bars) and temperature (line) (calculated as the average of the medians of all districts for each month).

3.1.3. Body weight

The two different storing methods resulted in significant differences in weevil weight. Frozen weevils were significantly less heavy [mean±s.d.] (n = 64, 0.56±0.23 g) than non-frozen weevils (n = 174, 1.15±0.31 g) that were dissected immediately after collection or kept alive in the laboratory for a week (Wilcoxon-Mann-Whitney test: $U = 786.0$, $p < 0.001$) (**Figure 8**). Average weight of non-frozen samples is significantly higher in female weevils (female 1.20±0.30 g v. male 1.05±0.31 g) (Wilcoxon-Mann-Whitney test: $U = 2047.5$, $p = 0.003$) (**Figure 8**). However, no significant differences in weight were found between the sexes when considering only frozen weevils (female 0.56±0.23 g v. male 0.56±0.24 g) (Wilcoxon-Mann-Whitney test: $U = 456.5$, $p = 0.83$). Considering that the non-frozen weevils are expected to be the most similar to the weevil's *in vivo* weight, only non-frozen weevils will be considered for weight analysis from now on.

Body weight of weevils from the district of Porto was significantly smaller than that of other districts (Kruskal-Wallis test: $H = 27.11$, $p < 0.001$), even when considering only non-frozen samples (Kruskal-Wallis test: $H = 12.91$, $p = 0.01$) (Aveiro 1.21±0.26 g, Porto

0.90±0.44 g, Braga 1.10±0.29 g, Viana do Castelo 1.24±0.28 g). No differences were found between the remaining districts of Aveiro, Braga and Viana do Castelo, considering only non-frozen samples (Wilcoxon-Mann-Whitney test: $U > 323.5$, $p > 0.09$).

No comparison in body weight differences between months was made considering that samples collected from 28th of September onwards ($n = 64$) were frozen and, consequently, consistent comparison between months could not be made.

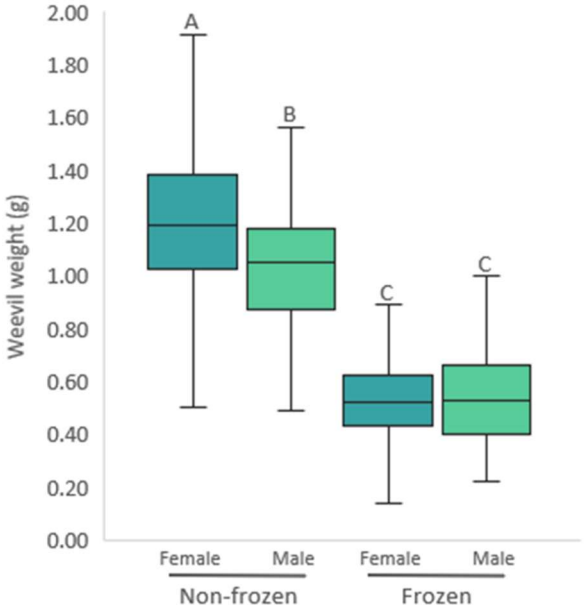

Figure 8. Boxplot of *Rhynchophorus ferrugineus* weight in frozen and non-frozen samples for males and females. Letters (A, B, C) represent different groups with significant differences with each other ($\alpha = 0.05$). Horizontal lines indicate the medians and the 25% and 75% quartile, whiskers below and above the boxes indicate the minimum and maximum values of the group, respectively. Outliers were removed to ease visualisation of remaining values.

3.1.4. Body length

No significant differences were found in length of frozen and non-frozen samples (frozen 3.38±0.38 cm v. non-frozen 3.37±0.45 cm) (Wilcoxon-Mann-Whitney test: $U = 5018.0$, $p = 0.87$). Thus, the data were pooled from both groups (frozen and non-frozen weevils) for this analysis which revealed that female weevils were significantly bigger than the male weevils (female 3.44±0.41 cm v. male 3.25±0.34 cm) (Wilcoxon-Mann-Whitney test: $U = 4279.5$, $p < 0.001$) (**Figure 9**).

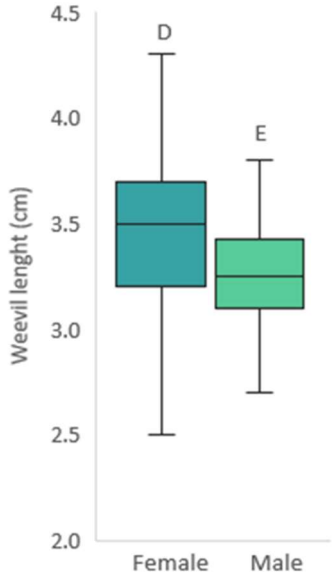

Figure 9. Boxplot of weevil length in males and females. Letters (D, E) represent different groups with significant differences with each other ($\alpha = 0.05$). Horizontal lines indicate the medians and the 25% and 75% quartile, whiskers below and above the boxes indicate the minimum and maximum values of the group, respectively. Outliers were removed to ease visualisation of remaining values.

Weevil length was significantly different between districts (Kruskal-Wallis test: $H = 10.68$, $p = 0.01$; Braga 3.23±0.39 cm, Aveiro 3.43±0.39 cm, Porto 3.36±0.38 cm, Viana do Castelo 3.43±0.35 cm). The district of Braga had significantly smaller weevils in comparison to Aveiro and Viana do Castelo (Wilcoxon-Mann-Whitney test: $U > 537.5$, $p < 0.01$) but no significant difference in the length of the weevil was found between the districts of Porto, Viana do Castelo and Aveiro (Wilcoxon-Mann-Whitney test: $U < 2153.5$, $p > 0.29$).

Total length was significantly different throughout the sampled months (Kruskal-Wallis test: $H = 11.40$, $p = 0.04$), however length of the body (without considering the snout) was not significantly different between the different sampled months (Kruskal-Wallis test: $H = 10.71$, $p = 0.06$) (**Figure 10**), which indicates that the observed difference was in the snout length and not total body size. In terms of total body length, weevils were significantly shorter in January (Wilcoxon-Mann-Whitney test: $U > 38.5$, $p < 0.04$).

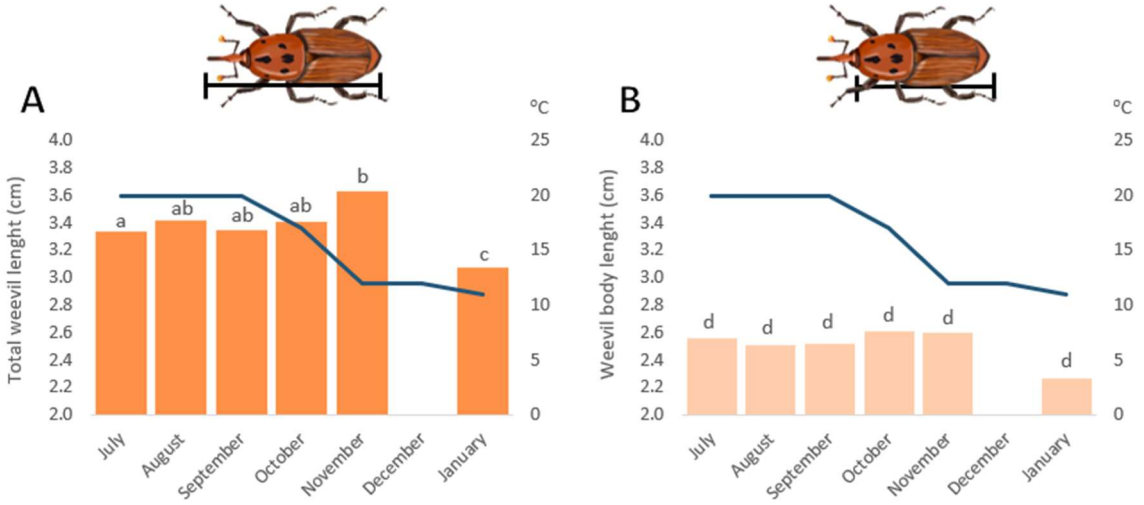

Figure 10. Average total length (cm) (A) and average body length (cm) (B) of sampled *Rhynchophorus ferrugineus* throughout sampling months from July 2021 to January 2022 (bars) and temperature (line) (calculated as the average of the medians of all districts for each month). Horizontal bars next to the weevil represent measured length for each graph. Letters (a, b, c) represent different groups with significant differences with each other ($\alpha = 0.05$). No samples were collected in December: the missing column indicates missing data, not zero.

3.1.5. Thoracic patterns

Diversity in number, and size of spots on the weevils' dorsal thorax was observed and photographed. Male and female weevils presented no sex-specific trend in pattern. Aveiro, Viana do Castelo, Braga, and most samples from Porto had similar pattern distribution and colour. Weevils from Botanical Garden in Porto presented darker elytra surface (**Figure 11**).

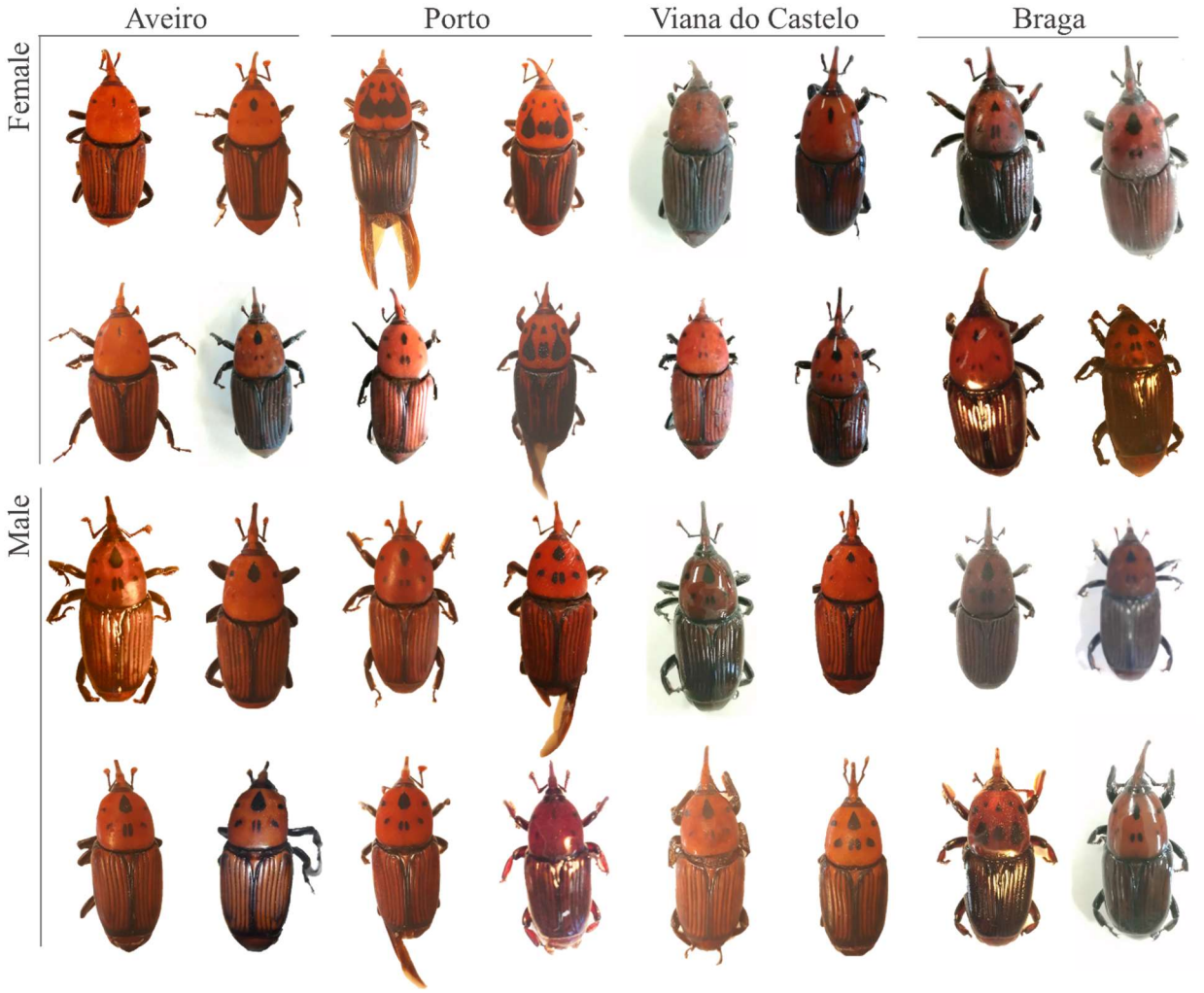

Figure 11. Thoracic pattern of sampled *Rhynchophorus ferrugineus*. Pictures are organised by sex (female top, male bottom) and by district (Aveiro (left), Porto, Viana do Castelo, and Braga (right). Images are not to scale.

3.2. Mites

3.2.1. Identified species

Seven species of mites were identified in association with the RPW: *Centrouropoda* sp. Berlese 1916, *Nenteria extremica* Kontschan, Mazza, Nannelli and Roversi 2014, *Curculanoetus rhynchophorus* Fain 1974, *Uroobovella* sp. Berlese 1905, *Acarus* sp. Linnaeus 1758, *Dendrolaelaps* sp. Halbert 1915, and one Mesostigmata identified only to the order level (**Table 3**, **Figure 12**).

Table 3. Taxonomic levels of mites identified in association with *Rhynchophorus ferrugineus.*

Kingdom	Phylum	Class	Order	Family	Genus	Species
Animalia	Arthropoda	Arachnida	Acariformes	Acaridae	*Acarus*	sp.
Animalia	Arthropoda	Arachnida	Astigmata	Histiostomatidae	*Curculanoetus*	*rhynchophorus*
Animalia	Arthropoda	Arachnida	Mesostigmata	Trematuridae	*Nenteria*	*extremica*
Animalia	Arthropoda	Arachnida	Mesostigmata	Uropodidae	*Centrouropoda*	*sp.*
Animalia	Arthropoda	Arachnida	Mesostigmata	Digamasellidae	*Dendrolaelaps*	sp.
Animalia	Arthropoda	Arachnida	Mesostigmata	Urodinychidae	*Uroobovella*	sp.
Animalia	Arthropoda	Arachnida	Mesostigmata			

Centrouropoda sp., the more described mite genus in association with the RPW, was identified and compared to the original description of deutonymphal *Centrouropoda almerodai* (Wisniewski *et al.*, 1992) and a recent revision of the genus taxonomy (Abo-Shnaf & Allam, 2019). Observed specimens had an idiossoma (the main body tagma of mites) 614.2±29.2 μm long and 439.7±36.0 μm wide (n = 10), which is very similar to *C. almerodai*'s description of 630 x 460 μm. However, the observed specimens did not match fully to the description of *C. almerodai*. For example, h4 and h2 are hair like setae on the mite's hypostome. *C. almerodai* is described as having setae h4 and h2 of equal length, however we observed h4 to be shorter than h2. *C. peruana* is described as having h4 shorter than h2, however other features did not match the full description of *C. peruana*. *Centrouropoda* sp. was often observed to attach to the host via anal pedicel, but it was also present often without any physical attachment.

N. extremica identification was based on its original description of deutonymphal life form (Kontschan *et al.*, 2014a). Observed specimens had an idiossoma 394.8±16.4 μm long and 297.7±19.4 μm wide (n = 10) and the original description was of 370-380 μm long and 280-290 μm wide. All described morphological details were identically found on observed specimens. This mite was often observed attached to the host via anal pedicel, but also free roaming.

Curculanoetus rhynchophorus was identified based on its original description of deutonymphal life form (Fain, 1974). Observed specimens had an idiossoma 239.1±16.3 μm long and 165.8±14.7 μm wide (n = 10) and the original description was of 235 μm long and 160 μm wide. All described morphological details were identically found on observed specimens.

No key to the genus *Uroobovella* sp. was found. Description was based on recent observations of mites from this genus (Farahani *et al.*, 2016; Porcelli *et al.*, 2009). Observed specimens had an idiossoma 886.3±30.9 μm long and 654.8±23.5 μm wide (n = 10). No recent observations of the genus indicated dimension of deutonymphs but approximate measurements, obtained from microphotograph scale from Farahani *et al.* (2016), were 850 μm long and 604 μm wide. All observed morphological details were identical to recent observations, however we lacked a more detailed description for identification to species level. *Uroobovella* sp. was often attached to the RPW via anal pedicel, but it was also observed without any pedicel attachment.

Identification of *Acarus* sp., was based on the genus key of Griffiths (1970). Mites matched genus criteria but not any specific species from this genus, possibly due to the fact that this was not a recent revision. Observed specimens had an idiossoma 199.7±16.0 μm long and 137.3±15.3 μm wide (n = 10).

Dendrolaelaps sp. identification was based on descriptions of deutonymphal life forms of the genus (Kinn, 1984; Lindquist, 1975; McGraw & Farrier, 1969; Womersley, 1954). However, observed specimens did not fully match any found descriptions of specific species. Observed specimens had an idiossoma 296.0±194.7 μm long and 194.7±5.9 μm wide (n = 10).

Mites identified as Mesostigmata were identified to order level based on the key to Acari orders from Krantz and Walter (2009). Observed mites had the morphological features to follow the taxonomic key “Key to the Superorders, Orders, and Suborders of Acari” but could not be identified any further. Specimens had an idiossoma 340.3±8.2 μm long and 189.9±21.0 μm wide.

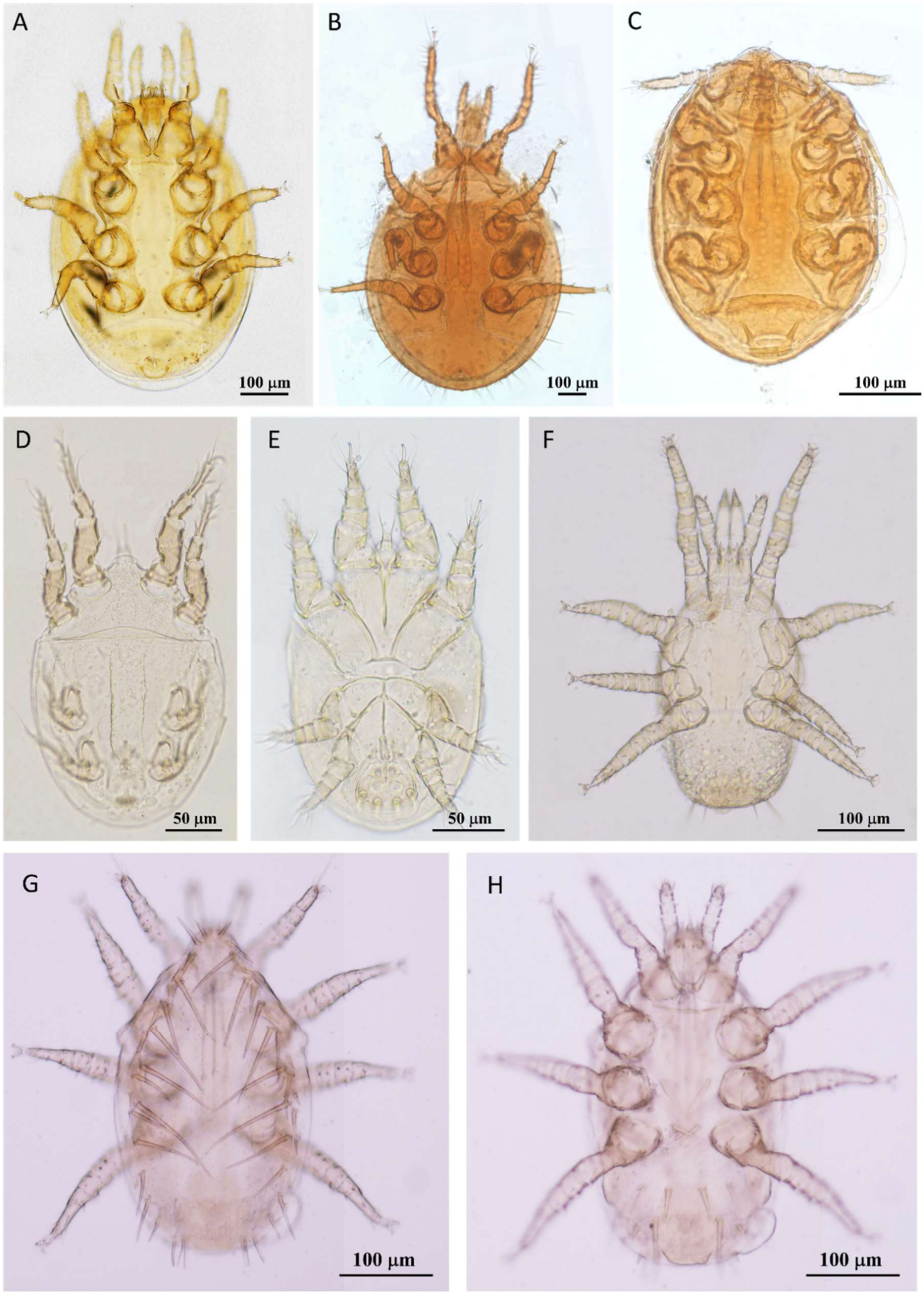

Figure 12. Microphotographs of mites found with association with *Rhynchophorus ferrugineus*: (A) *Centrouropoda* sp., (B) *Uroobovella* sp., (C) *Nenteria extremica*, (D) *Curculanoetus rhynchophorus*, (E) *Acarus* sp., (F) *Dendrolaelaps* sp., (G and H) Mesostigmata - dorsal view (G) and ventral view (H).

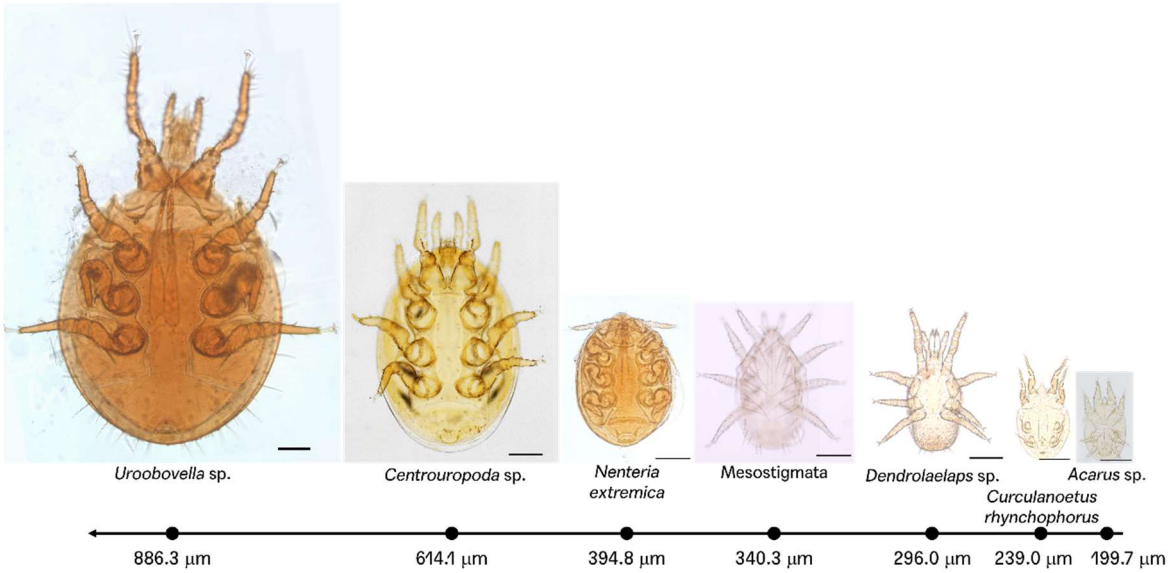

Figure 13. Microphotographs of mites identified on *Rhynchophorus ferrugineus* represented at the same scale and their respective mean length of idiossoma. Bars in microphotographs represent 100 µm.

3.2.2. Molecular analysis

Chelex 10% extractions, in contrast to thermal-shock extractions, consistently produced PCR amplification products. In addition, Chelex 10% is less time-consuming and dispendious, therefore, after the first extractions, only Chelex 10% protocol was performed. However, despite the obtained fragments, the quality of the DNA produced was doubtful, considering that high-quality sequences were not obtained.

Samples with both one and five mites yielded amplified sequences and so we proceeded to do the DNA extractions with one and three mites per isolate considering that fewer mites in the same sample can possibly result in more accurate sequences due to potential intra-species variation.

Amplifications with primers for the ITS sequence were not successful, only primers for COI and 18S resulted in amplification sequences.

ITS2 was initially amplified from 8 *Centrouropoda* sp. mite extractions (3 samples with one mite, one sample with 5 mites, 4 samples per extraction method) and 7 of these extractions were successfully amplified. However, the obtained sequences did not yield clear DNA sequences for comparison with the sequence database and subsequent amplifications

with *Centrouropoda* sp., *Nenteria extremica*, *Acarus* sp., *Dendrolaelaps* sp., *Uroobovella* sp., and Mesostigmata did not efficiently amplify the samples. Amplification of COI, with LCO1490/HCO2198 primers, was initially done with 8 *Centrouropoda* sp. mite extractions (3 samples with one mite, one sample with 5 mites, 4 samples per extraction method), all of which were successfully amplified. Subsequent amplifications with other mite types were successful. The 18S region successfully amplified samples with 1 *Centrouropoda* sp. mite, and samples with three mites of *Dendrolaelaps* sp., *N. extremica* and *Acarus* sp.

3.2.2.1. Phylogeny

Most obtained sequences from mite DNA for the COI region were not clear enough for identification via comparison with existing databases either through direct comparison in BLAST tool or through the construction of a phylogenetic tree. However, sequences from the 18S region yielded clearer sequences with which it was possible to build a phylogenetic tree for analysis (**Figure 14**).

The number of sequences found in GenBank was rather low (non-existing for *Centrouropoda* sp. or any species from the genus *Nenteria*, and only one sequence of the 18S (*Uropoda orbicularis* (AY620926.1)) was found from the Uropodidae family), and none matched 100% with any of the sequences obtained. Therefore, sequences from the same genus or family were introduced to test phylogenetic proximity to the samples from this study and to ease the identification of clades. A sequence from the Astigmata order (HG996221.1) was introduced in the phylogenetic tree (**Figure 14**) as an outgroup to separate sequences to the order level between Astigmata, Mesostigmata and Acariformes.

The phylogenetic analysis confirmed morphological identification at the family level (**Figure 14**). *Acarus* sp. was in proximity with sequences of the same genus, inserted into the Acaridae family and Acariformes order. *Dendrolaelaps* sp. is positioned in assigned genus clade. Although there are currently no sequences in current sequence database for the 18S region of *Centrouropoda* sp. and *N. extremica*, in the phylogenetic tree they are positioned within the morphologically assigned families: Uropodidae and Trematuridae families (**Table 3**), respectively.

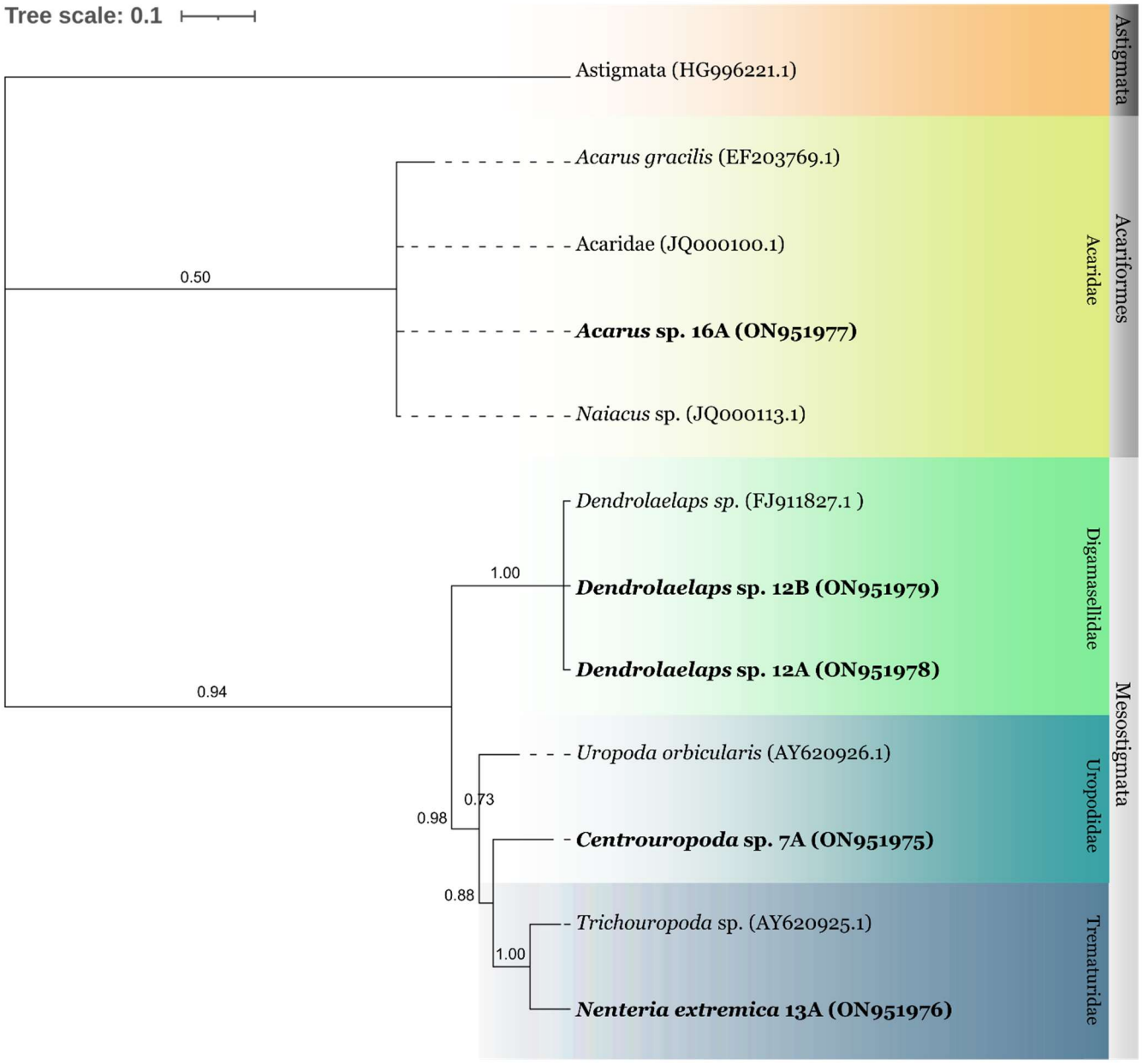

Figure 14. Phylogenetic tree with sequences obtained from mites found in association with *Rhynchophorus ferrugineus*. Names in **bold** are sequences obtained in this study. Each colour represents a family, gray boxes represent different orders. GenBank accession number is indicated between parenthesis. Bootstrap values are indicated in respective branches.

3.2.3. Species diversity

Mite species diversity was on average 3.5±1.4 mite species per weevil. Species diversity was not significantly different between sexes (female 3.4±1.4 species, male 3.5±1.3 species) (Wilcoxon-Mann-Whitney test: $U = 5973.0$, $p = 0.35$).

Diversity was not significantly different between districts (Aveiro 3.4±1.3 species, Porto 3.5±1.2 species, Braga 3.4±1.5, Viana do Castelo 3.6±1.5) (Kruskal-Wallis test: $H = 0.419$, $p = 0.94$).

In terms of temporal variation, species diversity was significantly different between sampling months (July 3.7±1.3 species, August 3.8±1.4 species, September 3.2±1.4 species, October 3.4±0.9 species, November 2.4±1.4 species, January 4.2±0.75 species) (Kruskal-Wallis test: $H = 13.85$, $p = 0.02$), with the highest average values associated with July, August, October, and January. Although it is important to note that November ($n = 7$) and January ($n = 6$) had a low number of weevils, which might render observed statistical differences inaccurate.

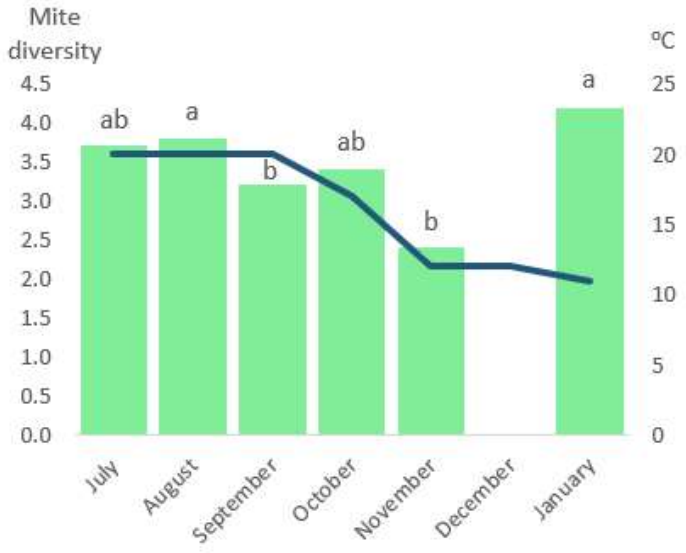

Figure 15. Species diversity of mites associated with *Rhynchophorus ferrugineus* throughout sampling months from July 2021 to January 2022 (bars) and temperature (line) (calculated as the average of the medians of all districts for each month). Letters (a, b) represent different groups with significant differences with each other ($\alpha = 0.05$). No samples were collected in December: the missing column indicates missing data, not zero.

3.2.4. Prevalence

Considering that all inspected weevils (n = 238) were associated with mites, mite Prevalence was 100 % for both sexes, all districts, and all sampling months.

Different mite species had different total Prevalence (Prevalence across all samples), in female and male weevils and in different districts. However, no significant differences were observed in Prevalence between male and female weevils, for any species (Chi-square test: $\chi 2 < 1.35$, $p > 0.25$) (**Table 4**).

Table 4. Prevalence for each mite species in female and male weevils. The Prevalence was compared with chi-square test ($\alpha = 0.05$).

	Prevalence (% (*n*))			
	All weevils	Female weevils	Male weevils	*χ2 (p)*
All species	100.0 (238)	100.0 (155)	100.0 (83)	*
Centrouropoda sp.	87.0 (207)	85.2 (132) a	90.4 (75) a	0.02 (0.88)
Nenteria extremica	52.9 (126)	52.9 (82) a	53.0 (44) a	0.97 (0.35)
Acarus sp.	68.5 (163)	70.3 (109) a	65.1 (54) a	0.17 (0.68)
Dendrolaelaps sp.	38.2 (91)	36.8 (57) a	41.0 (34) a	0.18 (0.67)
Uroobovella sp.	24.8 (59)	26.5 (41) a	21.7 (18) a	0.67 (0.41)
Curculanoetus rhynchophorus	45.0 (107)	40 (62) a	54.2 (45) a	0.08 (0.78)
Mesostigmata	30.3 (72)	31.6 (49) a	27.7 (23) a	1.35 (0.25)

χ2: chi-square test value; *: no statistics are computed because Prevalence is constant; *χ2* (*p*) values in **bold** are significant ($\alpha = 0.05$); Different letters in the same row represent significantly different groups.

Prevalence of *N. extremica* and *C. rhynchophorus* was significantly different between districts (Chi-square test: $\chi 2 < 10.76$, $p > 0.01$). being lower in Aveiro for *N. extremica* and higher in Aveiro for *Uroobovella* sp. (**Table 5**).

Table 5. Prevalence for each mite species in samples districts of Aveiro, Porto, Braga and Viana do Castelo. The Prevalence was compared with chi-square test ($\alpha = 0.05$).

	Prevalence (% (*n*))					
	All weevils	Aveiro	Porto	Braga	Viana do Castelo	*χ2 (p)*
All species	100.0 (238)	100.0 (111)	100.0 (44)	100.0 (53)	100.0 (30)	*
Centrouropoda sp.	87.0 (207)	86.5 (96) a	88.6 (39) a	88.7 (47) a	83.3 (25) a	1.91 (0.59)
Nenteria extremica	52.9 (126)	44.1 (49) *a*	61.4 (27) bc	56.6 (30) ac	66.7 (20) bc	**10.76 (0.01)**
Acarus sp.	68.5 (163)	72.1 (80) a	72.7 (32) a	56.6 (30) a	70.0 (21) a	5.19 (0.16)
Dendrolaelaps sp.	38.2 (91)	32.4 (36) a	40.9 (18) a	41.5 (22) a	50.0 (15) a	0.99 (0.80)
Uroobovella sp.	24.8 (59)	30.6 (34) a	15.9 (7) b	20.8 (11) ab	23.3 (7) ab	**8.60 (0.04)**
Curculanoetus rhynchophorus	45.0 (107)	48.6 (54) a	45.5 (20) a	41.5 (22) a	36.7 (11) a	0.66 (0.88)
Mesostigmata	30.3 (72)	28.8 (32) a	25.0 (11) a	37.7 (20) a	30.0 (9) a	0.88 (0.83)

χ2: chi-square test value; *: no statistics are computed because Prevalence is constant; *χ2* (*p*) values in **bold** are significant ($\alpha = 0.05$); Different letters in the same row represent significantly different groups.

3.2.5. Mite load

Average mite load per host was 375.8±357.8 (1 – 1684) mites/host. There were no significant differences in mite load between sexes (female 393.5±373.7 mites/host v. male 347.6±338.1 mites/host) (Wilcoxon-Mann-Whitney test: $U = 6062.5$, $p = 0.47$).

The two different storing methods did not have significant differences in total mite load (non-frozen 402.6±374.9 mites/host v. frozen 307.4±328.7 mites/host) (Wilcoxon-Mann-Whitney test: $U = 4401.0$, $p = 0.10$).

No significant difference was found in mite load between districts (Aveiro 376.3±348.0 mites/host, Porto 468.3±425.0 mites/host, Braga 339.2±331.0 mites/host, Viana do Castelo 302.7±357.8 mites/host) (Kruskal-Wallis test: $H = 4.63$, $p = 0.20$).

Mite load per host was significantly different between sampling months (July 402.8±331.8 (7 – 1252) mites/host, August n = 475.7±383.6 (2 – 1564) mites/host, September n = 314.6±335.7 (4 – 1588) mites/host, October n = 404.9±387.2 (10 – 1684) mites/host, November = 211.9±237.3 (1 – 546) mites/host, January n = 394.7±458.0 (45 – 1272) mites/host) (Kruskal-Wallis test: $H = 11.2$, $p = 0.048$)) (**Figure 16**). September and November had significantly few mites than other months (Wilcoxon-Mann-Whitney test: *U*

> 2.0, $p < 0.04$). No value can be calculated for December as no weevils were collected in this month.

Figure 16. Mite load /host on *Rhynchophorus ferrugineus* throughout sampling months (bars) and temperature (line) (calculated as the average of the medians of all districts for each month). Different letters indicate significantly different months. Letters (a, b) represent different groups with significant differences with each other ($\alpha = 0.05$). No samples were collected in December: the missing column indicates missing data, not zero.

3.2.6. Mite load distribution per body part

All inspected surface body parts were associated with mite presence (**Figure 17, Table 6**). There were no significant differences in mite load per body part between sexes (Wilcoxon-Mann-Whitney test: $U = 6040.0$, $p = 0.44$).

Mite load was significantly different between all body parts (Friedman test: $\chi^2 = 706.8$, $p = 0.00$) except between the thorax and the neck (Wilcoxon signed ranks test: $Z = -1.72$, $p = 0.08$). Mite load on the elytral surface and membranous wings was significantly greater than those which were observed in the other body regions (280.5±275.7 mites/host) (Wilcoxon signed ranks test: $Z < -11.94$, $p = 0.00$) (**Table 6**).

Second highest mite load was observed in the abdomen (73.6±121.8 mites/host), followed by the legs (10.7±34.3 mites/host), thorax (4.9±15.1 mites/host), head and antenna (4.7±10.6 mites/host) and neck (3.1±8.9 mites/host) (**Table 6**).

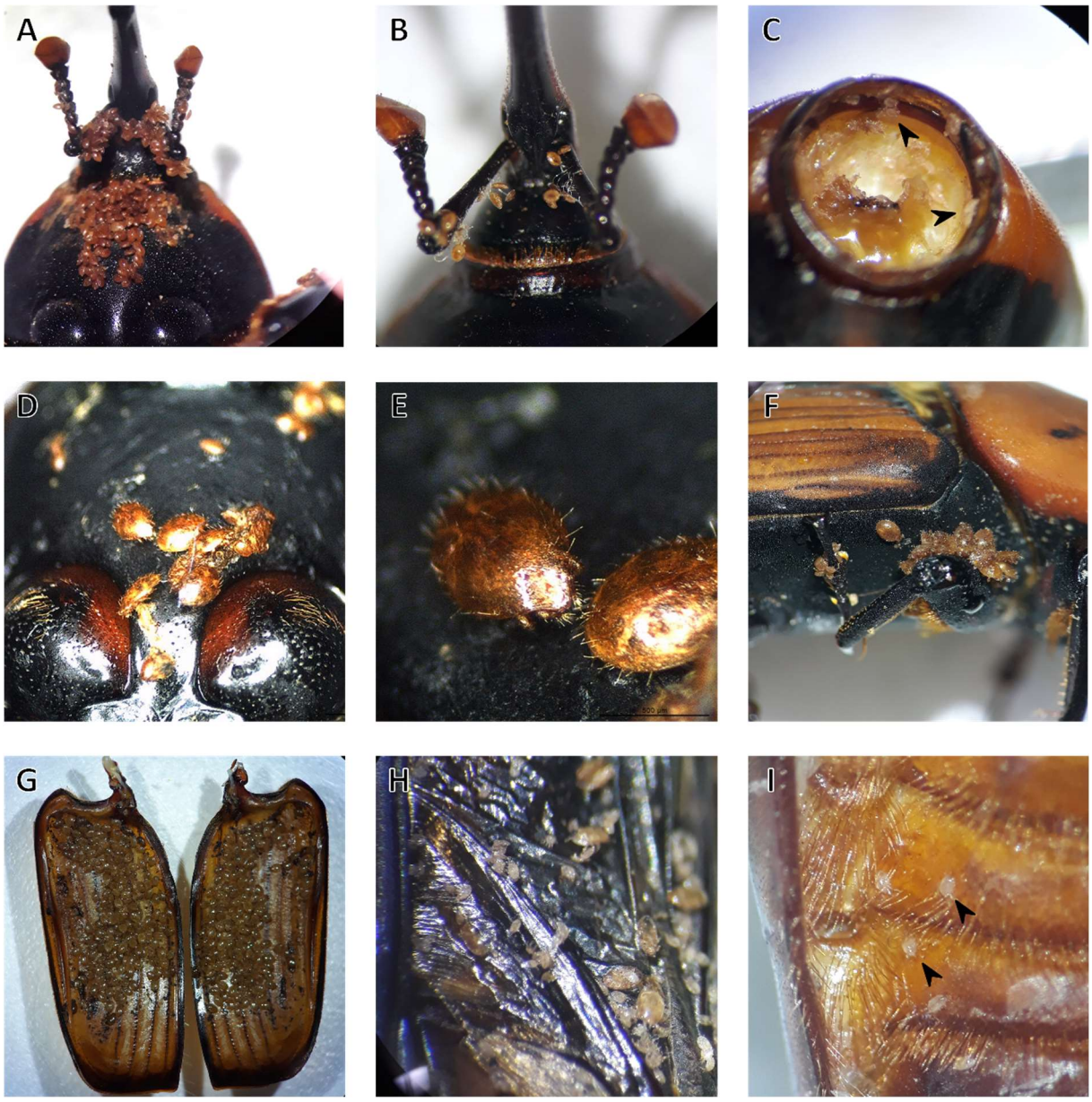

Figure 17. Mites (black arrows) on inspected body parts of *Rhynchophorus ferrugineus*. A: Head, antenna and upper thorax, B: head and antenna, C: neck, D and E: thorax, F: legs, G: elytra, H: membranous wings, I: abdomen.

3.2.7. Correlation of mite load and weevil body size

There were significant differences in the weevil's body weight between frozen and non-frozen samples (see section 3.1.3). Therefore, correlation analysis between this variable and mite load only considered non-frozen weevils. A significant and positive correlation between the weevil's mite load and its body weight was observed (Spearman correlation: $r_s = 0.25$, $p = 0.001$). If only considering frozen weevils, no significant correlation between weight and mite load was observed (Spearman correlation: $r_s = 0.03$, $p = 0.84$).

Considering all the data together (frozen and non-frozen weevils), no significant correlation between mite load and total length (Spearman correlation: $r_s = -0.04$, $p = 0.53$) or length without nostrum was found (Spearman correlation: $r_s = 0.07$, $p = 0.30$). Similarly, if considering only non-frozen or only frozen samples, no significant correlation between the weevil's mite load and respective length was observed (Spearman correlation: non-frozen $r_s = 0.01$, $p = 0.87$ v. frozen $r_s = 0.12$, $p = 0.33$).

3.2.8. Correlation of mite load and species diversity

A positive moderate correlation between mite load and species diversity was observed (Spearman correlation: $r_s = 0.54$, $p < 0.001$) which indicates that species diversity was higher when mite load was higher too.

3.2.9. Mite abundance

Mite species were significantly different in mite abundance (Friedman test: $\chi^2 = 427.2$, $p < 0.001$). *Centrouropoda* sp. was the most abundant species (142.9±165.3 mites/host), followed by *Acarus* sp. (111.5±220.1 mites/host), *C. rhynchophorus* (78.9±169.1 mites/host), *N. extremica* (15.9±52.5 mites/host), *Uroobovella* sp. (10.7±51.2 mites/host), *Dendrolaelaps* sp. (9.8±36.8 mites/host), and Mesostigmata (7.8±37.6 mites/host).

There were no differences in the abundance between sexes for *Centrouropoda* sp., *N. extremica*, *Acarus* sp., *Dendrolaelaps* sp., *Uroobovella* sp., and Mesostigmata (Wilcoxon-Mann-Whitney test: $U < 5808.5$, $p < 0.21$). However, *C. rhynchophorus* abundance was significantly higher in male weevils (female 69.4±167.0 mites/host v. male 96.6±172.7 mites/host) (Wilcoxon-Mann-Whitney test: $U = 5421.5$, $p = 0.03$) (**Figure 18**).

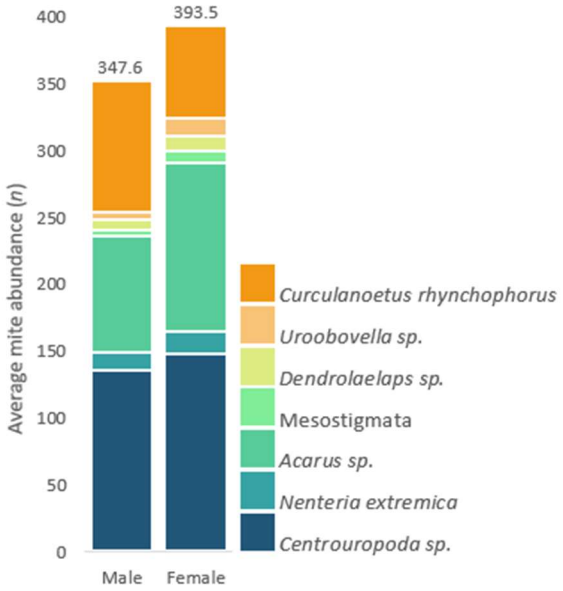

Figure 18. Mite abundance on female and male *Rhynchophorus ferrugineus*. Bars represent average mite abundance of mite species on female and male weevils with each colour representing a different species. Number on top of each bar is average mite abundace per sex.

In addition, there were no significant differences in abundance of *Centrouropoda* sp., *Acarus* sp., Mesostigmata, *Dendrolaelaps* sp., *Uroobovella* sp. or *C. rhynchophorus* between districts (Kruskal-Wallis test: $H < 4.57$, $p > 0.21$). However, there was a significant difference in *N. extremica* abundance between the districts of Aveiro v. Porto (Wilcoxon-Mann-Whitney test: $U = 1961.5$, $p = 0.04$) and Aveiro v. Viana do Castelo (Wilcoxon-Mann-Whitney test: $U = 1234.0$, $p = 0.02$). *Nenteria extremica* was significantly more abundant in Porto (32.0±89.5 mites/host), than Viana do Castelo (25.2±68.3 mites/host) and Aveiro (11.9±36.5 mites/host).

In terms of variation in mite abundance between different months, there were no significant differences in mite abundance of *Centrouropoda* sp., *N. extremica*, *Acarus* sp. and Mesostigmata between different sampling months (Kruskal-Wallis test $H < 9.72$, $p > 0.08$). However, *Uroobovella* sp. was significantly more abundant in July, August and September (Kruskal-Wallis test: $H = 11.91$, $p = 0.04$), and *C. rhynchophorus* was significantly more abundant in July, August, October and January (Kruskal-Wallis test: $H = 11.91$, $p = 0.04$) (**Figure 19**).

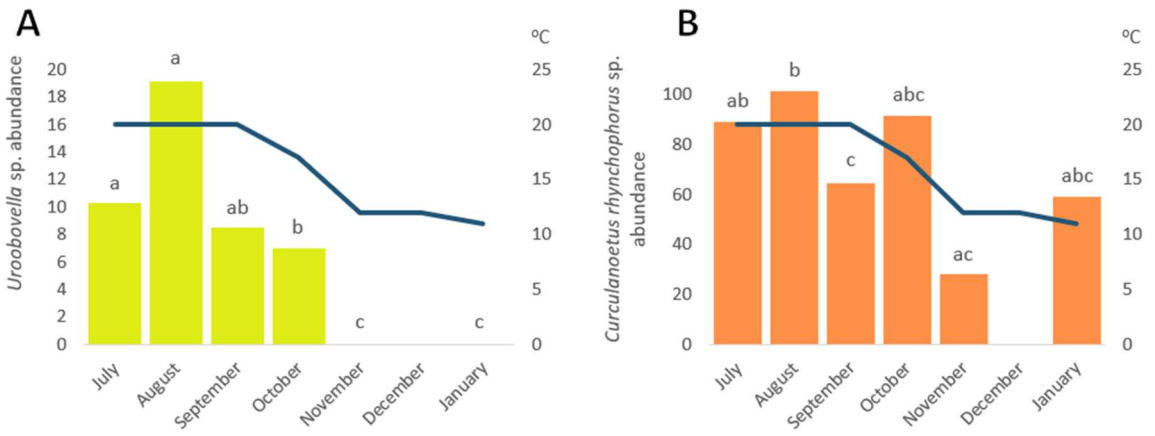

Figure 19. Mite abundance of *Uroobovella* sp. (A) and *Curculanoetus rhynchophorus* (B) associated with *Rhynchophorus ferrugineus* throughout sampling months from July 2021 to January 2022 (bars) and temperature (line) (calculated as the average of the medians of all districts for each month). Letters (a, b, c) represent different groups with significant differences with each other ($\alpha = 0.05$). No samples were collected in December: the missing column indicates missing data, not zero.

Table 6. Mite load for all body parts and mite abundance for each body part for each mite species.

	Mite load	Mite abundance						
	mean ± s.d. (range)	mean ± s.d. (range)						
Body part	All species	*Centrouropoda* sp.	*Nenteria extremica*	*Acarus* sp.	Mesostigmata	*Dendrolaelaps* sp.	*Uroobovella* sp.	*Curculanoetus rhynchophorus*
Total body	375.8 ± 357.8 (0-1684)	142.9 ± 165.3 (0-720)	15.9 ± 52.5 (0-469)	110.1 ± 213.0 (0-1201)	7.8 ± 37.6 (0-500)	9.5 ± 36.8 (0-354)	10.7 ± 51.2 (0-471)	78.9 ± 169.1 (0-1025)
Head & antenna	4.7 ± 10.6 (0-70)	0.3 ± 3.9 (0-60)	3.4 ± 9.0 (0-70)	0.1 ± 0.7 (0-10)	0.0 ± 0.1 (0-1)	0.2 ± 3.2 (0-50)	0.4 ± 1.8 (0-15)	0.3 ± 3.0 (0-44)
Neck	2.8 ± 6.8 (0-50)	0.0 ± 0.1 (0-1)	0.1 ± 0.8 (0-10)	0.4 ± 2.1 (0-17)	0.1 ± 1.9 (0-30)	2.2 ± 6.3 (0-50)	0.0 ± 0.1 (0-2)	0.0 ± 0.2 (0-3)
Legs	9.4 ± 25.8 (0-204)	0.3 ± 2.0 (0-30)	2.7 ± 12.4 (0-128)	4.3 ± 17.0 (0-200)	0.0 ± 0.1 (0-1)	0.0 ± 0.2 (0-2)	1.4 ± 11.5 (0-150)	0.6 ± 3.8 (0-50)
Thorax	4.9 ± 15.1 (0-161)	0.3 ± 2.1 (0-23)	0.3 ± 2.0 (0-22)	3.3 ± 12.6 (0-135)	0.0 ± 0.1 (0-2)	0.5 ± 3.8 (0-50)	0.4 ± 3.0 (0-40)	0.1 ± 1.2 (0-17)
Wings & elytra	280.5 ± 275.7 (0-1390)	135.6 ± 161.2 (0-720)	7.3 ± 39.2 (0-322)	57.0 ± 138.6 (0-1100)	5.7 ± 32.8 (0-450)	3.2 ± 22.9 (0-300)	8.0 ± 49.4 (0-469)	63.7 ± 136.6 (0-786)
Abdomen	73.6 ± 121.8 (0-700)	6.5 ± 17.6 (0-160)	2.1 ± 16.5 (0-180)	45.1 ± 105.5 (0-700)	1.9 ± 8.1 (0-70)	3.4 ± 27.2 (0-351)	0.5 ± 3.5 (0-41)	14.1 ± 54.2 (0-517)

3.2.10. Mite abundance distribution for body part

Different species were more abundantly associated with specific body parts (Friedman test: $\chi^2 > 27.97$, $p < 0.001$). *Centrouropoda* sp., *N. extremica*, Mesostigmata, and *C. rhynchophorus* were significantly more abundant on the membranous wings and elytra (Wilcoxon signed ranks test: $Z < -2.91$, $p < 0.02$). *Acarus* sp. and *Dendrolaelaps* sp. were equally more abundant on the membranous wings, in the elytra and the abdomen (Wilcoxon signed ranks test: $Z < -0.09$, $p > 0.932$). *Uroobovella* sp. was equally abundant on the legs and membranous wings and elytra (Wilcoxon signed ranks test: $Z = -1.63$, $p = 0.10$) (**Figure 20**).

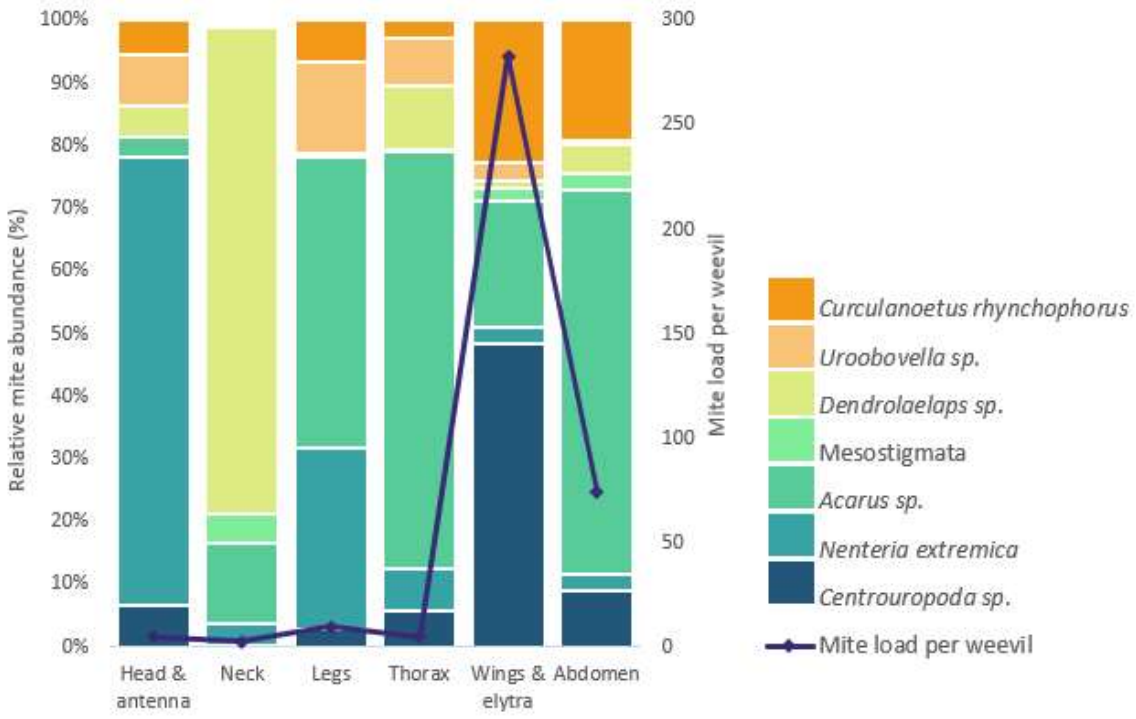

Figure 20. Relative mite abundance (bars) per body part (each bar) per species of mite (each color) and mite load per weevil for each body part (line).

Most of the mites of the species *Centrouropoda* sp., *Uroobovella* sp., and *N. extremica* found on the head and antenna, were attached to the weevil via anal pedicel (**Figure 21**).

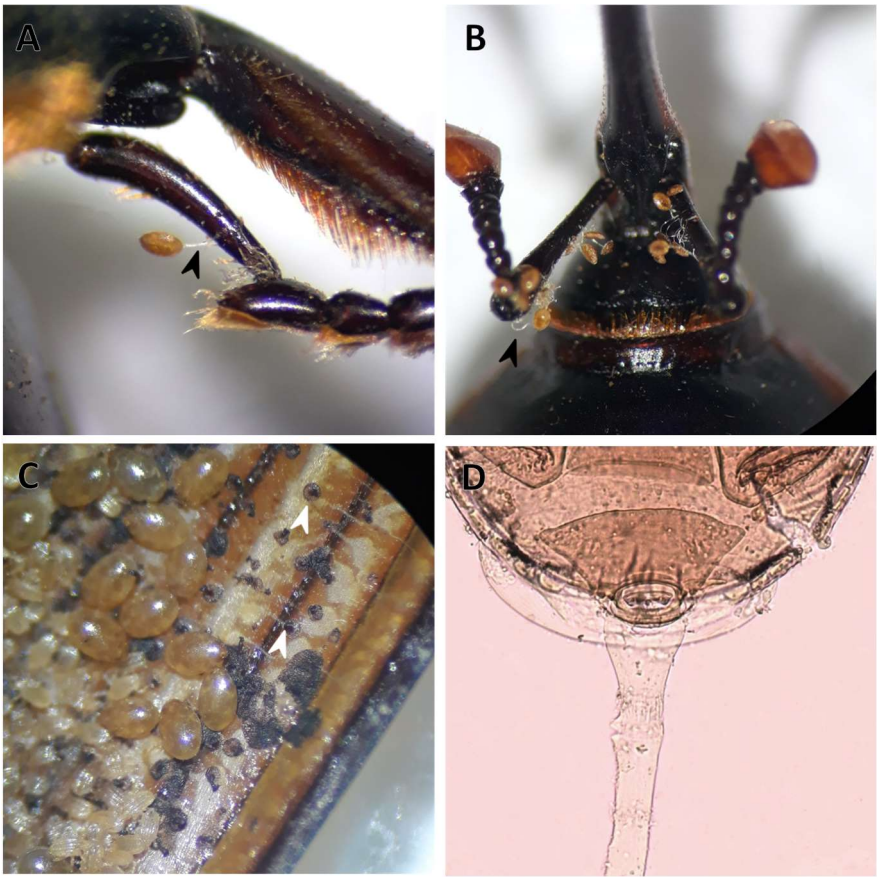

Figure 21. Mites attached via anal pedicel (black arrows) to *Rhynchophorus ferrugineus* on leg (A) and antenna (B). Remaining portion of anal pedicel (white arrows) after mite detachment on elytra (C). *Nenteria extremica* mite with anal pedicel (D).

Mites identified on the neck crevice were mainly *Dendrolaelaps* sp. These mites were observed in the inner part of the joint between the head and the thorax, in a particularly sheltered area (**Figure 22**).

The more abundant mite species identified on the weevil's legs, thorax and abdomen were *Acarus* sp. These mites were found distributed across external leg surface, but significant agglomerates were found on the leg crevice penetrating the weevil's abdomen and thorax. In the thorax, mites accumulate in the sub-thoracic surface that is in partial contact with the surface of the elytra (**Figure 22**).

Centrouropoda sp. was the more abundant species under the membranous wings and elytra (135.6±161.2 (0 – 720) mites/host) followed by equally abundant *Acarus* sp. (57.9±138.6 (0 – 1100) mites/host) and *C. rhynchophorus* (63.7±136.6 (0 – 786) mites/host). Membranous wings and elytra were by far the more populated area on the RPW's body. *Centrouropoda* sp. was found to occupy central areas and mites with smaller dimensions (Mesostigmata, *Dendrolaelaps* sp, *C. rhynchophorus*, *Acarus* sp.) were found on the external crevice of the sub-elytral space (**Figure 22**).

3.2.11. Correlation of mite abundance with species diversity

A positive and significant correlation was observed between mite abundance for each species and species diversity across all mite species (Spearman correlation *Centrouropoda* sp. $r_s = 0.36$, $p < 0.001$; *N. extremica* $r_s = 0.43$, $p < 0.001$; *Acarus* sp. $r_s = 0.45$, $p < 0.001$; Mesostigmata $r_s = 0.44$, $p < 0.001$; *Dendrolaelaps* sp. $r_s = 0.44$, $p < 0.001$; *Uroobovella* sp. $r_s = 0.41$, $p < 0.001$; *C. rhynchophorus* $r_s = 0.36$, $p < 0.001$), which means that species diversity is higher when mite abundance, for each species, is higher.

Figure 22. *Dendrolaelaps* sp. mite (black arrows) accumulation in neck (A, B) and *Acarus* sp. (black arrows) on sub-thoracic area (C, D) of *Rhynchophorus ferrugineus*. Mite distribution on *Rhynchophorus ferrugineus* sub-elytral space (E, F, G). Bigger and smaller dimension mites occupy different areas of the elytra with bigger mites populating the interior space (green circle) and smaller dimension mites occupying the peripheral and crevice space (orange circle) (E, F). Mite populations often overlap and occupied the same area (G).

3.3. Fungi

3.3.1. Identified species

Five genera of fungi were found in association with the RPW. Four were identified through observations of morphological characteristics in cultures in PDA (**Figure 23**) and DNA sequencing (a phylogenetic tree was made – **Figure 24**): *Alternaria* sp., *Scopulariopsis* sp., *Fusarium* sp., *Penicillium* sp.; and one species was identified via observation of the spores in mites associated with the RPW but it was not possible to cultivate on PDA: *Curvularia* sp. (**Table 7**, **Figure 23**).

Table 7. Taxonomic levels of fungi identified in association with *Rhynchophorus ferrugineus*.

Kingdom	Phylum	Class	Order	Family	Genus	Species
Fungi	Ascomycota	Sordariomycetes	Microascales	*Microascaceae*	*Scopulariopsis*	sp.
Fungi	Ascomycota	Sordariomycetes	Hypocreales	*Nectriaceae*	*Fusarium*	sp.
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	*Trichocomaceae*	*Penicillium*	sp.
Fungi	Ascomycota	Dothideomycetes	Pleosporales	*Pleosporaceae*	*Curvularia*	sp.
Fungi	Ascomycota	Dothideomycetes	Pleosporales	*Pleosporaceae*	*Alternaria*	sp.

Alternaria sp. was found in association with samples from Viana do Castelo; *Fusarium* sp. from samples from Porto, Aveiro and Braga; *Penicillium* sp. from Aveiro, Braga and Viana do Castelo; and *Scopulariopsis* sp. from Aveiro (**Table 8**). *Curvularia* sp. was identified on *Centrouropoda* sp. and *Acarus* sp. in Aveiro and Porto, and on Mesostigmata only in Aveiro.

Table 8. Sampled fungal organisms and respective month and district of sampling.

Organism	Isolate	Sampling month (2021)	Sampling district
Scopulariopsis sp. Bainier	Av1	July	Aveiro
Scopulariopsis sp. Bainier	Av2	July	Aveiro
Scopulariopsis sp. Bainier	Av3	July	Aveiro
Alternaria sp. Nees	Vi1	August	Viana do Castelo
Fusarium sp. Link	Pt1	August	Porto
Fusarium sp. Link	Pt2	August	Porto
Fusarium sp. Link	Pt3	August	Porto
Fusarium sp. Link	Av4	August	Aveiro
Fusarium sp. Link	Br5	October	Braga
Penicillium sp. Link	Av3	August	Aveiro
Penicillium sp. Link	Av7	September	Aveiro
Penicillium sp. Link	Av8	September	Aveiro
Penicillium sp. Link	Vi9	September	Viana do Castelo
Penicillium sp. Link	Vi10	September	Viana do Castelo
Penicillium sp. Link	Br6	October	Braga
Curvularia sp. Boedijn			

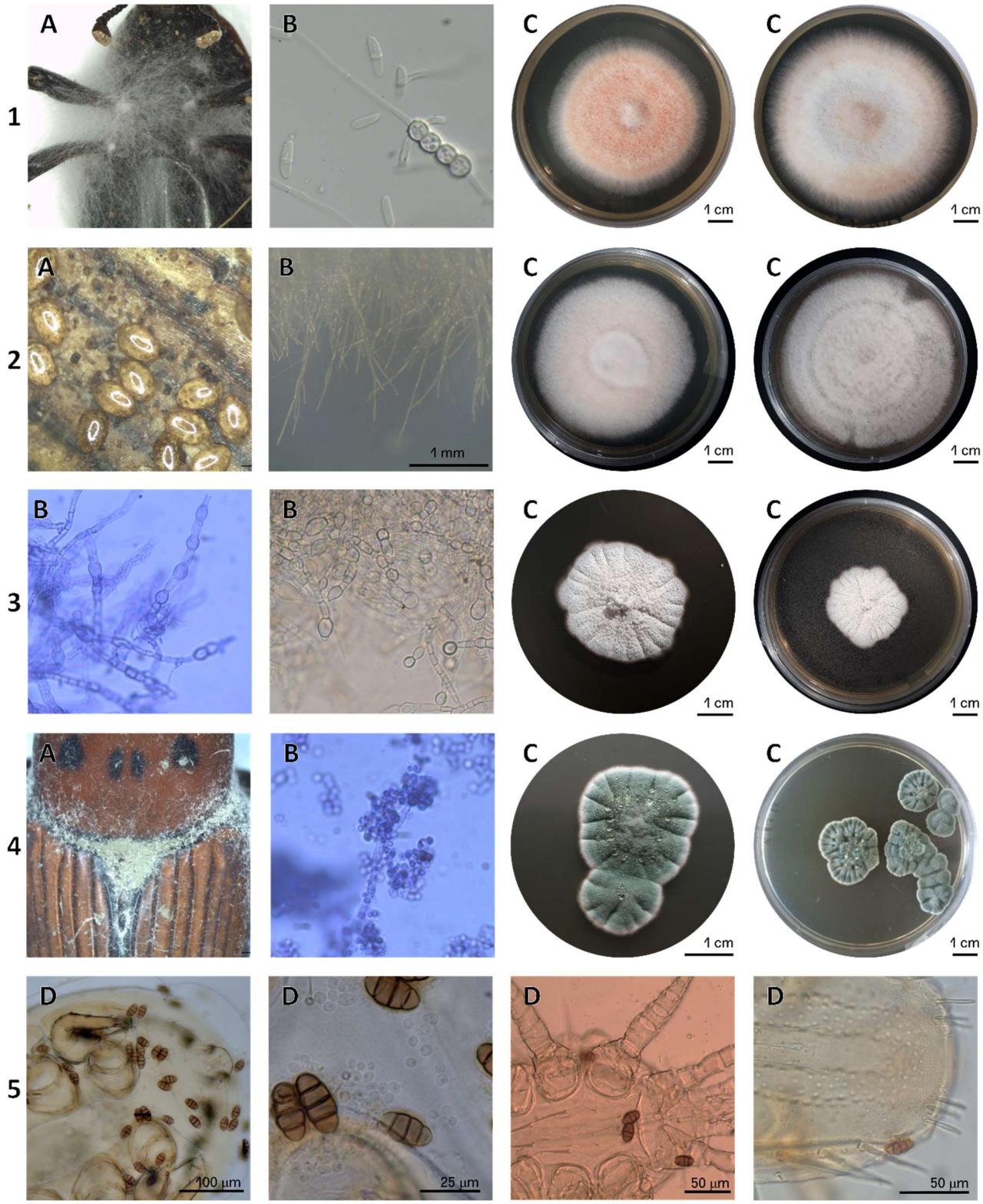

Figure 23. Fungi identified in association with *Rhynchophorus ferrugineus* and its mites. Each row is representing each species of identified fungi: 1: *Fusarium* sp., 2: *Alternaria* sp., 3: *Scopulariopsis* sp., 4: *Penicillium* sp., 5: *Curvularia* sp.. A: Visible fungi growth on *R. ferrugineus*, B: Microphotograph of fungal mycelia and/or spores, C: Fungal growth on 9 cm Petri dish with potato dextrose agar, frontal view, D: Fungal conidia presence on mites.

3.3.2. Molecular analysis

All obtained sequences used for identification are from the 18S region. Although the ITS region amplification was attempted, no fungal DNA extractions were successfully amplified with ITS primers. All obtained sequences matched the GenBank database with >99.3% matched percent identity.

The three obtained sequences matched sequences from the 18S region identified as *Scopulariopsis* sp., *Scopulariopsis crassa*, *Scopulariopsis brevicaulis* and *Doratomyces stemonitis* with >99.9% percentage identity match (**Annex B7**). *Scopulariopsis* sp. and *Doratomyces* sp. have very similar conidiophores, however, *Doratomyces* sp. conidiophores grow in a stalk-like structure (the synnema) that is absent in *Scopulariopsis* sp. (Webster & Weber, 2007). Observed fungi had no stalk-like growth of conidiosphores (**Figure 23**), therefore these were identified as *Scopulariopsis* sp. Considering that both species of *Scopulariopsis* genus matched the sequences equally the identification was only considered to the genus.

Fusarium sp. yielded five sequences with between 857 bp and 1032 bp. The five obtained sequences matched sequences from the 18S identified as *Fusarium* sp., *Fusarium solani* and *Fusarium oxysporum* with 100% identity match and 100% query cover (**Annex B9**). Considering that samples equally matched *Fusarium* sp., *Fusarium solani* and *Fusarium oxysporum* sequences, we only consider identification to genus level.

Penicillium sp. yielded six sequences with between 946 bp and 1025 bp. The six obtained sequences matched sequences from the 18S region identified as *Penicillium* sp., *Penicillium brevicompactum*, and *Penicillium crustosum* with >99.0% identity match and 100% query cover (**Annex B10**). Considering the match of the same sequences to various species from this genus, identification was only considered to the genus.

Alternaria sp. yielded a consensus sequence of 1029 bp. The obtained sequence matched *Alternaria* sp., *Alternaria tenuissima*, *Alternaria alternata*, *Alternaria solani* e *Alternaria japonica* with 100% identity match and 100% query cover (**Annex B8**). Since all matches were with species from the genus *Alternaria* sp. this identification was considered.

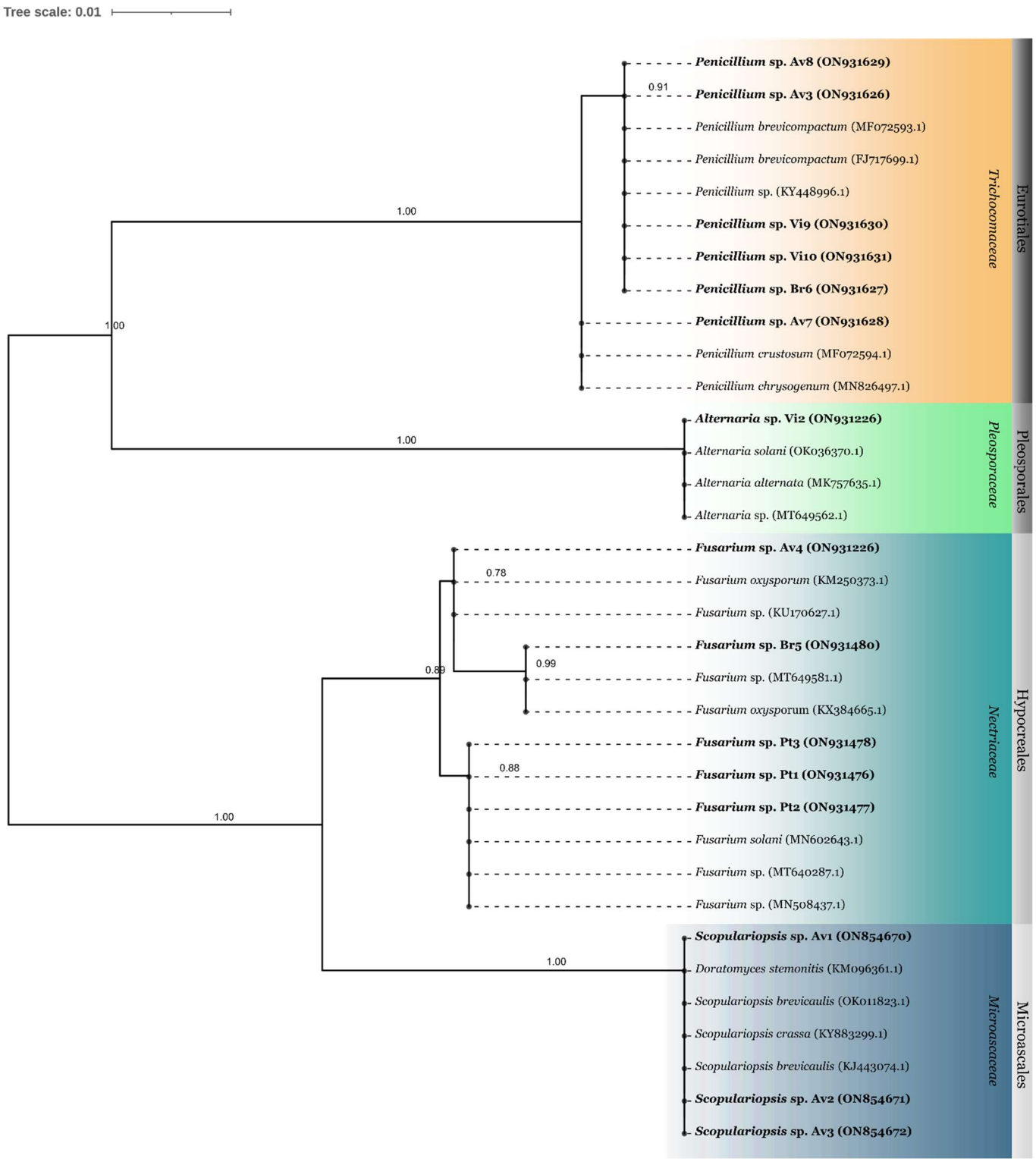

Figure 24. Phylogenetic tree with sequences obtained from fungi found in association with *Rhynchophorus ferrugineus*. Names in **bold** are sequences obtained in this study. Each colour represents a family, gray boxes represent different orders. Code between parenthesis indicates the GenBank accession number for each sequence. Bootstrap values are indicated in respective branches.

4. Discussion

This dissertation is the first work that characterizes RPW populations and their associated organisms (mites and fungi) in northern Portugal, and that provides molecular characterisation of the mite species associated with the RPW. Considering that this insect is a pest with substantial impact in the country, and an effective and sustainable control method is yet to be developed, any added contributions to this field are highly valuable and may lead to useful knowledge for their effective control.

4.1. Weevils

Sex ratio of the sampled weevils was 1 male: 1.9 females (see section 3.1.1). Aggregation pheromone traps have been found to consistently attract more female than male weevils by other authors in several countries (Abbas *et al.*, 2006; Al-Saoud, 2013; Wahizatul Afzan Azmi, 2014; El Garhy, 1996; Haris *et al.*, 2014; Kaakeh *et al.*, 2001; Ramos *et al.*, 2015), e.g. 1 male: 2.1 females in Portugal (Ramos *et al.*, 2015), 1 male: 1.5 females in the United Arab Emirates (UAE) (Abbas *et al.*, 2006), 1 male: 2 females in Egypt (El Garhy, 1996). Despite these results, this does not necessarily reflect a higher proportion of female weevils in the natural populations, given that pheromone traps may sample as little as 30% of the local population, and other studies, using different capture methods, have found no difference in sex ratio of sampled weevils (Abbas *et al.*, 2006; Senafi *et al.*, 2020). Indeed, sampling methods that are not pheromone based do not present the same high presence of female weevils (Ávalos *et al.*, 2014). Poorjavad *et al.* (2009) found that higher proportion of pheromone-attracted females may result from their higher innate attraction to the aggregation pheromones for oviposition. Mated females, as opposed to males and non-mated females, are more attracted to aggregation pheromones possibly due to the fact that they are in active search of new healthy palms to lay eggs (Poorjavad *et al.*, 2009). These observations indicate that capturing weevils with pheromone traps might be a productive method for pest control given that a single female can lay hundreds of eggs (Haris *et al.*, 2014), implying that the removal of a few females from the environment can result in a strongly decrease of the negative effects on the palm tree in the vicinity.

A higher number of weevils fell into the pheromone traps in the warmer months of July to September, with a maximum which was reached in September (44% of total sample) (see

section 3.1.2). El Garhy (1996) had similar results in Egypt, with more weevils captured during the summer months, when the average temperature was above 14 ºC. Other studies also consistently reported higher sampling with pheromone traps and overall RPW activity when temperatures were higher and rainfall was lower (Wahizatul Afzan Azmi, 2014; Haris *et al.*, 2014; Vidyasagar *et al.*, 2000). However, when temperatures reach more than 39 ºC during the day, RPW have been found to reduce movement and seclude from heat in palm trees and surrounding soil (Abbas *et al.*, 2000).

Given that the RPW is more active in warmer temperatures (up to 39 ºC) (Abbas *et al.*, 2000), the global rising in average temperatures (Lindsey & Dahlman, 2020) might result in a niche expansion and further spread of this invasive pest beyond the current invaded regions such as, central European countries. The RPW has also been found to have metabolic mechanisms to tolerate lower temperatures in the winter (León-Quinto & Serna, 2022), which might contribute for the species to tolerate the cold winter and then thrive and spread in warmer seasons.

The activity of adult RPW was observed in almost all months of sampling (all except December) (see section 3.1.2), even in the winter months, which indicates a good adaptation of these populations to the Portuguese climate, which potentiates its effect as a pest, during almost all year.

Female weevils, considering only non-frozen samples, were found to be heavier and bigger than male weevils (weight: female 1.20±0.30 g v. male 1.05±0.31 g; length: female 3.44±0.41 cm v. male 3.25±0.34 cm) (see section 3.1.3). The longer body size of female weevils has been described in literature (female 3.28 cm v. male 3.08 cm (Al-Ayedh, 2008); female 4.15 cm v. male 3.90 cm (Viado & Bigornia, 1949)).

As with most insect species, the bigger weight of sampled females, has been described in the literature in different localities, such as Spain (female 1.18±0.03 g v. male 1.04±0.02 g) (Ávalos *et al.*, 2014) and Saudi Arabia (female 1.27±0.04 g v. male 1.13±0.05 g) (Hoddle *et al.*, 2015). However, other studies, such as in Saudi Arabia, reveal no significant difference between male and female weevils (female 1.46±0.0 g v. male 1.48±0.08 g) (Al Ansi *et al.*, 2020).

In our study, storing methods were found to correlate with significant differences in the weevil's weight (frozen 0.56±0.23 g v. non-frozen 1.15±0.31 g) (see section 3.1.3). Frozen samples were, not only frozen, but also kept in the lab on apple diet for a longer period than non-frozen samples. Salama and Abdel-Razek (2002) observed different weights of adult RPW when specimens were fed with different diets, where weevils had higher weight with a banana diet (2.19 g for both female and male), followed by sugar cane diet (female 1.69 g v. male 1.41 g) and then "semi-artificial diet" (female 1.35 g v. 0.69 g). Another study that fed weevils a meridic diet, mainly consisting of agar, yeast, wheat, and corn flour revealed lower weights (female 0.57 g v. male 0.54 g) (El-Shafie *et al.*, 2013), which were similar to the weight of the frozen samples. Therefore, it is also possible that the observed differences in weight were a result not only from the preservation process (frozen v. non-frozen), but also from the different diets fed to the weevils.

This indicates the importance of choosing an adequate diet and storing methods of weevils and of detailing diet and storing methods in research studies to rule out the influence of these factors on weight or other parameters.

A vast diversity of thoracic pattern variation in RPW has been described (Rugman-Jones *et al.*, 2013). In our studied weevils we also observed a diversity of patterns (see section 3.1.5). Observed differences in number and size of black thoracic spots have been documented in other populations (Rugman-Jones *et al.*, 2013) and do not necessarily indicate novelty in observed populations.

4.2. Mites

Out of the seven species found, six of these recorded genera (*Acarus* sp., *C. rhynchophorus*., *N. extremica*, *Centrouropoda* sp., *Dendrolaelaps* sp., *Uroobovella* sp.), have been previously documented in association with the RPW (Dilipkumar *et al.*, 2015). The order Mesostigmata has been described extensively in association with the RPW (Dilipkumar *et al.*, 2015), however, the species assigned as Mesostigmata was not considered as "previously described" given that it was only identified to the order level and, therefore, it was not possible to ensure with certainty that the observed species was found on RPW in other studies.

The six mites identified at genus level were found to be in deutonymphal life stage. Deutonymphs are the phoretic life form - a facultative dispersal stage (Bajerlein & Witaliński, 2014). Previous descriptions of the mentioned species in association with the RPW were also identified as deutonymphs (Al-Deeb *et al.*, 2011; Allam *et al.*, 2013; El-Sharabasy, 2010; Farahani *et al.*, 2016; Kontschan *et al.*, 2014a; Slimane-Kharrat & Ouali, 2019).

This study is the first that tests the efficacy of genomic tools for complementing morphological identification of mites associated with the RPW. Although sequence amplification was obtained when using primers for the COI region, resulting sequences did not have sufficient quality for successful identification via BLAST tool (see section 3.2.2). This might be due to the diversity found in mite physical characteristics, that may contribute to the difficulty to use a unique extraction method which can be universally applied. For example, *Uroobovella* sp. is significantly bigger and has a stronger outer layer than *Acarus* sp., which indicates that it possibly needs an adaptation of the extraction methods.

However, the 18S region amplification yielded quality sequences of mite DNA that could be contextualised taxonomically to the family level (see section 3.2.2.1). Although identification to the genus or species level would be ideal, using this region for identification to the family level is already a useful tool (considering that for example, mite identified as Mesostigmata was only identified to the order level) and presents novelty in the molecular study of RPW-associated mites.

One of the challenges of molecular identification is the lack of existing sequences for observed mites in current databases. For example, *Centrouropoda almerodai*, one of the more universally described mites in association with the RPW (Dilipkumar *et al.*, 2015), and *C. rhynchophorus* had no sequences for any DNA region on the GenBank database. The sequences uploaded in the context of this work are the first ones of the *Centrouropoda* sp. genus.

Previous studies report the difficulty in morphological-based identification due to scarcity of organised taxonomic literature and also previous erroneous identifications by other authors (Dilipkumar *et al.*, 2015). Therefore, the betterment of mite DNA extraction protocols and PCR amplification primers for these mites could be of extreme relevance considering that it may provide a solution to overcome difficulties in the morphological

identification. Further studies, that explore different DNA extraction methods (such as commercial kits) and testing of different primers for different regions, are warranted for the widespread adoption of molecular tools in future identification of RPW-associated mites.

Mites were present in quite high diversity with an average 3.5±1.4 species of mites per weevil in a total of 7 identified species (see section 3.2.3). Farahani *et al.* (2016) found only 3 different species in Iran, El-Kady *et al.* (2017) 10 genus in Egypt, Slimane-Kharrat and Ouali (2019) 3 species in Tunisia, Al-Deeb *et al.* (2011) 2 species in UAE, Al-Dhafar and Al-Qahtani (2012) 3 species in Saudi Arabia, Dilipkumar *et al.* (2015) 5 species in Malaysia, and Abolafia and Ruiz-Cuenca (2020) 1 species in Spain. The relatively high number of species in the samples is likely not a result of the distance between sampling sites (135.9 km maximum distance), given that no significant difference was found between diversity of mites for different districts, or the species distribution between districts except for *N. extremica* between Aveiro, Viana do Castelo and Porto.

Mite species found on the weevils' body could have two sources: transportation with the weevil from the old to the new location, or mites originating from the new location. Considering that almost all mite species found have been associated with the RPW, it is possible that the observed mites have been transported with the weevil from other localities to the studied regions. Thus, it is not possible to conclude with certainty that the species diversity is a result of diverse local mite communities. Given the current widespread of RPW populations it is possible that the observed populations originate from multiple locations and therefore harbour mites from different regions, increasing the local numbers of mite species. In order to access this, further studies that investigate the mite diversity in non-infected palms and phylogenetic studies of mite populations could be performed.

Mite species diversity was observed to fluctuate between different months (see section 3.2.3), being the lowest in September and November. These results do not reflect a seasonal variation of mite diversity considering that October had highest diversity. Hassan *et al.* (2011) observed higher species diversity, and more deformed adults, in the winter, possibly due to the fact that the RPW spends more time secluded with focus on reproduction in the colder months and can, consequently, associate with mites that are roaming the infested host. These results and conclusion are somewhat impaired by the low number of RPW individuals collected in the months of November (n = 7) and January (n = 6).

In addition to a high species diversity, sampled weevils also had a relatively high average number of mites on their body (377.5±361.6 mites/host) (3.2.5). Lokela *et al.* (2021) found an average of 30 mites per female weevils and 20 per male weevil in the Democratic Republic of Congo on *Rhynchophorus phoenicis*, Farahani *et al.* (2016) found an average of 62 mites/host in Iran, Atakan *et al.* (2009) found an average 213 mites /female host and 189 mites /male hosts in Turkey, and Dilipkumar *et al.* (2015) an average of 300-400 mites/host in Malaysia. It is interesting to note the above-mentioned study that is in the RPW's native habitat (Southeast Asia – in Malaysia) (Dilipkumar *et al.*, 2015), is associated with higher mite load, which indicates that these mite species are well adapted to Portugal's climate and habitats, as they are in its native origin.

Total mite load was slightly higher in female weevils although not significantly different (female 393.5±373.7 mites/host v male 347.6±338.1 mites/host). Other studies have reported higher mite load on female weevils. Lokela *et al.* (2021) found 30 mites/host on females and 20 mites/host on males on *Rhynchophorus phoenicis*; Al-Deeb *et al.* (2011) found a maximum of 598 mites/host on females and maximum 381 mites/host on males.

Similarly to species diversity, mite load was lower in the months of September and November. These observations could be a result of differing timing in mite life cycle, where some mite families could be more secluded during specific months and others might be in their phoretic phase. However, no correlation between temperature and these parameters was observed, considering that October had a higher mite load than September and November.

Considering that female weevils had significantly higher weight, size, and slightly (but not significant) higher mite load, it is possible that bigger weevil body dimensions might result in higher mite load, due to more available area for occupation. A positive and significant correlation between mite load and the weevil's body weight was observed, considering only the non-frozen samples. However, no significant correlation with body length was seen. These observations may indicate that either weevil width, which was not measured for these samples, is the defining factor that correlates to mite load, or the mites prefer weevils with higher body weight. The host's weight might be an indication of overall fitness and therefore be a sensible criterion for host selection by mites.

There was no correlation between weight and mite load when considering only the frozen weevils (see section 3.2.7) which indicates a significant variation in the weevil's

weight as a result of freezing or different diet, or eventual loss of mites during the freezing process, not allowing for a correlation analysis that includes frozen samples.

Mites were found associated with all external body parts of the weevil. The maximum mite load was found on the inner surface of the elytra and membranous wings (280.5±275.7 mites/host). This finding is consistent with previous studies that note the higher accumulation of mites on the sub-elytral space (Al-Deeb *et al.*, 2011; Dilipkumar *et al.*, 2015; Mazza *et al.*, 2011). Besides being a warmer and more humid environment, this area is the only external surface where mites are sheltered from being brushed against palm-tree tissue or the wind during flight. It is probable that mites preferably occupy the sub-elytral area before occupying other less favourable spaces. The thorax and neck had similar average mite load. These two regions are nearby and provide comparable lack of shelter from outside contact. Therefore, it is expectable that similar mite load was found in these two body parts and not between other sites.

The accumulation of high number of mites on the elytra and wing area might lead to flight capacity impairment on the host. Bajerlein and Bloszyk (2004) reported that this happens to some families of beetles when at high densities of more than 100 mites. Our results reveal that specimens can carry up to 1684 mites per host (see section 3.2.5), which could possibly entail a fitness cost for the weevil. However, it is relevant to note that the traps used in this study only capture weevils that have the ability to fly to the trap, which indicates that weevils that might have their flight ability significantly impaired by mite presence were not sampled in this study, suggesting the potential for a greater impact on host's fitness than the observed mite load suggests. However, Mazza *et al.* (2011) observed higher presence of mites and lower longevity in trap-collected over plant-collected individuals, which suggests otherwise.

The presence of mites in elevated number on other insects has been shown to entail fitness cost in foraging activities, general movements (Al-Deeb *et al.*, 2011; Hyatt, 1959), flight ability (Kinn, 1966), flight altitude and speed (Kinn, 1970). Other host-mite associations have been considered of parasitic nature (implying a negative impact on the host) (Al-Deeb *et al.*, 2011; Cardoza *et al.*, 2008; Dilipkumar *et al.*, 2015; Houck & Cohen, 1995; Mazza *et al.*, 2011). It is possible that mites that have been long called phoretic could be parasitic, in case of their presence in large numbers, due to their role in flight hindrance

(Mazza *et al.*, 2011). Moreover, Kontschan *et al.* (2014b) also suggest that mites can feed on RPW larvae, which would increase their parasitic characteristic. Further behavioural studies about the specificity of these interactions are warranted in order to conclude with certainty about the impact of the mite's presence and their potential as biocontrol agents.

Female and male weevils revealed similar species abundance with the exception of *C. rhynchophorus* that had significant differences between sexes (female 69.4±167.0 mites/host v. male 96.6±172.7 mites/host) (see section 3.2.9). Therefore, the difference between males and females is not a result of differing anatomy considering that this area is similar but only different in dimension. *Curculanoetus rhynchophorus* was first described in association with *Rhynchophorus phoenicis* (Fain, 1974) and has been found in association with the RPW in the United Arab Emirates (Al-Deeb *et al.*, 2011) and China (Yu, 2019). However, none of these results mention a preference for the host's sex. Further studies of *C. rhynchophorus* populations in the RPW will possibly allow to discriminate the criteria for host choice of this mite.

All mite species, with the exception of *N. extremica*, were found to be equally abundant in the studied districts (see section 3.2.9). *Nenteria extremica* was found to be more abundant in the district of Porto. Uropodina mite group (to whom *N. extremica* belongs) populations have been found to decrease in number and diversity with increasing habitat degradation (Napierała *et al.*, 2015). However, this might not be the cause of the differences in abundance in our samples given that the traps placed in the district of Porto were placed in urban areas, that are typically more degraded, as opposed to Viana do Castelo and Aveiro that were in the vicinity of more vast green areas. Eventually this difference in abundance is conditioned by other factors (i.e., proximity to shore, humidity, seasonal temperature variation).

Most of the observed *Centrouropoda* sp., *N. extremica* and *Uroobovella* sp. specimens were attached to the weevil via anal pedicel. The anal pedicel is a glue-like structure that is projected from the anal area of deutonymphal mites (**Figure 21**). This attachment structure has been previously described in Uropodoid mites, which include *Centrouropoda* sp., *Uroobovella* sp., and *N. extremica* (Farahani *et al.*, 2016; Knee *et al.*, 2012a). Considering that the anal pedicel provides a strong attachment method, it is likely to be the reason why *N. extremica* was the more abundant species on the head and antenna, a very exposed body part.

Dendrolaelaps sp. was the more abundant species found on the neck (2.2±6.3 mites/host (0 – 50)). Unlike Uropodoid mites, which can attach via anal pedicel, or *Acarus* sp. and *C. rhynchophorus*, which can attach via sucker plate (Al-Deeb *et al.*, 2011; Hughes, 1957), *Dendrolaelaps* sp. are not physically attached to the weevil's body. Therefore, these small mites need more protection against external agents or the weevil's body movements. Considering that the sheltered neck area offers shield by providing a physical barrier, this might be the reason why these species accumulate in this region.

Acarus sp. was the more abundant species found in association with the legs (4.3±17.0 (0 – 200) mites/host), thorax (3.3±12.6 (0 – 135) mites/host) and abdomen (45.1±105.5 mites/host (0 – 700)) of the weevil. This species was found distributed on the legs' external surface, the joint cavity between the legs and the body, the sub-thoracic surface and in the more peripheral areas of the abdomen. Even though *Acarus* sp. has a somewhat strong attachment through their sucker plates, a more guarded environment is beneficial which explains the preference for more sheltered body parts of the host. The more reduced dimensions of this mite, which was the smallest recorded, also make these an advantageous location. It also could be hypothesised that the leg hairs on male weevils could offer advantageous shelter. However, no significant differences in *Acarus* sp. abundance were found between male and female weevils which indicates no evident advantage of leg hairs in sheltering for *Acarus* sp. mites.

Centrouropoda sp., the second observed largest mite, was the more abundant species under the membranous wings and elytra (135.6±161.2 (0 – 720) mites/host), followed by equally abundant *Acarus* sp. (57.9±138.6 (0 – 1100) mites/host) and *C. rhynchophorus* (63.7±136.6 (0 – 786) mites/host). The separation of bigger mites on the central areas of the elytra and smaller mites on peripheral areas indicates a need for segregation between mite species that might result from interspecific competition for attachment space to the host.

4.3. Fungi

Five genera of fungi were identified in association with the RPW: *Scopulariopsis* sp., *Alternaria* sp., *Fusarium* sp., *Penicillium* sp., and *Curvularia* sp (see section 3.3.1). Only *Scopulariopsis* sp. had not been previously associated with the RPW (Wahizatul A Azmi, 2013; Porcelli *et al.*, 2009; Ramos *et al.*, 2015). *Penicillium* sp., *Alternaria* sp. and *Curvularia* sp. have been associated with mites (Hubert *et al.*, 2004; Porcelli *et al.*, 2009).

Fusarium sp., *Alternaria* sp. and *Penicillium* sp. are generally classified as opportunistic saprophytes. *Fusarium* sp. and *Alternaria* sp. are plant pathogens that have been observed to be transported by arthropods (Heitmann *et al.*, 2021), *Penicillium* sp. and *Alternaria* sp. produce mycotoxins that can be pathogenic to agricultural crops (Sweeney & Dobson, 1998), *Curvularia* sp. and *Penicillium* sp. are the cause of infectious diseases in agricultural cultivars (namely the leaf spot of maize) (Akinbode, 2010; Garcia-Aroca *et al.*, 2018) and human health in particular for immune-compromised patients (De la Monte & Hutchins, 1985; Pitt, 1994). The observed association of these fungi with the RPW indicates that the threat of this weevil goes beyond being a threat to palm trees through direct impact but also as a vector for pathogenic agents that might menace surrounding flora and agricultural crops and human health. Yet, these fungi are described as ubiquitous, and their spread might not be caused exclusively by the presence of RPW.

Fusarium sp. has been documented as a pathogen of oil palm trees (Flood, 2006). Therefore, considering we found this fungus in association with the weevil, the weevil-fungi complex might be responsible for palm tree damage and death in Portugal. It could be that the weevils render the host palm tree more vulnerable to opportunistic infection by *Fusarium* sp., which indicates that a combined control method of an insecticide with a fungicide might be more effective, considering their combined effect in palm tree degradation, as suggested by Ziedan *et al.* (2022).

This fungi has also been observed to cause infection in humans and approximately 70% of *Fusarium* infections in humans are caused specifically by *F. oxysporum* and *F. solani* (Nucci & Anaissie, 2007), therefore, identifying RPW-associated fungi to the species level is important in order to specify the specific threat that these fungal strains represent.

Some species of the *Scopulariopsis* genus have been found to cause severe skin and respiratory infections in immunosuppressed humans (Iwen *et al.*, 2012; Kriesel *et al.*, 1994; Steinbach *et al.*, 2004). Hence, similarly to *Penicillium* sp. and *Curvularia* sp., the transportation of these fungi to urban areas (often colonised by RPW) can potentially endanger human health.

Mites from the *Acarus* sp. genus have been found to feed on *Penicillium* sp. species that are present in soil (Sinha & Mills, 1968) and have been found to have moderate entomopathogenic effect on nymphs of the arthropod Triatominae family (da Costa *et al.*,

2003). This could indicate that more abundant *Acarus* sp. populations in association with the RPW could potentially benefit the weevil in keeping it free of this potentially entomopathogenic fungus. In addition, considering that this strain has been described to have negative impact on agricultural crops (Sweeney & Dobson, 1998), *Acarus* sp. mite could have a positive impact on phytopathogen control.

However, it is relevant to note that our data is limited and pertains only to presence and not to abundance or prevalence of fungi in RPW populations. Considering that the identified fungi might be present in few specimens of RPW populations, further studies with insight on these parameters are needed in order to conclude with certainty of the real impact the RPW might have as a fungal carrier.

Although the 18S region is described as a sequence for higher taxonomic level identification (Raja *et al.*, 2017), a significant difference was found between reported sequences to identify up to the genus level, as made clear from our phylogenetic analysis (**Figure 24**). However, further studies with the ITS region or with primers specific to the identified genus would be interesting to further specify the mycobiome associated with the RPW in this region. For example, primers Bt2a/Bt2b of the beta-tubulin region for identification of *Penicillium* sp. (Glass & Donaldson, 1995), primers ef1/ef2 or the translation elongation factor region for identification of *Fusarium* sp. (Geiser *et al.*, 2004), and primers AAF2/AAR3, ADF2/ADR1 and ARF2/ARR3 for *Alternaria* sp. species (Konstantinova *et al.*, 2002). Furthermore, the difficulty in amplifying sequences with the selected ITS primers could be due to the specific primers selected. Other primers have been described for fungal barcoding for the ITS region (Raja *et al.*, 2017) that could be explored for species-level identification.

4.4. Further species interactions

4.4.1. *Centrouropoda* sp. and nematodes

Centrouropoda sp. has been found to predate on the entomopathogenic nematode *Steinernema carpocapsae* (Morton & Garcia-del-Pino, 2011). *Steinernema* sp. nematodes are currently the more widespread control method against RPW infestation in Europe. This genus of mite was present in the highest number (142.9±165.3 mites/host), and is one of the

most described internationally in association with the RPW (Dilipkumar *et al.*, 2015). The fact that this mite feeds on the nematode that comprises the biocontrol solution leads us to think that the mite's presence in weevil wild populations might yield a positive outcome for the weevil, potentially establishing a mutualistic interaction based on phagophilia changing its role from parasite to beneficial mutualist. Therefore, this mite could have both a positive (phagophilia) and negative (flight impairment) impact on the weevil's fitness. However, this observation was made based on *in vitro* assays. *In vivo* experiments, where mite-nematode interactions take place in a palm tree, have yet to be designed. This complex network of interactions has been described for other insects by Halliday (2019), where, so thought, parasitic mites have a positive impact on the host by negatively impacting other parasites. This study also argues that the large number of symbiotic species associated with the RPW will make it difficult to isolate the effect of any one species as being either harmful or beneficial to its host also because the impact of specific relationships might fluctuate between harmful, neutral and beneficial depending on the host's fitness, habitat quality status and/or number of associated organisms.

5. Future work

Although this work has been extensive and novel in terms of describing current populations of the RPW and associated mites and fungi in northern Portugal, the results we obtained point us to future work that could be developed to further understand these communities.

Ongoing work is happening namely to expand the sampled population to other regions of the country, such as Faro, the first locality with documented presence of the RPW (Boavida & Franca, 2008), Madeira, an insular region with a temperate climate that might reveal contrasting diversity of mites and seasonal presence of RPW, and other localities from central Portugal, such as Lisbon.

Studies of mite populations from palm trees that the RPW has not infected could be interesting to assess whether mite population are brought on exclusively by the RPW or if they are already existing in palm trees. This could also help discriminate if the mite populations result from palm-tree or RPW importation to the country.

Identify fungi to species level with different primers and to study the specific nature of the interaction of identified fungal strains with *P. canariensis*. This could be done with bioassays with different treatments of *P. canariensis*, with control (no insect and no fungi inoculation), with RPW (inoculation of RPW larvae into the plant) and with RPW + fungus (for example *Fusarium* sp.). We would then assess the plant's immune response and survival to see if the fungus has a synergistic effect with RPW. It could be possible that the RPW render the palm tree more vulnerable to fungus infection.

6. Conclusions

This study is pioneer and will now allow for the advancement of pest management studies in the Portuguese context, also in Europe. It recorded the activity of the RPW in almost all periods of sampling, which included several winter months, where it was not expected to reveal activity. Several associated mites and fungi were also recorded during almost all the sampling months. Mite group comprises seven different species, which is a high diversity comparable to the one found in the RPW original locality, south-eastern Asia, with the most abundant mite being *Centrouropoda* sp. We also recorded five different species of fungi, one of which, *Scopulariopsis* sp., is for the first time associated with the RPW. Most of the identified fungal genera are associated with pathogenic capacity toward agricultural crops and immunocompromised humans, revealing the importance of RPW pest control beyond the scope of plant protection. The association of *Fusarium* sp. strains also warrants the need for combined pest control with a fungicide, considering that has been documented to cause damage to infected palm trees. The more abundantly observed mite, *Centrouropoda* sp., has also been observed to feed on the nematode genus that is ubiquitously used in Portugal as a control method against the weevil which might be one of the causes of the inefficacy of current methods and calls for a redesign of current pest management procedures. Our results suggest the potential that a new control method with *Centrouropoda* sp. and a *Fusarium* sp. fungicide might yield positive results for palm tree disinfestation. Furthermore, the success of using molecular tools for identification of mites up to the family taxonomical level, indicates great potential for future identification of RPW-associated mites, presenting a possible solution to previous difficulties in mite identification.

7. References

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Annex

Annex A. Communications

Annex A1. Abstract of oral communication done in May 2022 at the "Young Researchers Meeting of U. Porto, IJUP 2022" awarded *distintion* for best presentation in Bioogical Sciences.

19512 | Phoretic and parasitic organisms associated with *Rhynchophorus ferrugineus* Olivier, 1790 in Northern Portugal

Matos, Inês, CIIMAR
Silva, Diogo, Faculdade de Ciências
Oliveira, João, Faculdade de Ciências
Pereira, Nuno, Faculdade de Ciências
Gonçalves, Cláudia, Faculdade de Ciências
Alves, Rita, Faculdade de Ciências
Rangel, Luís, CIIMAR
Santos, Maria João, Faculdade de Ciências
Ayra-Pardo, Camilo, CIIMAR

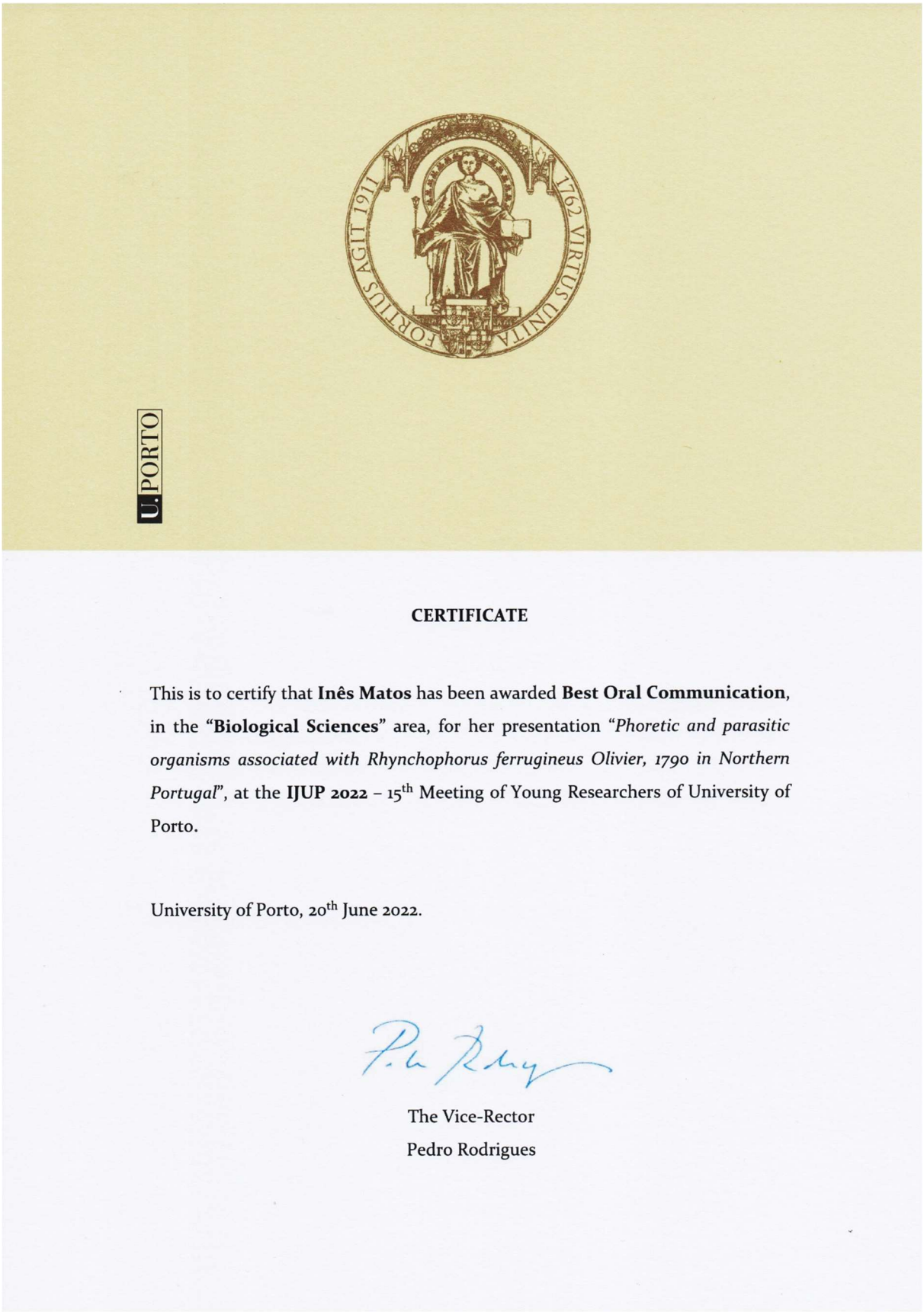
Abstract

The red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier, 1790, is native to Southeast Asia and has been spread beyond its native habitat, becoming a significant pest of the Canary Island date palm (*Phoenix canariensis*) in Portugal. This weevil is associated with several organisms, such as mites and fungi. Our objective was to document the presence of these organisms, and to detail the species presence and distribution on RPW in Northern Portugal. RPW specimens were collected via pheromone traps from July 2021 to January 2022 in 4 districts of Northern Portugal. Weevils were dissected and inspected for mites. Also, fungal spores and mycelium from weevils and mites with visible fungal growth were placed in potato dextrose agar plates, and strains were isolated through serial plating. Mites were counted and identified morphologically, and fungal species were identified based on culture morphology and ITS2 rDNA sequences.

Mite prevalence in RPW was 100%. All body parts of the host were associated with mites, but the highest average intensity was found under the elytra with 308 mites per weevil (mpw). We found 7 types of mites: *Centrouropoda* sp., *Curculanoetus rhynchophorus*, *Uroobovella* sp., *Acarus* sp., *Nenteria* sp., and Mesostigmata type 1, and Mesostigmata type 2. A total of 4 fungal genera were found, i.e. *Scopulariopsis* sp., *Alternaria* sp., *Fusarium* sp., and *Penicillium* sp.

Our study showed a high parasitic diversity of RPW-associated mites. The high-intensity levels of mpw may also entail fitness costs to RPW. Some mite types have not been described before and could represent new associations with the RPW. The fungi that we isolated from RPW could also be affecting the mites and be pathogenic to other hosts. The latter suggests that these fungi could be acting as biocontrol agents or using the RPW-mite complex as a vector.

Annex A2. Diploma of awarded *distinction* of best oral presentation in Biological Sciences at the "Young Researchers Meeting of U. Porto, IJUP 2022".



U.PORTO

CERTIFICATE

This is to certify that **Inês Matos** has been awarded **Best Oral Communication**, in the **"Biological Sciences"** area, for her presentation *"Phoretic and parasitic organisms associated with Rhynchophorus ferrugineus Olivier, 1790 in Northern Portugal"*, at the **IJUP 2022** – 15th Meeting of Young Researchers of University of Porto.

University of Porto, 20th June 2022.

The Vice-Rector
Pedro Rodrigues

Annex A3. Abstract of poster presented in may 2022 at the "Young Researchers Meeting of U. Porto, IJUP 2022".

19649 | World distribution of mites associated with the Red Palm Weevil (*Rhynchophorus ferrugineus* Olivier, 1790)

Silva, Diogo, Faculdade de Ciências
Matos, Inês, CIIMAR
Santos, Maria João, Faculdade de Ciências
Ayra-Pardo, Camilo, CIIMAR

Abstract

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* Olivier, 1790, is a major pest of palm trees. It is native to South-East Asia and has been introduced in Europe, showing a preference for the Canary Island date palm (*Phoenix canariensis*). There are several organisms associated with the RPW. Mites are one of the major groups associated with the RPW and are often considered phoretic organisms, but several studies have shown that some mites might cause harm to this weevil´s life cycle. We aim to review and document the world distribution of mite species in RPW. We reviewed 36 scientific papers from 1981 to 2020 about mites associated with RPW. Information about the mites, such as family, genera, species, locality and year of the collection, was compiled in a summary table.

According to our analysis, associated mites were found in 18 countries, Egypt with the highest species diversity (31 species). A total of 49 species, from 20 different families were identified. The most diverse families were Uropodidae with 6 species, and Urodinychidae and Laelapidae with 5 species of mites each. *Centrouropoda almerodai* and *Uroobovella marginata* were reported in 8 and 7 different localities, respectively. We found that the same mite species has been detected found associated with RPW in different localities. Our findings suggest that mites, while attached to this weevil´s body, are reallocated from their original locality following the RPW dispersion route.

Annex A4. Abstract of poster "Site distribution analysis of mites from *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionoidea): first report in Portugal" to be presented in august 2022 at the "ICOPA 2022: 15th International Congress of Parasitology in Copenhagen".

Site distribution analysis of mites from *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionoidea): first report in Portugal

Submission Topic: AS01 Living with parasites / AS01.2 Climate change, changing world, biodiversity, environments, sustainability

Authors: Inês Matos[1,2,3,]; Diogo Silva[1,2]; João Oliveira[1,2]; Nuno Pereira[1,2]; Claudia Gonçalves[1,2]; Rita Alves[1,2]; Luis Rangel[1,2]; Maria João Santos[1,2]; Camilo Ayra-Pardo[1,2].

[1]FCUP - Porto University, Biology Department, Porto, Portugal, [2]CIIMAR, Animal Pathology, Matosinhos, Portugal, [3]Aveiro University, Biology Department, Aveiro, Portugal

Introduction

The red palm weevil (RPW), Rhynchophorus ferrugineus Olivier, 1790, is native to Southeast Asia and has been spread beyond its native habitat, becoming a significant pest of the Canary Island date palm (*Phoenix canariensis*). This weevil is associated with mites whose interaction type is yet to be unravelled. We aimed to document the presence and distribution of mites on RPW in Northern Portugal.

Methods

Pheromone traps were placed across 4 districts of Northern Portugal (Porto, Aveiro, Braga, and Viana) from July2021 to January 2022, amounting to 174 adult weevils. Weevils were frozen, dissected and inspected for mites. Mites were counted and identified under a microscope.

Results

The prevalence in RPW was 100%. All body parts of the host were associated with mites, but the highest average intensity was found under the elytra with 308 mites per weevil (mpw). While the abdomen had average72 mpw and the remaining body parts only 24 mpw. We found 5 species and 2 undetermined species of mites: Centrouropoda sp., Curculanoetus rhynchophorus, Uroobovella sp., Acarus sp. and Mesostigmata Type 1 were more prevalent on the wings and elytra, while Nenteria sp. and Mesostigmata type 2 were, respectively, more prevalent on the head and antenna and neck.

Conclusions

Our study indicated a 100% prevalence and a high parasitic diversity of RPW-associated mites. It also showed the wings and elytra as the main infection sites. Our findings suggest a possible compromise of RPW fitness due to high-intensity levels of mites per weevil.

Acknowledgements: FCT - PTDC/ASP-PLA/6228/2020, PTDC/ASP-PLA/6228/BI_Lic_2021-019, CEECIND/03501/2017, UIDB/04423/2020, UIDP/04423/2020.

Annex A5. Abstract of poster "Identification of pathogenic fungi associated with the Red Palm Weevil-mite complex in Portugal" to be presented in august 2022 at the "ICOPA 2022: 15th International Congress of Parasitology in Copenhagen".

Identification of pathogenic fungi associated with the Red Palm Weevil-mite complex in Portugal

Submission Topic: AS01 Living with parasites / AS01.2 Climate change, changing world, biodiversity, environments, sustainability

Authors:Inês Matos[1,2,3]; Diogo Silva[1,2]; Luis Rangel[1,2]; Maria João Santos[1,2]; Camilo Ayra-Pardo[1,2];

[1]FCUP -Porto University, Biology Department, Porto, Portugal, [2]CIIMAR, Animal Pathology, Matosinhos,Portugal, [3]Aveiro University, Biology Department, Aveiro, Portugal

Introduction

The red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier, 1790, native of Southeast Asia, has spread to become a pest of the Phoenix canariensis palm tree. This weevil, and its associated mites, are known to carry fungi. We aim to detail the fungi species present on the RPW and its associated mites in Portugal.

Methods

RPW specimens were collected via pheromone traps in northern Portugal from July 2021 to January 2022 in 4 districts (Aveiro, Braga, Viana, Porto). Fungal spores and mycelium from weevils and mites with visible fungal growth were placed in potato dextrose agar plates and strains were isolated through serial plating. Fungal species were identified based on morphology and ITS2 rDNA sequences.

Results

A total of 4 fungal genera were found, i.e. *Scopulariopsis* sp., *Alternaria* sp., *Fusarium* sp., and *Penicillium* sp. *Scopulariopsis* sp. is known to protect the tick *Dermacentor variabilis* against *Metarhizium anisopliae* infection while, on the other hand, it can also be pathogenic to other mites. *Alternaria* sp. and *Fusarium* sp. are plant and human pathogens, and *Penicillium* sp. has been found to grow better on chitin rich-medium, indicating its potential as an entomopathogen.

Conclusions

Our findings suggest that the RPW and its parasitic mites can be vectors of fungi that can be pathogenic to plants, humans, and their own parasites. On the other hand, they can carry fungi that may compromise their own populations, suggesting prospective novel biocontrol agents. In general, the study of fungi associated with the RPW-mite parasitic complex could be a research field with broad potential in both the field of biocontrol and pathogen spread.

FCT - PTDC/ASP-PLA/6228/2020, PTDC/ASP-PLA/6228/BI_Lic_2021-019,

CEECIND/03501/2017,UIDB/04423/2020, UIDP/04423/2020.

Annex A6. Abstract of poster "World distribution of mites associated with *Rhynchophorus ferrugineus* Olivier, 1790 (Red Palm Weevil)" to be presented in august 2022 at the "ICOPA 2022: 15th International Congress of Parasitology in Copenhagen".

World distribution of mites associated with Rhynchophorus ferrugineus Olivier, 1790 (Red Palm Weevil)

Submission Topic: AS01 Living with parasites / AS01.2 Climate change, changing world, biodiversity, environments, sustainability

Authors: Diogo Silva[1,2]; Inês Matos[1,2,3]; Maria João Santos[1,2]; Camilo Ayra-Pardo[1,2];

[1]FCUP - Porto University, Biology Department, Porto, Portugal, [2]CIIMAR, Animal Pathology, Matosinhos, Portugal, [3]AveiroUniversity, Biology Department, Aveiro, Portugal

Introduction
Rhynchophorus ferrugineus Olivier, 1790, commonly known as Red Palm weevil (RPW), is a major pest of palm trees and is native to South-East Asia. Upon its arrival to Europe, it has shown preference for the Canary Island date palm (Phoenix canariensis). This weevil has interactions with several organisms. Although mites associated with the RPW are often considered phoretic organisms, several studies have shown that some mites might cause harm to this weevil's life cycle. We aim to review and document the world distribution of mite species in PW

Methods
36 scientific papers from 1981 to 2020 about mites associated with RPW were analysed. Information about the mites, such as family, genera, species, locality, and year of the collection, was compiled in a summary table.

Results
According to our analysis, associated mites were found in 18 countries, Egypt being the one with the highest species diversity (31 species). A total of 49 mite species, from 20 different families were identified. The most diverse families were Uropodidae with 6 species, and Urodinychidae and Laelapidae with 5 species each. *Centrouropoda almerodai* and *Uroobovella marginata* were reported in 8 and 7 different localities respectively.

Conclusions
We found that the same mite species are associated with RPW in several different localities. Our results suggest that mites have been reallocated from their original location while attached to the RPW.

Acknowledgements: FCT - PTDC/ASP-PLA/6228/2020, PTDC/ASP-PLA/6228/BI_Lic_2021-019,UIDB/04423/2020, UIDP/04423/2020.

Annex A7. Abstract of presentation "Distribution of mites on *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionidae) in Northern Portugal and its consequences on public health" to be presented in September 2022 at the "17th Yes Meeting Scientific Competition".

Distribution of mites on *Rhynchophorus ferrugineus* Olivier, 1790 (Coleoptera: Curculionidae) in Northern Portugal and its consequences on public health

Authors: João Oliveira1,2, Inês Matos1,2,3, Diogo Silva1,2, Luis Filipe Rangel1,2, Maria João Santos1,2, Camilo Ayra Pardo1,2

1. FCUP- University of Porto, Department of Biology, Porto, Portugal; 2. CIIMAR, Animal Pathology Laboratory, Matosinhos, Portugal, 3. Aveiro University, Biology Department, Aveiro, Portugal.

Introduction

The red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier, 1790, is a species native to Asia. It's currently distributed throughout the world, being documented in Portugal. Several mite species are phoretic in association with RPW. Inhalation or contact with the by-products of mite metabolism can cause allergic reactions in sensitive individuals. This study aimed to investigate the mite species and their distribution on RPW in northern Portugal.

Methods and materials

From July 2021 to January 2022, pheromone traps were placed in 4 districts in northern Portugal (Aveiro; Braga; Porto; Viana). 37 weevils were collected, frozen, and dissected under a magnifying glass, for inspection of mites. The mites were counted and identified under a microscope.

Results

Mite's prevalence on RPW was 100%. All weevil's body parts were associated with mites, and the highest average intensity was verified under the wings and elytra, with 196 mites per weevil (mpw). In total, 5 types of mites were found (*Acarus* sp.; *Centrouropoda* sp.; *Curculanoetus* sp.; *Nenteria* sp.; *Uroobovella* sp.) and 2 still undetermined species (Mesostigmata sp.1 and sp.2.). The most abundant mites on the weevil were *Centrouropoda* sp. and *Acarus* sp., with an average, respectably, of 92 and 78 mpw. The average mpw was 251 in females and 513 in males. Mites were more prone to male weevils, although *Uroobovella* sp. was the only one who only appeared in females.

Conclusion and discussion

A great diversity of mite species was found on RPW. The prevalence of mites in RPW was 100%, and the average number of mites per individual was 293, with the highest prevalence in males. The wings and elytra were most frequently infested, which could affect the flight ability and fitness of RPW and indicate a parasitic relationship between mites and weevils. These mites can be vectors of disease, but their effects on the population remain unresolved.

Annex B. Supplementary information

Annex B1. Table of primers used, respective sequence, target region, target organisms and source.

Primer	Direction	Sequence (5'-3')	Target region	Target organisms	Source
ITS2-F1	forward	ATATGCTTAAATTCAGCGGG	ITS1+5.8S+ITS2	Mites	Navajas *et al*. (1998)
ITS2-R1	reverse	GGGTCGATGAAGAACGCAGC	ITS1+5.8S+ITS2	Mites	Navajas *et al*. (1998)
LCO1490	forward	GGTCAACAAATCATAAAGATATTGG	COI	Mites	Folmer *et al*. (1994)
HC02198	reverse	TAAACTTCAGGGTGACCAAAAAATCA	COI	Mites	Folmer *et al*. (1994)
18e	forward	CTGGTTGATCCTGCCAGT	18S	Mites	Hillis and Dixon (1991)
18R	reverse	CTACGGAAACCTTGTTACG	18S	Mites	Whipps *et al*. (2003)
WormA	forward	GCGAATGGCTCATTAAATCAG	18S	Mites	Littlewood and Olson (2014)
WormB	reverse	CTTGTTACGACTTTTACTTCC	18S	Mites	Littlewood and Olson (2014)
ITS1F	forward	CTTGGTCATTTAGAGGAAGTAA	ITS	Fungus	White *et al*. (1990)
ITS4	reverse	TCCTCCGCTTATTGATATGC	ITS	Fungus	White *et al*. (1990)
NS1	forward	GTAGTCATATGCTTGTCTC	18S	Fungus	White *et al*. (1990)
NS4	reverse	CTTCCGTCAATTCCTTTAAG	18S	Fungus	White *et al*. (1990)

Annex B2. List of sequences published in GenBank and respective accession number.

- *Scopulariopsis* sp. isolate Av1 ON854670
- *Scopulariopsis* sp. isolate Av2 ON854671
- *Scopulariopsis* sp. isolate Av3 ON854672
- *Alternaria* sp. isolate Vi2 ON931226
- *Fusarium* sp. isolate Pt1 ON931476
- *Fusarium* sp. isolate Pt2 ON931477
- *Fusarium* sp. isolate Pt3 ON931478
- *Fusarium* sp. isolate Av4 ON931479
- *Fusarium* sp. isolate Br5 ON931480
- *Penicillium* sp. isolate Av3 ON931626
- *Penicillium* sp. isolate Br6 ON931627
- *Penicillium* sp. isolate Av7 ON931628
- *Penicillium* sp. isolate Av8 ON931629
- *Penicillium* sp. isolate Vi9 ON931630
- *Penicillium* sp. isolate Vi10 ON931631
- *Centrouropoda* sp. isolate 7A ON951975
- *Nenteria extremica* isolate 13A ON951976
- *Acarus* sp. isolate 16A ON951977
- *Dendrolaelaps* sp. isolate 12A ON951978
- *Dendrolaelaps* sp. isolate 12B ON951979

Annex B3. Description and GenBank accession number of obtained sequences of *Centrouropoda* sp. isolates from this study (*) and more significant matches to existing sequences in GenBank.

Accession number	Lenght	Description	Scientific name	Query cover	Percent identity	E value
ON951975*	1718	*Centrouropoda* sp. isolate 7A 18S ribosomal RNA gene, partial sequence	*Centrouropoda* sp.	-	-	-
AY620926.1	1734	*Uropoda orbicularis* 18S ribosomal RNA gene, partial sequence	*Uropoda orbicularis*	85.0%	84.6%	0.0
KY922099.1	1756	*Polyaspis* sp. AD1351 voucher UMMZ BMOC 08-0807-008 AD1351 18S ribosomal RNA gene, partial sequence	*Polyaspis* sp.	96.0%	87.2%	0.0

Annex B4. Description and GenBank accession number of obtained sequences of *Nenteria extremica* isolates from this study (*) and more significant matches to existing sequences in GenBank.

Accession number	Lenght	Description	Scientific name	Query cover	Percent identity	E value
ON951976*	1720	*Nenteria extremica* isolate 13A 18S ribosomal RNA gene, partial sequence	*Nenteria extremica*	-	-	-
AY620925.1	1762	*Trichouropoda* sp. AL5867 18S ribosomal RNA gene, partial sequence	*Trichouropoda* sp.	99.0%	90.5%	0.0

Annex B5. Description and GenBank accession number of obtained sequences of *Acarus* sp. isolates from this study (*) and more significant matches to existing sequences in GenBank.

Accession number	Lenght	Description	Scientific name	Query cover	Percent identity	E value
ON951977*	1695	*Acarus* sp. isolate 16A 18S ribosomal RNA gene, partial sequence	*Acarus* sp.	-	-	-
JQ000113.1	1525	*Naiacus* sp. AD305 18S ribosomal RNA gene, partial sequence	*Naiacus* sp.	89.0%	97.8%	0.0
EF203769.1	1516	*Acarus gracilis* 18S ribosomal RNA gene, partial sequence	*Acarus gracilis*			
JQ000100.1	1746	Acaridae gen. n. sp. n. AD469 18S ribosomal RNA gene, partial sequence	Acaridae (family)	100.0%	96.4%	0.0

Annex B6. Description and GenBank accession number of obtained sequences of *Dendrolaelaps* sp. isolates from this study (*) and more significant matches to existing sequences in GenBank.

Accession number	Lenght	Description	Scientific name	Query cover	Percent identity	E value
ON951978*	1673	*Dendrolaelaps* sp. isolate 12A 18S ribosomal RNA gene, partial sequence	*Dendrolaelaps* sp.	-	-	-
ON951979*	1587	*Dendrolaelaps* sp. isolate 12B 18S ribosomal RNA gene, partial sequence	*Dendrolaelaps* sp.	-	-	-
FJ911827.1	1576	*Dendrolaelaps* sp. 2 APGD-2010 18S ribosomal RNA gene, partial sequence	*Dendrolaelaps* sp.	94.0%	96.3%	0.0

Annex B7. Description and GenBank accession number of obtained sequences of *Scopulariopsis* sp. isolates from this study (*) and more significant matches to existing sequences in GenBank.

Acession code	Lenght	Description	Scientific name	Query cover	Percent identity	E value
ON854670*	1020	*Scopulariopsis* sp. isolate Av1 18S small subunit ribosomal RNA gene, partial sequence	*Scopulariopsis* sp.	-	-	-
ON854671*	1020	*Scopulariopsis* sp. fungal isolate Av2 18S small subunit ribosomal RNA gene, partial sequence	*Scopulariopsis* sp.	-	-	-
ON854672*	1020	*Scopulariopsis* sp. fungal isolate Av3 18S small subunit ribosomal RNA gene, partial sequence	*Scopulariopsis* sp.	-	-	-
KY883299.1	1035	*Scopulariopsis crassa* culture CGMCC:3.17942 18S ribosomal RNA gene, partial sequence	*Scopulariopsis crassa*	100%	>99.9%	0.0
KY883298.1	1034	*Scopulariopsis crassa* culture CGMCC:3.17941 18S ribosomal RNA gene, partial sequence	*Scopulariopsis crassa*	100%	>99.9%	0.0
KJ443074.1	1666	*Scopulariopsis brevicaulis* strain G413 18S small subunit ribosomal RNA gene, partial sequence	*Scopulariopsis brevicaulis*	100%	>99.9%	0.0
KM096361.1	1544	*Doratomyces stemonitis* strain MF570 18S ribosomal RNA gene, partial sequence	*Doratomyces stemonitis*	100%	>99.9%	0.0
OK011823.1	1292	*Scopulariopsis brevicaulis* isolate 1-83 small subunit ribosomal RNA gene, partial sequence	*Scopulariopsis brevicaulis*	100%	>99.9%	0.0

Annex B8. Description and GenBank acession code of obtained sequences for each *Alternaria* sp. isolate from this study (*) and more significant matches to existing sequences in GenBank.

Accession number	Lenght	Description	Scientific name	Query cover	Percent identity	E value
ON931226*	1029	Vi2 *Alternaria* sp. 18S small subunit ribosomal RNA gene, partial sequence	*Alternaria* sp.	-	-	-
MT649562.1	1650	*Alternaria* sp. isolate 70 small subunit ribosomal RNA gene, partial sequence	*Alternaria* sp.	100%	100%	0.0
MN075309.1	1102	*Alternaria tenuissima* isolate BAT4 small subunit ribosomal RNA gene, partial sequence	*Alternaria tenuissima*	100%	100%	0.0
MK757635.1	1053	*Alternaria alternata* isolate BAA2 small subunit ribosomal RNA gene, partial sequence	*Alternaria alternata*	100%	100%	0.0
OK036370.1	1058	*Alternaria solani* isolate AMaNaL small subunit ribosomal RNA gene, partial sequence	*Alternaria solani*	100%	100%	0.0
GQ354822.1	1664	*Alternaria japonica* strain HDJZ-ZWM-06 18S ribosomal RNA gene, partial sequence	*Alternaria japonica*	100%	100%	0.0

Annex B9. Description and GenBank acession code of obtained sequences for each *Fusarium* sp. isolate from this study (*) and more significant matches to existing sequences in GenBank.

Accession number	Lenght	Description	Scientific name	Query cover	Percent identity	E value
ON931476*	976	*Fusarium* sp. isolate Pt1 18S small subunit ribosomal RNA gene, partial sequence	*Fusarium* sp.	-	-	-
ON931477*	1036	*Fusarium* sp. isolate Pt2 18S small subunit ribosomal RNA gene, partial sequence	*Fusarium* sp.	-	-	-
ON931478*	1032	*Fusarium* sp. isolate Pt3 18S small subunit ribosomal RNA gene, partial sequence	*Fusarium* sp.	-	-	-
MT640287.1	1630	*Fusarium* sp. MAS2 isolate ABD1 small subunit ribosomal RNA gene, partial sequence	*Fusarium* sp.	100%	100%	0.0
MN602643.1	1671	*Fusarium solani* isolate XXTF8 small subunit ribosomal RNA gene, partial sequence	*Fusarium solani*	100%	100%	0.0
MN508437.1	1712	*Fusarium* sp. MAS2 strain SZBNS18 small subunit ribosomal RNA gene, partial sequence	*Fusarium* sp.	100%	100%	0.0
ON931479*	922	*Fusarium* sp. isolate Av4 18S small subunit ribosomal RNA gene, partial sequence	*Fusarium* sp.	-	-	-
KU170627.1	1715	*Fusarium* sp. S9 18S ribosomal RNA gene, partial sequence	*Fusarium* sp.	100%	100%	0.0
KM250373.1	1342	*Fusarium oxysporum* isolate SPS-03 18S ribosomal RNA gene, partial sequence	*Fusarium oxysporum*	100%	100%	0.0
ON931480*	857	*Fusarium* sp. isolate Br5 18S small subunit ribosomal RNA gene, partial sequence	*Fusarium* sp.	-	-	-
MT649581.1	1654	*Fusarium* sp. isolate 89 small subunit ribosomal RNA gene, partial sequence	*Fusarium* sp.	100%	100%	0.0
KX384665.1	1206	*Fusarium oxysporum* strain 150403-45 M07 LO NS1.ab1 18S ribosomal RNA gene, partial sequence	*Fusarium oxysporum*	100%	100%	0.0

Annex B10. Description and GenBank acession code of obtained sequences for each *Penicillium* sp. isolate from this study (*) and more significant matches to existing sequences in GenBank.

Accession number	Lenght	Description	Scientific name	Query cover	Percent identity	E value
ON931626*	967	*Penicillium* sp. isolate Av3 18S small subunit ribosomal RNA gene, partial sequence	*Penicillium* sp.	-	-	-
ON931627*	966	*Penicillium* sp. isolate Br6 18S small subunit ribosomal RNA gene, partial sequence	*Penicillium* sp.	-	-	-
ON931628*	965	*Penicillium* sp. isolate Av7 18S small subunit ribosomal RNA gene, partial sequence	*Penicillium* sp.	-	-	-
ON931629*	967	*Penicillium* sp. isolate Av8 18S small subunit ribosomal RNA gene, partial sequence	*Penicillium* sp.	-	-	-
ON931631*	1064	*Penicillium* sp. isolate Vi10 18S small subunit ribosomal RNA gene, partial sequence	*Penicillium* sp.	-	-	-
MF072593.1	1001	*Penicillium brevicompactum* strain 001 small subunit ribosomal RNA gene, partial sequence	*Penicillium brevicompactum*	100%	99.90%	0.0
KY448996.1	1278	*Penicillium* sp. strain OUCMDZ-4920 18S ribosomal RNA gene, partial sequence	*Penicillium* sp.	100%	99.90%	0.0
FJ717699.1	1351	*Penicillium brevicompactum* isolate PenC 18S ribosomal RNA gene, partial sequence	*Penicillium brevicompactum*	100%	99.90%	0.0
ON931630*	967	*Penicillium* sp. isolate Vi9 18S small subunit ribosomal RNA gene, partial sequence	*Penicillium* sp.	-	-	-
MF072594.1	1107	*Penicillium crustosum* strain 011 small subunit ribosomal RNA gene, partial sequence	*Penicillium crustosum*	100	100	0.0
MN826497.1	1654	*Penicillium chrysogenum* strain DY115-F2 small subunit ribosomal RNA gene, partial sequence	*Penicillium chrysogenum*	100	100	0.0