INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Design and characterization of molecular markers for the detection of predation of *Trioza erytreae*

Rym Moudjeber

Dissertation submitted to Escola Superior Agrária de Bragança to obtain the Degree of Master in Biotechnological Engineering

Supervised by

Paula Cristina dos Santos Baptista

Vitor Ramos

Bragança
2022

ACKNOWLEDGEMENT

It is a pleasant duty to express in a few lines the gratitude that I owe to all those who have contributed from far and near to the development of this work, that they find here my deep respect and my deep gratitude.

I address my sincere thanks to my thesis directors all the professors and all the people who by their words, their writings, their advice and their criticisms guided my reflections and agreed to meet me and answer my questions during my research.

Professor Paula Baptista, my supervisor, for the trust that she gave me by agreeing to supervise this work, for her multiple advice and for all the hours she spent with this work. Her energy and confidence were the driving forces for me when I felt overwhelmed and stressed. I took great pleasure in working with her.

Professor Vitor Ramos for all the time he devoted to me, without his help the progress of my thesis would've been impossible; as well as for his patience and his precious advice.

Professor Mahmoudi Hassen that made all this experience possible by suggestion IPB for a second master degree, I am grateful for the opportunity he gave me.

I would like to especially thank my parents Dr Moudjeber Djamel Eddine and Dr Latifa Moudjeber, they are the reason I am here today no words can express the gratitude and the love I have for both them. Thank you for believing in me even when I didn't! and beyond everything sorry for the continuous stress you've had to deal with . To my sisters "Asma, Nawel" for their advice as well as their unconditional support, they have always believed in me and have never left me especially during the past difficult moments and for all their sacrifices throughout my life.

I also take this opportunity to thank my uncle Khaled Moudjeber and his wife Afaf Moudjeber for their support, their love, for being there during all the difficult moments, for believing in me and continually pushing me to be the best version of myself.

I consider myself one of the lucky ones cause I've been blessed with such a supporting family, I can't thank you enough and hope you are proud.

To my friends, Samar and Momo thank you for your help, encouragements, prayers and all you did they find here the expression of my gratitude.

This work is supported by the project PRE-HLB: Preventing HLB epidemics for ensuring citrus survival in Europe. H2020-SFS-2018-2 Topic SFS-05-2018-2019-2020 - New and emerging risks to plant health (nº 817526).

Dedication

To my dear father To my dear mother and sisters Who find here the proof of all my gratitude and my eternal love and appreciation.

Table of Contents

Abbreviation list

Bp: base pairs

COI: mitochondrial Cytochrome Oxidase subunit 1.

DNA: Deoxyribonucleic Acid.

EPPO: European and Mediterranean Plant Protection Organization.

HLB: Huanglongbing.

LSU Large subunit ribosomal ribonucleic acid

NCBI: The National Center for Biotechnology Information.

PCR: Polymerase Chain Reaction.

SSU Small subunit ribosomal ribonucleic acid

Index of figures

Index of tables

Abstract

Trioza erytreae (Hemiptera, Psylloidea) is an important citrus pest being also one of the vectors of *Candidatus* Liberibacter spp., the causal bacterial agents of citrus HUANGLONGBING (greening) disease, deemed as the most devastating disease for citrus crops. In 2014, *T. erytreae* was detected in NW Iberian Peninsula, being the first detection of the pest in continental Europe. The spreading of *T. erytreae* towards Iberian Peninsula is being fast, threatening the citrus production in Europe. Due to the progressively strict EU policies on pesticides, their harmful effects and not successful eradication of *T. erytreae* attempts, the use of insecticides is regarded as impracticable. Thus, biocontrol using natural enemies is seen as an environmentally sound and effective approach to limited this pest. Hence, the main goal of this work was to develop PCR-based diagnostic methods that can detect for the presence of DNA from *T. erytreae* in the gut of arthropod predators. Fifteen *T. erytreae* species-specific primer sets targeting the mitochondrial COI, SSU and LSU regions were designed and tested for their specificity and sensitivity against phylogenetically-related psyllids. Among the 15 primers sets tested, two targeting the COI gene (T3F - T2R) and the rrnL gene (LSU_F2LSU_R1) showed to be highly specific and sensitive to detected *T. erytreae* DNA, inclusively when mixed at equal ratios with DNA of closely-related taxa of *Trioza*. The small size of the fragment amplified by these two primer sets (199 bp and 291 bp, for T3F - T2R and LSU_F2-LSU_R1, respectively), suggested their suitability to detected fragmented *T. erytreae* DNA in the gut of potential predators. Despite these promising results, the feasibility of these primer sets to recognize predators collected in natural settings must be validated in future works.

Keywords: African citrus psyllid, HUANGLONGBING, natural enemies, COI, SSU, LSU.

Resumo

Trioza erytreae (Hemiptera, Psylloidea) é uma importante praga dos citrinos, sendo também um dos vetores de *Candidatus* Liberibacter spp., o agente causal da doença huanglongbing (greening) dos citrinos, e considerada como sendo a mais devastadora na citricultura. Em 2014, foi detetada a presença de *T. erytreae* no noroeste da Península Ibérica, tendo sido esta a primeira vez reportada na Europa continental. A dispersão de *T. erytreae* na Península Ibérica esta a decorrer de forma rápida, ameaçando a produção de citrinos na Europa. Devido às políticas cada vez mais rigorosas da UE sobre o uso de pesticidas, os seus efeitos nocivos e tentativas fracassadas de erradicação de *T. erytreae*, tem levado à procura de alternativas. Assim, o biocontrolo usando inimigos naturais é visto como uma abordagem ambientalmente correta e eficaz para limitar esta praga. Assim, o principal objetivo deste trabalho foi desenvolver um método de diagnóstico baseados em PCR que permita detetar a presença de DNA de *T. erytreae* no intestino de predadores artrópodes. Quinze conjuntos de primers específicos de *T. erytreae* foram desenhados nas regiões mitocondriais COI, SSU e LSU, e testados quanto à sua especificidade e sensibilidade contra psilídeos filogeneticamente próximos de *Triosa*. De entre os 15 conjuntos de primers testados, dois desenhados no gene COI (T3F - T2R) e no gene rrnL (LSU_F2-LSU_R1) mostraram elevada especificidade e sensibilidade ao DNA de *T. erytreae*, permitindo a sua deteção quando misturado em proporções iguais com DNA de espécies taxonomicamente relacionados com *Trioza*. O reduzido tamanho do fragmento amplificado por estes dois conjuntos de primers (199 pb e 291 pb, para T3F - T2R e LSU_F2-LSU_R1, respetivamente), sugere a sua adequação para detetar fragmentos de DNA de *T. erytreae* no intestino de potenciais predadores. Apesar destes resultados promissores, a viabilidade dos dois conjuntos de primers para reconhecer predadores coletados em ambientes naturais deve ser validada em trabalhos futuros.

Palavras-chave: Psila-africana-dos-citrinos, HUANGLONGBING, inimigos naturais, COI, SSU, LSU

1. Framework and objectives

1. Framework and objectives

Trioza erytreae (Del Guercio, 1918) (Hemiptera, Psylloidea, Triozidae), also known as the African citrus psyllid, is a biting-sucking insect that causes considerable direct damage to *Citrus* spp. fruits and other ornamental hosts. This insect, is also one of the insect vectors of *Candidatus* Liberibacter spp., the causal bacterial agent of Huanglongbing/Citrus Greening Disease (HLB) (Bové, 2006), considered the most devastating disease affecting citrus worldwide (Duran-Vila et al., 2015; Cocuzza et al., 2017). Indeed, this disease is responsible to cause huge losses in fruit production and quality, mainly due to its rapid dispersal, severity and fast progression of symptoms as well as difficulty of preventing new infections and absence of durable control mechanisms (Timmer et al., 2003).

The native distribution of this sap-sucking insect is considered to be throughout the Afrotropic ecozone (Cocuzza et al., 2017). However, *T. erytreae* was observed in no continental Europe for the first time in 1994, on the Madeira archipelago (Portugal), and then reported for Canary Islands (Spain) in 2002 (Cocuzza, 2017). In 2014, it was detected in NW Iberian Peninsula, being the first detection of the pest in continental Europe. The spreading of *T. erytreae* towards both the south of Portugal and of Spain and eastern Spain is being fast, threatening the citrus production in Europe. So far, the presence of HLB in the Iberian Peninsula was not detected (Wang, 2020). Therefore, the containment and control of *T. erytreae* are extremely important to prevent its spreading and thus to reduce the risks of a possible outbreak of HLB. Due to the progressively strict EU policies on pesticides, their harmful effects and not successful eradication of *T. erytreae* attempts, the use of insecticides is regarded as impracticable. Therefore, there is an urgent need to develop more effective and environmentally friendly tools to control this pest. This strategy has long been promoted by the EU (Directive 2009/128/EC) and more recently, it forms part of the European Green Deal, in particular of the Farm to Fork Strategy, which aims to reduce pesticide use in the EU by 50% by 2030.

The exploitation of natural enemies of *T. erytreae*, such as predators, could be an environmentally sound and effective approach to reduce or mitigate this pest. However, the identification of predator-prey relationships cannot be always done by direct observation in field conditions (Symondson, 2002). In this regard, the molecular identification of prey in predator diets by using polymerase chain reaction (PCR)-based techniques has been proven to be highly effective in identifying natural enemies of several insect pests (Rejili et al., 2016; Albertini et al., 2018; Rodrigues et al., 2022). Hence, the main goal of this work was to develop PCR-based diagnostic methods that can detect for the presence of DNA from *T. erytreae* in the gut of arthropod predators.

2. Introduction

2. Introduction

2.1. Trioza erytreae

Trioza erytreae (Del Guercio, 1918) (Hemiptera, Psylloidea, Triozidae), also known as the African citrus psyllid, is considered one of the most devastating pests of citrus plants in Africa and Middle East (Van den Berg et al., 1990). In addition to feeding damage, *T. erytreae* is a vector of the causal agent of the African form of citrus HUANGLONGBING (greening) disease, *Candidatus* Liberibacter spp., which is one of the most devastating citrus diseases in the world (Lee et al., 2015). This pest, with origin in SubSaharan Africa, was recently found in mainland Europe, in particular in the northwestern Iberian Peninsula (Cocuzza et al., 2017). *T. erytreae* spread rapidly, being its geographic range expanding throughout the Iberian Peninsula, and the severity of *T. erytreae* attacks have been increasing, threatening the citrus production in Europe (Arenas-Arenas et al., 2018).

2.1.1. *T. erytreae*, recent spread and threats

The psyllid *T. erytreae* was first reported in Southern Africa in 1897 (Van den Berg et al., 1990). The occurrence of the species (Figure 1) in Sub-Saharan African and Middle East regions is long known (Cocuzza et al., 2017). It is sensitive to hot, dry conditions, being favored by cooler, moist areas above 500-600 m altitude, where citrus growth flushes tend to be prolonged (Cocuzza et al., 2017; Aidoo et al., 2019). Whereas the native distribution of the species is deemed as to be throughout the Afrotropic ecozone, *T. erytreae* was observed in non-continental areas of Europe for the first time in 1994, on the island of Porto Santo (Madeira, Portugal) (Cocuzza et al., 2017). Later, in 2002, its occurrence was also reported in Tenerife (Canary Islands, Spain) (Cocuzza et al., 2017). This pest was then detected in 2014 in northwestern Iberian Peninsula, in mainland Spain (in Galicia) and mainland Portugal (in the region of Porto), which represent the first detection of the African citrus psyllid in continental Europe (Cocuzza et al., 2017; Arenas-Arenas et al., 2018; 2019).

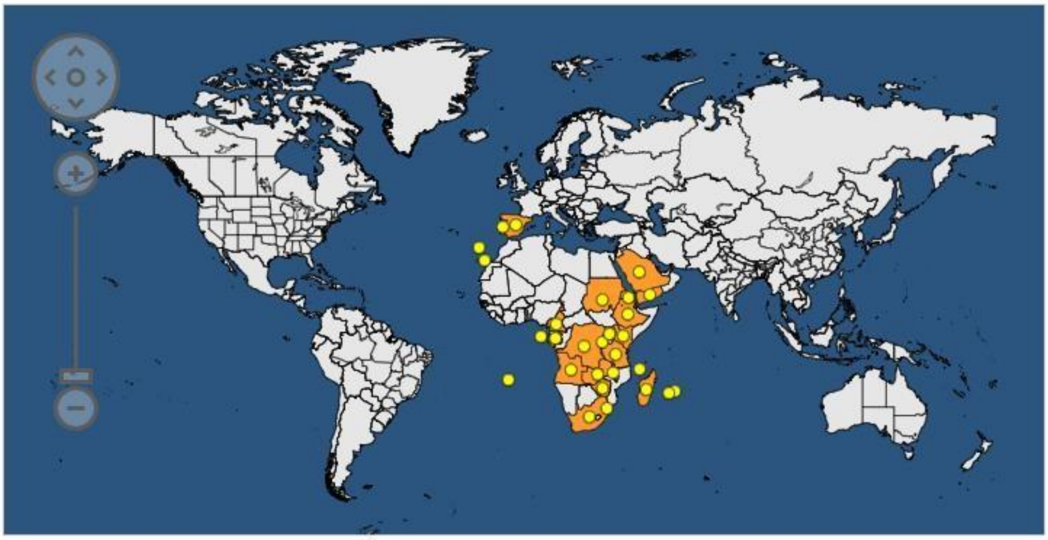

Figure 1. Current known distribution of *Trioza erytreae* worldwide. Yellow dots represent presence. Source: EPPO, 08-11-2022.

The route that *T. erytreae* took to reach the Macaronesia Islands and NW Iberian Peninsula remains unknown, but the broad expansion of the species, in a relatively short period of time, might indicate that it was due to man activities (Cocuzza et al., 2017). In new areas of colonization, *T. erytreae* can observe a rapid dispersion triggered by the search for new feeding and oviposition sites (Cocuzza et al., 2017). Since this pest is closely associated with plants in the Citrus family, its presence in the Iberian Peninsula represents a serious threat to citriculture (Arenas-Arenas et al., 2018). From its first appearance in Portugal, in 2014, *T. erytreae* showed a considerably spread both towards the north and the south (Arenas-Arenas et al., 2018; see also Figure 2). The spreading towards the south is being fast, what is a matter of main concern, since the main citrus producing areas are located in the southern (Algarve in Portugal; Huelva and Seville in Spain) and eastern (Murcia, Valencia and Catalonia) regions of the Iberian Peninsula (Cocuzza et al., 2017; Arenas-Arenas et al., 2018). The fact that it was already detected in the region of Lisbon (Figure 2) means that the pest is less than 200 km apart of citrus orchards from Algarve and Huelva.

Since *T. erytreae* is the natural vector of the African HLB, which may seriously impact the citriculture sector (Arenas-Arenas et al., 2018), and given its recent geographic expansion (Figure 2), the psyllid is now listed as an A2 quarantine pest by EPPO (The

European and Mediterranean Plant Protection Organization), meaning that the pest is locally present in the EPPO region (EPPO, 2019).

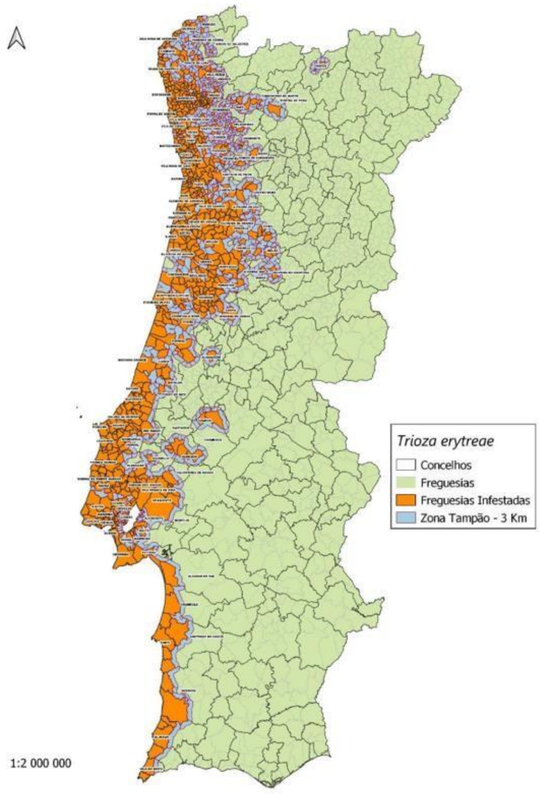

Figure 2. Map showing the spread of *Trioza erytreae* in Portugal, data from 17/06/2022. Source: Portuguese Ministry of Agriculture, Rural Development and Fisheries, 2022.

2.1.2. Taxonomy and phylogeny

The genus *Trioza* Förster (1848) belongs to *Triozidae* low 1888, which with almost 1000 species from 70 genera is the second largest family of *Psylloidea*, the superfamily of true bugs (Cocuzza et al., 2017; Percy et al., 2018). *Trioza urticae* L. is the type species for the genus and is phylogenetically placed in a sub-clade (group M) at the base of the clade of *Triozidae* (Percy et al., 2018). *Trioza erytreae* (Del Guercio, 1918) is believed to be native to SE Africa and was originally described from specimens collected in lemon trees, in *Eritrea* (Cocuzza et al., 2017). In his taxonomic revision on *Trioza*, (Hollis,1984) placed *T. erytreae* in the *erytreae*-group along with nine other species, although stating that this “a difficult group to define…[that] may be artificial”. However, he recognized

T. erytreae as the only species of the group known to develop on Rutaceae plants (Hollis, 1984). In the phylogeny-based revision of psyllids performed by Percy and co-authors (2018), *T. erytreae* is placed in a clade (Group D) together with some other *Trioza* spp. (including *T. chenopodii*, *T. percyae, T. tricornuta*) and species from other genera such as *Aacanthocnema*, *Anomocephala*, *Casuarinicola*, *Cerotrioza*, *Kuwayama* and *Megatrioza*. There are two other species assigned to *Trioza* that are known to feed and develop on *Citrus* plants (Cocuzza et al., 2017). These are *T. citroimpura* Yang and Li 1984 and *T. litseae* Bordage 1898 (=*T. eastopi* Orian 1972), being both easily distinguishable morphologically from *T. erytreae* (Cocuzza et al., 2017).

2.1.3. Biology and ecology

T. erytreae is found in specific host plants belonging to one particular taxonomic family, the Rutaceae or Citrus family, which includes important edible fruit crops belonging to the genus *Citrus* L. such as orange (*Citrus sinensis*), lemon (*Citrus limon*), grapefruit (*Citrus paradisi*), key lime (*Citrus aurantiifolia*) and pomelo (*Citrus maxima*) (King et al., 2008). The pest seems to have a specific preference for lemon and lime trees, although it is also found in other Rutaceae species, such as sweet and sour orange, mandarin and grapefruit (Green et al., 1971). *T. erytreae* populations are favored by cooler, moist areas above 500-600 m altitude, where citrus growth blooms usually are longer (Schwarz et al., 1970).

Whenever adults, *T. erytreae* individuals are easily recognizable (Figure 3, Cocuzza et al., 2017). According to Espinosa and Hodges (2009) and (Cocuzza et al.,2017) adults are winged, although they are pretty much sedentary. Wings always remain clear. Males are slightly smaller than females, both being around 4 mm long, and their abdomen tip is blunt, while that from females is sharp (Del Guercio, 1918). Females always predominate in the field. Their life span is between 17 and 50 days (Del Guercio, 1918). Breeding occurs 2-4 times each day (Cocuzza et al., 2017). Eggs are cylindrical with a sharp point that is driven through the leaf epidermis. They are yellowish when just laid, which may occur immediately after mating (Cocuzza et al., 2017). Eggs have an incubation period of 6-15 days and turn orange when they mature (Cocuzza et al., 2017). Nymphal development (five instars) takes 17 to 43 days, which should occur at temperatures higher than 10-12°C (Cocuzza et al., 2017). During the five instars, nymphs of *T. erytreae* is ventrally downward, slightly elongated, with visible marginal waxy white filaments or with another color going from pale yellow after hatching to olive green or dark grey in a final stage, with a pair of dark patches occurring in some populations dorsally on the abdomen. The characteristic galls on an infested citrus leaf are made by the nymphs. At emergence adults are light green, turning brown as they mature (Cocuzza et al., 2017). A detailed morphological description of *T. erytreae* can be found in (Hollis., 1984), while some morphometrics data are summarized in Table 1.

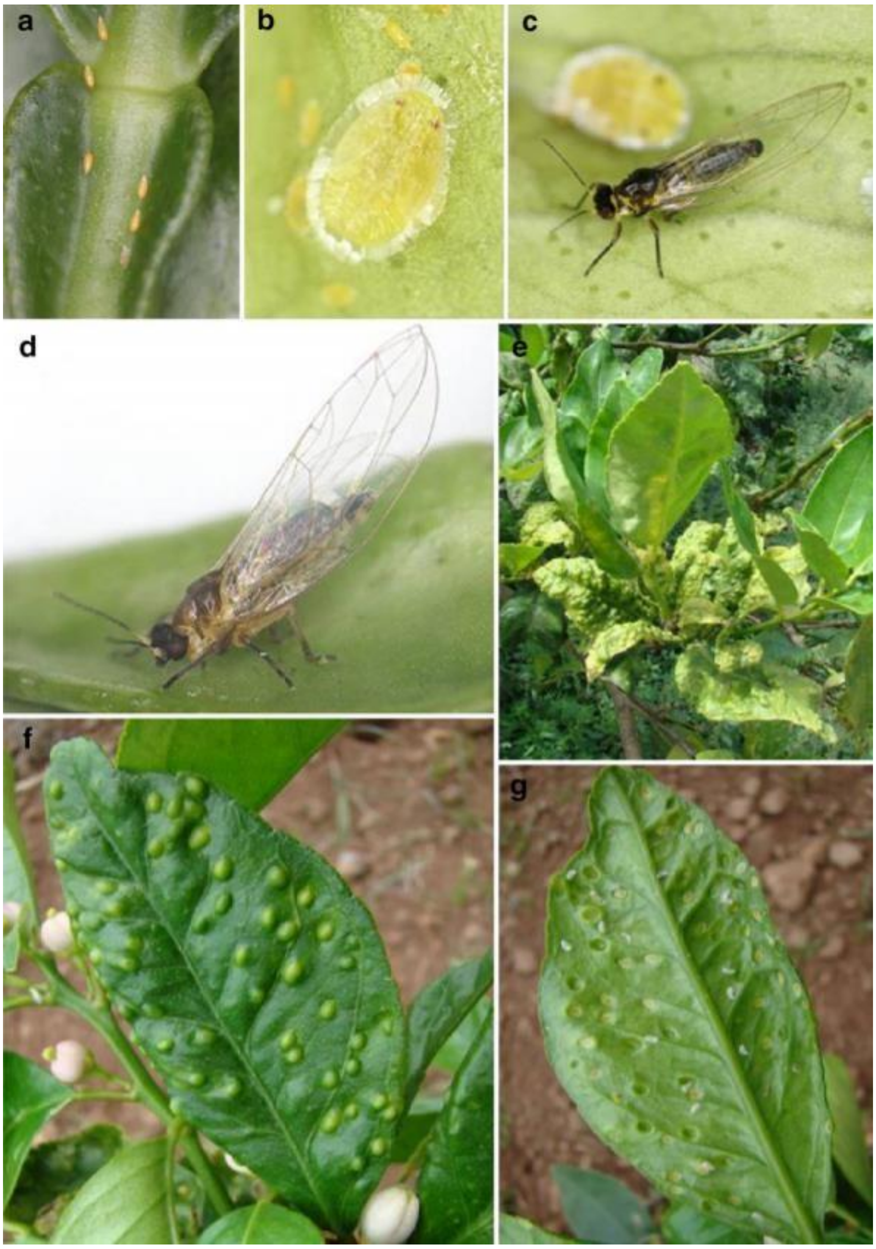

Figure 3. *T. erytreae* on citrus: (**a**) eggs, (**b**) fifth instar nymph, (**c**) adult male, (**d**) adult female, (**e**) heavily distorted leaves in a sprout seriously infested, (**f**) typical pit galls, protruding to the upper face of the leaves, (**g**) lower face of an infested leaf, with nymphs settled at each gall. Source**:** Cocuzza et al. (2017).

Table 1. *Trioza erytreae*: main morphological measures (mm) and ratios of fifth instar nymphs and adults (modified after Cocuzza et al., 2017).

	Fifth instar nymphs		Adults
Body length	1.38-1.66	Head width/ male	0.37-0.49
Body width	0.87-1.12	Male proctiger height	0.17-0.22
Antennal length	0.21-0.25	Male length	0.20-0.23
Forewing pad length	0.87-0.85	Female proctiger length	0.39-0.44
Caudal plate length	0.51-0.59	Female anal pore length	0.11-0.17

2.2. Huanglongbing/Citrus Greening Disease (HLB)

The psyllid itself leads to damage or deformation of leaves, which become chlorotic, stunted and/or galled (Van den Berg, 1990). But, for many years, *T. erytreae* was disregarded as a pest in native regions because of its minor direct damage on adult citrus trees (Cocuzza et al., 2017). Later, it was recognized as being one of the vectors of the bacteria *Candidatus* Liberibacter spp., that trigger off the most destructive and dangerous disease of citrus plants in the world – the Huanglongbing/Citrus Greening Disease (HLB) (Catling, 1970; Bové, 2006). It causes plants decline and premature death, being one of the most devastating diseases of Asia and Africa (Gottwald et al., 2007).

In the past, more than one name was given to this disease, such as yellow shoot disease (“Huanglongbing”) in China, “likubin” in Taiwan, dieback in India; leaf mottle in the Philippines, vein phloem degeneration in Indonesia, and yellow branch, blotchymottle, or greening in South Africa (Gottwald et al., 2007). When it became clear that all these names were referring to the same disease, the name "greening" was widely adopted the scientific literature and later the original Chinese name of “Huanglongbing” was established as the official name (Gottwald et al., 2007).

The HLB symptoms are diverse, but some unique characteristic features can be observed (Gottwald et al., 2007): infected trees normally develop yellow shoots (hence the original name of the disease); the affected leaves will develop different chlorotic patterns, with yellow and green areas, resembling those induced by zinc deficiency (Figure 4). These patterns are asymmetrical on the two halves of the leaf. In later stages, leaf drop, and twig dieback will occur. Infected fruits are small, lopsided, and have a bitter taste, and fall prematurely and excessively, while those that remain on the tree do not color properly, remaining green on the shaded side (Schwarz, 1970; Bové et al., 2006). As they ripen, the stylar end remains green, hence the name "greening" (Gottwald et al., 2007).

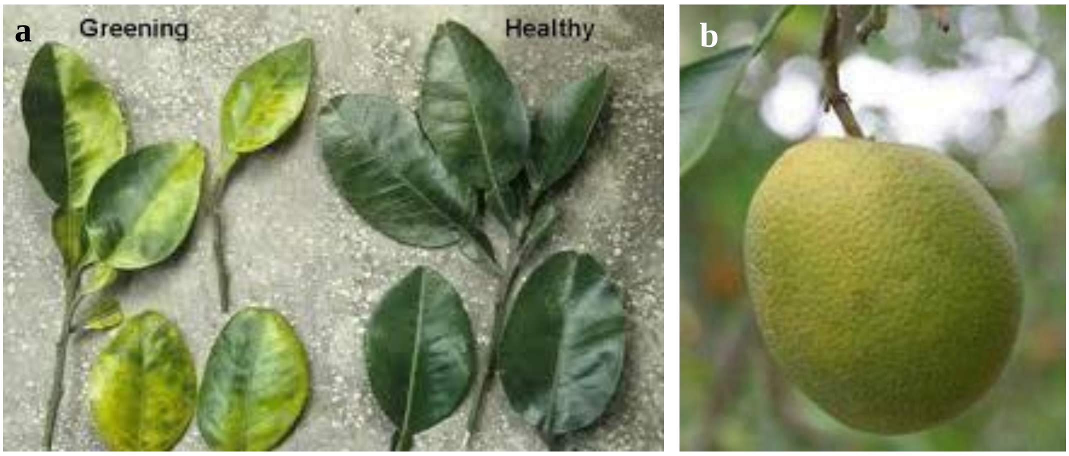

Figure 4. Symptoms of huanglongbing disease (HLB) in citrus tree. (**a**) Citrus leaves from a plant affected by HLB *vs* from a healthy plant; (**b**) Small lopsided fruit from greening-infected citrus tree. Source: University of Florida, 10/11/2022.

HLB is caused by three species of the genus *Candidatus* Liberibacter. *Candidatus Liberibacter* asiaticus (CLas), widespread in Asia and America, *Candidatus Liberibacter* africanus (CLaf), mainly restricted to Africa, and *Candidatus* Liberibacter americanus (CLam), only known from Brazil (Bové, 2006). Additional four subspecies of CLaf have also been recognized: *Candidatus* Liberibacter africanus subsp. capensis (CLafC), *Candidatus* Liberibacter africanus subsp. clausenae (CLafCl), *Candidatus* Liberibacter africanus subsp. zanthoxyli (CLafZ) and *Candidatus* Liberibacter africanus subsp. vepridis (CLafV) (Kumar et al., 2018). These three gram-negative intracellular αproteobacteria species, which colonize the phloem of their host plants, are transmitted by different insects. CLas and CLam are naturally transmitted by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Sternorrhyncha: Liviidae), while CLaf is transmitted by the

African citrus psyllid *Trioza erytreae* Del Guercio (Sternorrhyncha: Triozidae) (Bové, 2006). Either the African HLB (in Africa), or the Asian HLB (in India, Taiwan, the United States, Mexico, and Brazil) have already caused millions in losses, threatening the subsistence of the citriculture sector in those regions, as there is no technically feasible cure for affected plants (Gottwald et al., 2007; Cocuzza et al., 2017). So far, the presence of HLB in the Iberian Peninsula was not detected. Therefore, the containment and control of *T. erytreae* are extremely important to prevent its spreading and thus to reduce the risks of a possible outbreak of HLB (Urbaneja-Bernat et al., 2020).

2.3. Management of *T. erytreae*

The aforementioned emphasize the urgency to find an effective way to control and stop the spreading of *T. erytreae* in the Mediterranean region, something that should be attain sustainably, avoiding the creation of other socio-economic and eco-environmental problems. For instance, chemical control, which may be the quicker strategy to help to keep the population as low as possible, are costly, can trigger the emergence of other pests and may be detrimental to the environment and human health (Cocuzza et al., 2017). Moreover, the attempts of eradication of *T. erytreae* with insecticides in South Africa, Cameroon, Madeira and the Canary Islands were not successful (Cocuzza et al., 2017). Besides chemical-based control, efforts to manage the African HBL disease vector include use of quarantine measures, cultural measures, such as the removal of infected trees, and introduction of natural enemies as biocontrol agents (Van den Berg, 1990; Cocuzza et al., 2017; Aidoo et al., 2019).

Cocuzza et al. (2017) point up biocontrol as a valuable strategy to complement chemical control programs targeting the reduction and spread of *T. erytreae* populations, and thus of HLB. According to the authors, the introduction of natural enemies showing good biocontrol performances should be an effective way, especially in areas where the psyllid population is still low, and in buffer and endangered zones that are yet HLB-free areas. However, there is little information on natural enemies of psyllids, in general (Jerinić-Prodanović and Protić, 2013).

For *T. erytreae*, several parasitoids and predators have been listed, but most of them are native from South Africa (Annecke and Cilliers, 1963; Van den Berg et al., 1987). The list includes spiders, which appear to be the most important predators, lacewings (*chrysopids*), small beetles (*coccinellids*), hoverflies (*syrphids*), brown lacewings (*hemerobiids*), Hemiptera and predatory mites (Van den Berg et al., 1987). Hernández (2003) recorded coccinellids, spiders and lacewings as predators of *T. erytreae* in the Madeira and Canary Islands. According to (Cocuzza et al., 2017), the generalist predators from the Canary Islands preying upon *T. erytreae* are *Chrysoperla carnea* Stephens (Neuroptera, Chrysopidae), *Adalia bipunctata* (L.), *Brumus quadripustulatus* (L.), *Cryptolaemus montrouzieri* Mulsant, *Harmonia axyridis* (Pallas) (Coleoptera, Coccinellidae) and *Orius laevigatus* (Fieber) (Hemiptera, Anthocoridae). However, it was shown that this native fauna is neither sufficiently specific, nor numerous enough to control the pest populations present in citrus orchards from the Canary Islands (Cocuzza et al., 2017). The same trend is occurring in Galicia, mainland Spain (Otero et al., 2016; Cocuzza et al., 2017). The most effective parasitoid of *T. erytreae* seems to be *Tamarixia dryi* (=*Tetrastichus dryi*) (Waterston) (Hymenoptera, Eulophidae), a common and widespread wasp in its native Africa (Cocuzza et al., 2017). The released of this parasitoid in northern Spain and north-western Portugal in the fall of 2019 as a part of a classical biological control programme showed to successfully control *T. erytreae* (Pérez Rodríguez et al., 2019; Urbaneja-Bernat et al., 2019). However, there are still no results from its establishment in mainland Europe, and thus additional methods need to be implemented to reduce the spread of the vector in mainland Europe (Urbaneja-Bernat et al., 2020).

2.4. The use of PCR-based analyses to infer predator-prey interactions

As mentioned above, the exploitation of natural enemies of *T. erytreae*, such as predators, could be one strategy to control this pest. Indeed, several insects belonging to families Coccinellidae, Anthocoridae, Miridae, Chrysopidae, Hemerobiidae, Syrphdiae and Formicidae, and to the order Arachnida, have been reported to be potentially predators of *T. erytreae* (Gonzalez-Hernandez, 2003; Catling, 1970; van den Berg et al., 1987). Trophic relationships are generally assessed by examining the partially digested remains of consumers. However, such approach to infer predator-prey relationships in insects cannot be done, particularly in field conditions (Symondson, 2002). In this regard, the molecular identification of prey in predator diets by using polymerase chain reaction (PCR)-based techniques has been proven to be highly effective in identifying natural enemies of several insect pests (Rejili et al., 2016; Albertini et al., 2018; Rodrigues et al., 2022). Using taxon-specific primers, PCR techniques allow the detection of specific prey ingested in the predator's diet, since their DNA remains in the predator's gut before being eliminated by the digestion process (Symondson, 2002). Thus, the choice of target DNA region and the length of the sequence region to amplify, are two important issues that must be take into account during the design of taxon-specific primers.

The majority of recent studies have used an approach targeting multiple-copy, small and abundant DNA fragments in cells, such as mitochondrial DNA (mtDNA), as the degradation of prey DNA is expected to occur during the digestion process (Sousa et al., 2019). In insects, mtDNA is typically a small double-stranded circular molecule encoding 13 protein-coding genes, two rRNA genes (12S rRNA and 16S rRNA) and 22 tRNA genes, that are required for mitochondrial protein synthesis (Sint et al., 2011). The mitochondrial protein-coding gene cytochrome oxidase subunit I (COI) has been the most commonly targeted DNA barcoding markers in studies of gut contents (e.g., Rejili et al., 2016; Albertini et al., 2018; Rodrigues et al., 2022). In a recent work, this region was also showed to be suitable to detected *T. erytreae* in the gut of several potential predators (Molina et al., 2021). In contrast, the mitochondrial 12S and 16S rRNA genes have been less explored as genetic markers to assess predator–prey interactions s (Klarica et al., 2012). These two rRNA genes have slower substitution rates and are therefore generally better for the design of group-specific primers than COI gene (Ballard, 2000) Moreover, they have many indels (insertion/deletion mutations), making 12S rRNA and 16S rRNA useful species-specific markers (Hoogendoorn et al., 2001). Accordingly, the main goal of this work was to design and evaluate taxon-specific primers targeting in the mitochondrial COI, 12S rRNA and 16S rRNA genes, to be used for a PCR-based diagnostic method, to detect *T. erytreae* within potential predators.

3. Material and methods

3. Material and methods

To fulfil the aim of this work two main tasks were performed. The first one was devoted to the design of a species specific primer sets for *T. erytreae* detection. The second main task was devoted to the evaluation of the effectiveness and specificity of the newly designed primer sets and to optimize PCR conditions, by wet-lab procedures. For that purpose, several specimens of *T. erytreae* and from some of its relatives were use, after confirmation of their identity.

3.1. Design of *Trioza erytreae*-specific primers

A taxon-based search was performed in the NCBI database to check for sequences availability, particularly the eventual existence of (nearly) complete nuclear and/or mitochondrial genome sequences. Along with a preliminary literature survey, this allowed to recognize that the mostly used molecular marker for *Trioza* spp. and their relatives is the mitochondrial encoded cytochrome c oxidase I (COI). This was an expected result since COI is considered the primary barcode sequence for animal kingdom. Sixty partial or complete COI sequences from psyllids taxa were selected, comprising (1) *T. erytreae* specimens from different geographical origins (including from Madeira and Canary Islands), (2) other *Trioza* spp., and (3) phylogenetically close relatives, i.e., representatives from Group D and sister clades (Group A, B and C) from *Triozidae*, as defined in the study of Percy et al. (2018) (Figure 5). Also, it was possible to select and retrieve 24 mitochondrial genome sequences from the NCBI GenBank database from the superfamily Psylloidea, as illustrated in figure 5 by Percy et al. (2018). It included the mitogenomes from *T. erytreae* (NC_038142), from three other *Trioza* spp., and from several close relatives in the *Triozidae*. Multiple alignments either for COI or mitogenome sequences were then obtained using the ClustalW algorithm in MEGA X (Kumar et al., 2018). To evaluate the relationship between selected organisms, those alignments were also subjected to phylogenetic analyses. Neighbor-Joining (NJ) trees with 5000 bootstrap replicates were constructed to each multiple-alignment using the MEGA X. Phylogenetic trees were edited with Inkscape 0.92 (www.inkscape.org). The phylogenetic trees in figures 6 and 7 summarize the psyllids included in each alignment used for the design of the primers and showed the phylogenetic relationship among these organisms.

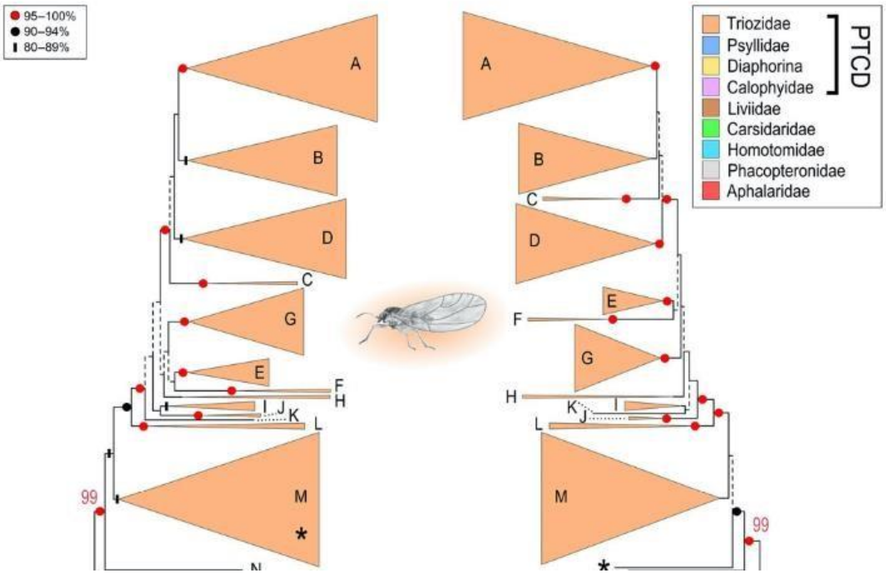

Figure 5. Best maximum likelihood trees generated using the all-taxa/all-nucleotide mitogenome dataset (left) and the all-taxa/conserved-codon mitogenome dataset (right), *Trioza erytreae* is included in clade D. Adapted from Percy et al. (2018).

The design of the primers was performed according to basic primer design rules, e.g., oligonucleotides length, G/C content, melting temperatures, self-dimer and hairpin formation, identification of highly conserved regions for the target organism, among other parameters (Dieffenbach et al., 1993). Oligonucleotide candidates were submitted to *in silico* analyses to ascertain their specificity by using Primer-BLAST (Ye et al., 2012). The thermodynamic properties' suitability of the primers was tested by using the online softwares OligoEvaluator (www.oligoevaluator.com) and PrimerSuite (www.primersuite.com), allowing to choose the better candidates. A shortlist of potentially suitable oligonucleotide candidates was thus obtained (Table 2), with four of them targeting the rrnL gene (16S), three the rrnS gene (12S), and six the COI gene (Figure 8). The primers were then synthesized at Frilabo (Portugal), and further tested.

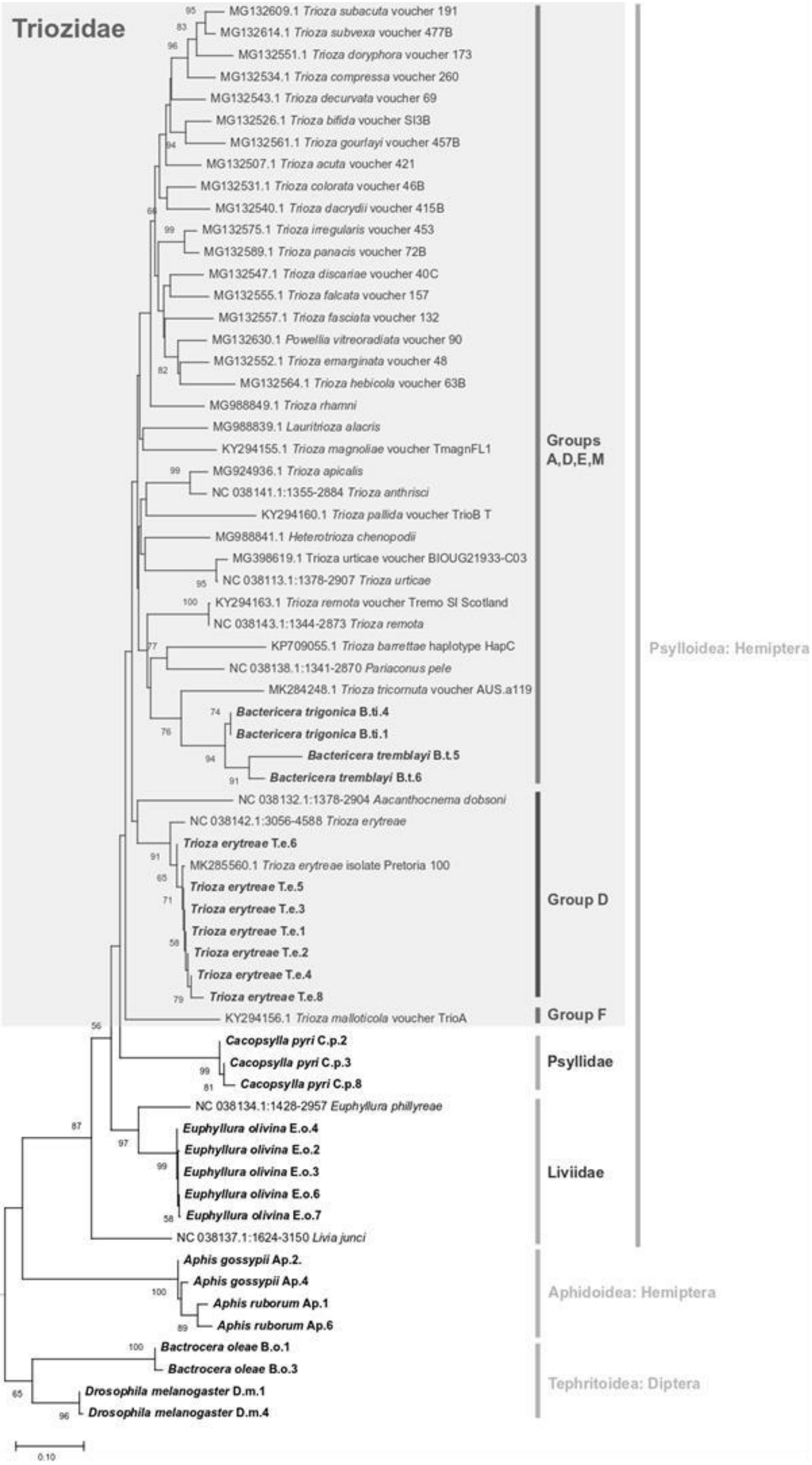

Figure 6. Neighbor joining phylogenetic tree of COI sequences from psyllids and other insects used in this work, either in the primer designing (sequences not bolded, with accession numbers) or in the following primer testing (in bold). Groups of Triozidae according to Percy et al. (2018)

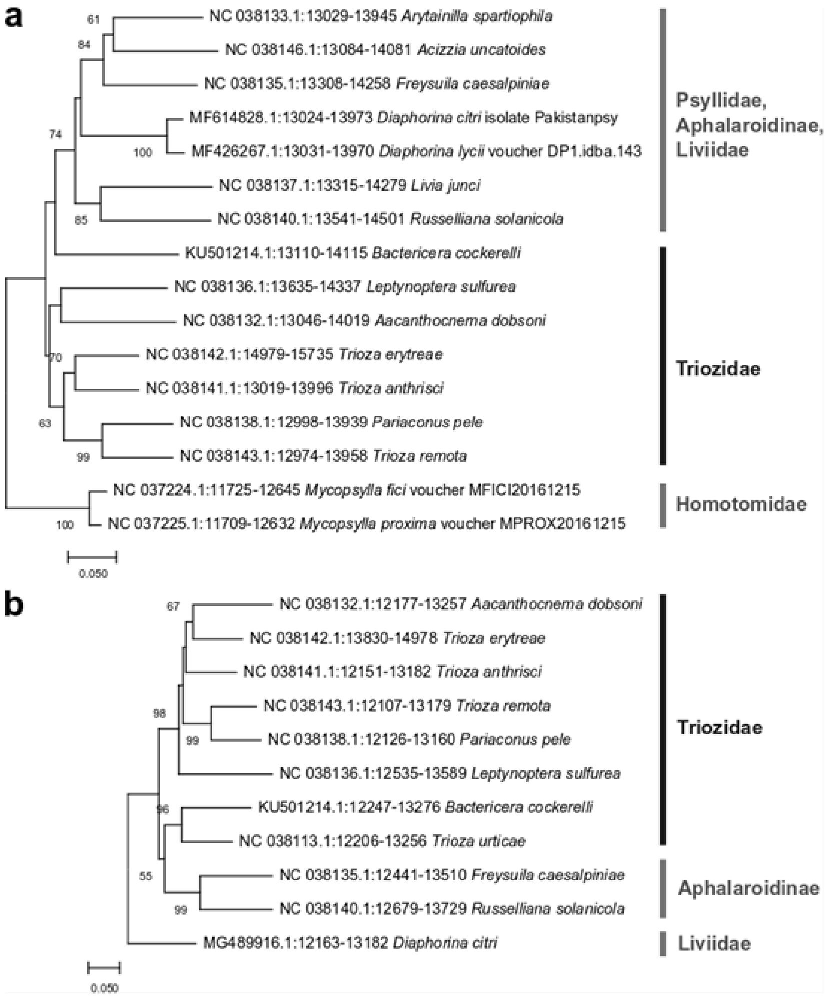

Figure 7. Neighbor joining phylogenetic trees for complete ITS+SSU (**a**) and LSU (**b**) sequences from the family Triozidae and related psyllids used for the primers designing. Sequences were retrieved from complete mitogenomes; nucleotide positions are indicated after accession numbers.

Table 2. Candidate primers designed and developed in this study to specifically amplify *Trioza erytreae*.

Target loci[&]	Primer name	Positions [#]	Sequence 5´- 3´
mt rrnL: 16S	LSU_F1	140-158	GACTTTATACATAAACACC
mt rrnL: 16S	LSU_F2	268-284	CTAAAATAGGATCTTCC
mt rrnL: 16S	LSU_R1	543-559	CCTGCTCAATGCTTAGG
mt rrnL: 16S	LSU_R2	543-561	AACCTGCTCAATGCTTAGG
mt ITS-rrnS: 12S	SSU_F1	56-77	GCTATTCATTTTCTTTCCAGGC
mt ITS-rrnS: 12S	SSU_R1	333-350	GTATGCCGCCGTCATGAG
mt ITS-rrnS: 12S	SSU_R2	333-358	ATTTACTTGTATGCCGCCGTCATGAG
mt COI	T1F	256-282	GCTCCAGATATAGCATTTCCTCGACTT
mt COI	T2F	335-356	AGTTGGGACAGGATGAACAGTT
mt COI	T3F	388-409	GAGGATATTCAGTAGATACTGC
mt COI	T1R	577-596	GCTAAAACAGGTAATGCCAA
mt COI	T2R	579-603	TGCTCCTGCTAAAACAGGTAATGCC
mt COI	T3R	635-656	AGGATCTCCTCCCCCGGCTGGA

[&] *mt*: mitochondrial; *rrnL* and *rrnS*: large (16S) and small (12S) subunit rRNA genes, respectively; *ITS*: internal transcribed spacer; *COI*: cytochrome c oxidase subunit I

[#] Positions according to the mitochondrial complete genome sequence of *Trioza erytreae* (NC_038142.1)

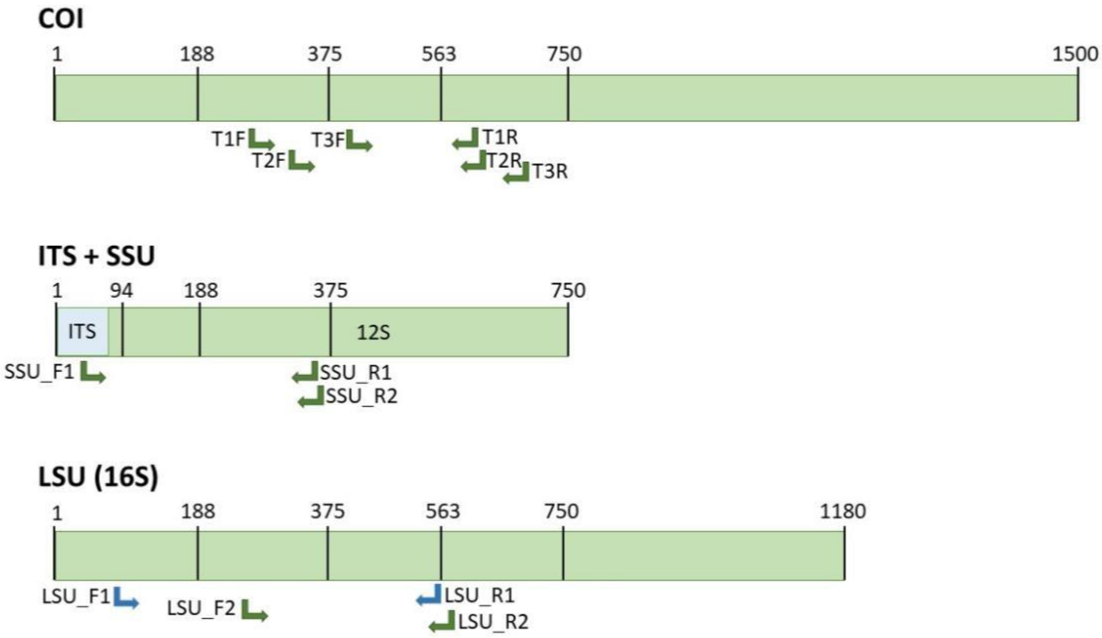

Figure 8. Schematic diagram of COI and rrnL and rrnS gene regions showing the binding sites of the candidate PCR primers developed and tested in this study. Nucleotide positions according to the loci retrieved from the complete mitochondrial genome of *Trioza erytreae* (NC_038142.1); COI depicted from positions 3056-4588 (1533 nt), rrnL (16S) from 13830-14978 (1186 nt), and ITS + rrnS (12S) from 14979-15735 (74 nt, ITS + 684 nt, 12S).

3.2. Molecular identification of arthropods

In order to evaluate the ability of the designed primer pairs to specifically amplify DNA from *Trioza*, a set of specimens of *T. erytreae* and other close relatives' specimens from the Triozidae or other families were molecularly identified, to be used as positive and negative control, respectively. Accordingly, five non-target species were selected, being three from the Triozidae family [*Bactericera tremblayi* (Wagner, 1961), *Bactericera trigonica* (Hodkinson, 1981) and *Bactericera nigricornis* (Foerster, 1848)], one from Psyllidae family [*Cacopsylla pyri* (Linnaeus, 1758)], and another one from the Liviidae family [*Euphyllura olivina* (Costa, 1839)]. These non-target species were selected based on Percy et al. (2018) classification. *B. tremblayi*, *B. trigonica* and *B. nigricornis* were obtained from the Consejo Superior de Investigaciones Científicas (CSIC) of Madrid (Spain). The other specimens were collected in the field with an entomological sweep net (38 cm in diameter), on the *Campus* of the Polytechnic Institute of Bragança (*C. pyri* specimens), and in olive orchards located at Mirandela (*E. olivina* specimens), during spring 2019. Adults of *T. erytreae* were collected in Caracoí (Portugal). All the insects were initially identified to the genus/species level using a binocular stereoscopic, preserved in absolute ethanol and stored at −20 °C, until subsequent DNA extraction.

To confirm the identification of the arthropods, a molecular-based approach was followed. All the insects were homogenized in liquid nitrogen, and the genomic DNA was extracted using the SpeedTools tissue DNA extraction kit (Biotools, Spain) following the manufacturer's instructions. Their identification was performed by sequencing a portion of mitochondrial COI sequence, using the universal primers LCO1490/HCO2198 (Folmer et al., 1994). Accordingly, amplifications were carried out in a MyCycler™ (BioRad) thermocycler, using 20 µl PCR reaction, containing 1x buffer, 2.5 mM of $MgCl_2$, 200 µm of dNTPs, 0.2 µm of each primer, and 1.25 U of Taq DNA polymerase (BIORON, Gmbh). The PCR program was set for an initial denaturation step at 94ºC for 3min, followed by 35 cycles of denaturation at 94ºC for 30s, primer annealing at 57ºC for 30s and extension at 72ºC for 20s followed by a final extension step at 72ºC for 7 min. PCR products (~710 bp) were further visualized in a 1% (v/v) agarose gel stained with 1X Gel Red™ nucleic acid gel dye (Biotium, California, USA). The amplified products were then purified and sequenced at Macrogen Inc. (Madrid, Spain). The DNA sequences were analysed and edited with MEGA v10.1.8 (Kumar et al., 2018) and the identification of each specimen was confirmed by querying the GenBank database using the Nucleotide Basic Local Alignment Search Tool (BLASTn) in NCBI's website (www.ncbi.nlm.nih.gov).

3.3. Evaluation of the specificity and sensitivity of the designed primers

Primer combinations (Table 3) were tested, their PCR conditions optimized, and their specificity and sensitivity evaluated. The specificity of the primers for *T. erytreae* was evaluated by using as template genomic DNA (gDNA) extracted from specimens of this species and from the five non-target species (*B. tremblayi*, *B. trigonica*, *B. nigricornis*, *C. pyri* and *E. olivina*). The sensitivity of the primers was assessed using gDNA from *T. erytreae* mix with gDNA of the five non-target species in the same ratio.

Table 3. Primer sets tested in this study and their estimated PCR product sizes.

Primer sets	Length (bp)
LSU_F1 - LSU_R1	419
LSU_F2 - LSU_R1	291
LSU_F1 - LSU_R2	421
LSU_F2 - LSU_R2	293
SSU_F1 - SSU_R1	294
SSU_F1 - SSU_R2	302
T1F - T1R	343
T1F - T2R	350
T1F - T3R	419
T2F - T1R	245
T2F - T2R	252
T2F - T3R	321
T3F - T1R	192
T3F - T2R	199
T3F - T3R	268

For all PCR assays, each primer pair was used in 10 µL reactions, containing 1x buffer, 2.5 mM of $MgCl_2$, 1.5 mg/mL of bovine serum albumin (BSA, Promega), 200 µM of dNTP’s, 0.2 µM of each primer, and 1.25 U Taq DNA polymerase (BIORON, GmbH). The PCR program was optimized by varying the annealing temperature (from 48ºC to 61ºC through gradient PCR). The annealing temperature should be low enough to allow both forward and reverse primers to bind to the single-stranded DNA, but not so low as to enable the formation of non-specific or intramolecular hairpins. Thereby, the annealing temperature is usually set as a few degrees (3-6) lower than the lowest Tm of the primers. Primer sets showing good performance at higher annealing temperatures were then subjected to further tests and optimizations to improve the specificity and sensitivity by varying the time of denaturation, annealing and extension. Optimized cycling protocols for the selected primer pairs are indicated in the results section.

4. Results and discussion

4. Results and discussion

This study developed a new PCR-based diagnostic assay for *T. erytreae* using the newly primers for three barcode regions of mitochondrial DNA, namely the large 16S (rrnL) and small 12S (rrnS) subunit rRNA genes as well as the standard barcode for invertebrates, cytochrome c oxidase subunit I COI. In total 15 primers were designed, being four targeting rrnL, three targeting rrnS and six the COI region, with lengths ranging from 17 to 27 bp. In theory, longer primers are expected to be less efficient during the annealing step, while shorter ones can be less specific (Grunenwald, 2003). Therefore, this highlights the importance for testing the specificity of the designed primers. Among the designed primers, 15 combinations were possible, that amplified DNA fragments with sizes ranging from 192 bp (targeting COI) to 421 bp (targeting rrnL) (Table 3).

4.1. Effectiveness of the primers to detected T. erytreae

The effectiveness of the designed primers to detected *T. erytreae*, was evaluated for the 15 primers pairs. Accordingly, a PCR was performed varying the annealing temperature from 48ºC to 61ºC. The results showed that all the primers pairs tested successfully yielded DNA fragments of the expected size, although a few amplicons showed faint and/or double bands depending on the annealing temperature (Table 4). Among these 15 primer pairs, five gave clear single bands, at all the annealing temperatures tested, including the highest ones. One of these pair of primers amplifies the rrnL (16S) region (LSU_F2 - LSU_R1, expected size: 291 bp), another one amplifies the rrnS (12S) region (SSU_F1/SSU_R1, expected size: 294 bp) and three amplify the COI region (T2F - T1R, expected size: 245 bp; T3F - T2R, expected size: 199 bp and T1F - T3R, expected size: 419 bp). Among these four primers set, were selected those that amplify a shorter fragment (LSU_F2 - LSU_R1, SSU_F1/SSU_R1, T2F - T1R and T3F - T2R) for further tested their specificity and sensitivity towards *T. erytreae.* Indeed, it is likely that during digestion, the prey DNA molecules are broken into smaller fragments. Thus, the smaller the amplification products size, the greater would be the success to detected *T. erytreae* DNA in the gut of potential predators. Previous studies have similarly reported that the size of amplification products affects prey DNA detection (Hoogendoorn et al., 2001.).

Table 4. Results of amplification observed in the different annealing temperatures tested for each primer set to improve the detection of *Trioza erytreae*.

Primer set	Annealing temperature				
	48.1 ºC	52.7 ºC	56.1 ºC	57.7 ºC	61.4 ºC
LSU_F1 LSU_R1	double bands	positive	positive	fainted band	fainted band
LSU_F2 LSU_R1	positive	positive	positive	positive	positive
LSU_F1 LSU_R2	fainted band	double bands	double bands	positive	positive
LSU_F2 LSU_R2	positive	positive	double bands	double bands	fainted band
SSU_F1 SSU_R1	positive	positive	positive	positive	positive
SSU_F1 SSU_R2	fainted band	fainted band	positive	positive	fainted band
T1F - T1R	double bands	double bands	positive	positive	fainted band
T1F - T2R	double bands	fainted band	fainted band	positive	positive
T1F - T3R	positive	positive	positive	positive	positive
T2F - T1R	positive	positive	positive	positive	positive
T2F - T2R	fainted band	positive	positive	positive	positive
T2F - T3R	positive	positive	fainted band	positive	positive
T3F - T1R	positive	fainted band	positive	fainted band	positive
T3F - T2R	positive	positive	positive	positive	positive
T3F - T3R	fainted band	positive	positive	fainted band	positive

PCR condition: initial denaturation for 3 min at 94°C, followed by 30 cycles at 94°C for 40 s, 48 to 61°C for 40 s, 72°C for 30s and a final extension at 72°C for 7 min.

4.2. Molecular identification of the arthropods

The COI sequences of the species specimens used in this study, confirmed the initial identification based on morphological traits (Table 5).

Table 5. Nucleotide Basic Local Alignment Search Tool (BLASTn) best-hit results for the different COI sequences (obtained with the universal primers) from specimens studied in this work.

Scientific name	**Score**	**Query cover**	**E-value**	**Identity (%)**	**Accession**
Bactericera tremblayi	1315	100%	0.0	98.40%	MT396422.1
Bactericera trigonica	822	99%	0.0	98.29%	MG988654.1
Bactericera nigricornis	898	100%	0.0	98.43%	MG924937.1
Cacopsylla pyri	782	100%	0.0	98.86%	MG988672.1
Euphyllura olivina	913	100%	0.0	99.60%	MT021797.1
Trioza erytreae	1242	100%	0.0	99.85%	MW286253.1

4.3. Specificity and sensitivity of the primers to detected *T. erytreae*

The specificity of the four pairs of primers previous selected was tested by using as templates genomic DNA extracted from *T. erytreae* and from five non-target species (including closely-related taxa of *Trioza*), such as *B. tremblayi*, *B. trigonica*, *B. nigricornis*, *C. pyri* and *E. olivina*. Accordingly, the primers sets were tested by using a PCR cycling consisting of an initial denaturation at 94°C (for 3 min), followed by 30 cycles at 94°C for 40 s, 61.4°C for 40 s, 72°C for 30s and a final extension at 72°C for 7 min. The results showed that when the four pair of primers were tested for the five nontarget species no clear PCR product was observed, while a positive amplification was noted when primers were tested with *T. erytreae* (Table 6). However, occasionally a weak PCR product was noted when primers sets SSU_F1-SSU_R1 and LSU_F2-LSU_R1 were tested with *C. pyri* and *B. tremblayi*, respectively. Similarly, the primer set T2F-T1R could occasionally detected the presence of *E. olivina* and *B. trigonica.* These results suggest that these three primer sets are not specific enough to detected *T. erytreae*. Curiously, no PCR product was amplified by these three primer sets when a mixture of DNA of the 5 non-target species at equal ratios was used. This result may be due to the competition of the target DNA for the same primers, resulting in absence of amplification. The primer set T3F-T2R was the only one that showed high specificity to DNA from *T. erytreae*.

The four primers sets also showed the capacity to detected DNA from *T. erytreae* when added to DNA of the five non-target species, at the same ratio (Table 6) (Figure 9). However, these results are not reliable for the primer sets SSU_F1-SSU_R1, LSU_F2LSU_R1 and T2F-T1R due to their lack of specificity to *T. erytreae* DNA.

In order to avoid the problem related to the lack of specificity, the conditions of the amplification were changed by increasing the time of the extension from 30s to 40s. The annealing temperature was kept the same (61.4ºC). This was the highest annealing temperature studied, and it is generally recognized that at high annealing temperature the primers maximally increased amplification specificity without sacrificing sensitivity (Ballantyne et al., 2008). The results showed that with these new PCR conditions, the specificity of the primers sets tested was improved, with exception for the primer set SSU_F1-SSU_R1 (Table 7).

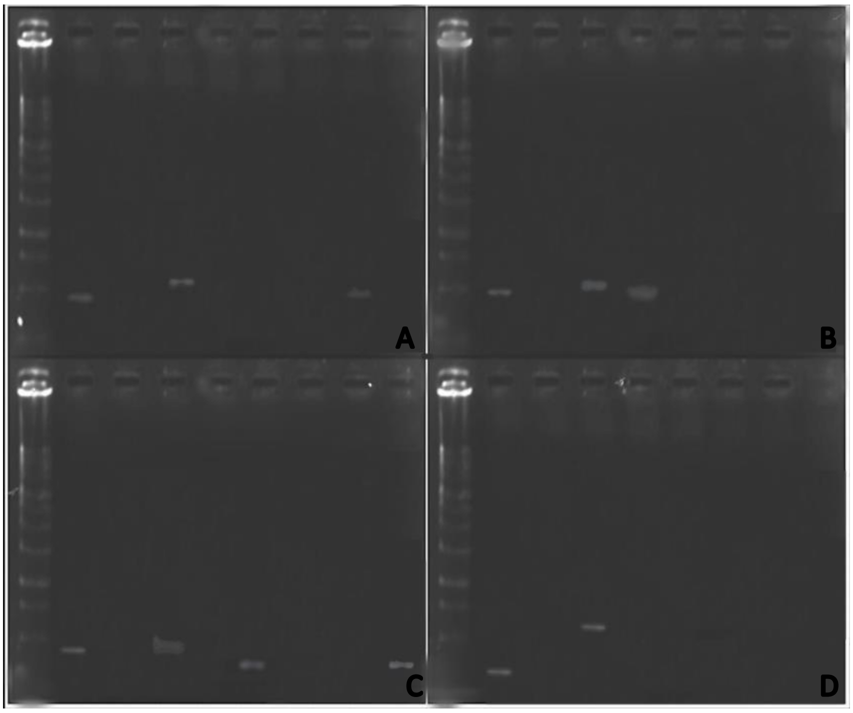

Figure 09. Agarose gel electrophoresis of amplification products of the PCR using primer pairs as followed: (A) LSU_F2 - LSU_R1, (B) SSU_F1/SSU_R1, (C) T2F - T1R and (D) T3F - T2R. Lane M: Molecular Weight Marker 1000 bp DNA Ladder (BIORON, GmbH). **line 01** Test 1: mix with gDNA of the five non-target species and *Trioza erytreae* in the same ratio, **line 02** Test 2: mix with gDNA of the five non-target species in the same ratio.**line 03** *T. erytreae* **line 04** *C. pyri* **line 05** *E. olivina* **line 06** *B. nigricornis* **line 07** *B. tremblayi* **line 08** *B. trigonica*

Table 6. Results of amplification observed for the different primer sets when tested with DNA of *Trioza erytreae* and non-target species.

Primer set	Test 1	Test 2	*T. erytreae*	*C. pyri*	*E. olivina*	*B. nigricornis*	*B. tremblayi*	*B. trigonica*
LSU_F2 LSU_R1	Positive	Negative	Positive	Negative	Negative	Negative	Faint band	Negative
SSU_F1 SSU_R1	Positive	Negative	Positive	Faint band	Negative	Negative	Negative	Negative
T2F T1R	Positive	Negative	Positive	Negative	Faint band	Negative	Negative	Faint band
T3F T2R	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative

Test 1: mix with gDNA of the five non-target species and *Trioza erytreae* in the same ratio. **Test 2**: mix with gDNA of the five non-target species in the same ratio.

PCR condition: initial denaturation for 3 min at 94°C, followed by 30 cycles at 94°C for 40 s, 61.4°C for 40s, 72°C for 30s and a final extension at 72°C for 7 min.

Table 7. Results of amplification observed for the different primer sets when tested with DNA of *Trioza erytreae* and non-target species.

Primer set	Test 1	Test 2	*T. erytreae*	*C. pyri*	*E. olivina*	*B. nigricornis*	*B. tremblayi*	*B. trigonica*
LSU_F2 LSU_R1	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
SSU_F1 SSU_R1	Positive	Faint band	Positive	Negative	Negative	Negative	Faint band	Negative
T2F T1R	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative
T3F T2R	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative

Test 1: mix with gDNA of the five non-target species and *Trioza erytreae* in the same ratio. **Test 2**: mix with gDNA of the five non-target species in the same ratio.
PCR condition: initial denaturation for 3 min at 94°C, followed by 30 cycles at 94°C for 40s, 61.4°C for 40 s, 72°C for 40s and a final extension at 72°C for 7 min.

Overall, among the four primers pairs, T3F-T2R showed to be the most sensitive and specific to DNA from *T. erytreae*, at any PCR conditions tested. This primer pair targets one region of the mitochondrial protein-coding genes, cytochrome c oxidase subunit COI. This region is recognized suitable for insect species identification because of their relatively larger universality and high level of inter-species variation (Magoda et al., 2022). In recent years, a steady number of DNA primers targeting this region have been developed (Elbrecht et al., 2019) and demonstrated to work effectively on different insect taxa. Moreover, COI has been the most widely used marker to detect prey DNA within predator's guts (King et al., 2008; Sint et al., 2011; Rejili et al., 2016; Albertini et al., 2018; Rodrigues et al., 2022). This is mainly due to the easily of amplified the COI region when present at low quantity and quality DNA in the gut samples because each cell contains hundreds to thousands of mitochondrial genomes and there is a positive correlation between gene copy number and detection success (Agusti et al., 2003). Recently, (Molina et al., 2021) have designed from the COI region a pair of specific primers that successfully detect *T. erytreae* in the gut of a wide range of predators, by amplifying a fragment of 194 bp. Thus, the COI seems to be an optimal target for species-specific detection of predation of *T. erytreae*. In our study, the primer set T3F-T2R target a relatively short sequence region (i.e., 199 bp) of the COI, suggesting to be able to detect denatured or fragmented DNA samples in the gut of predators. During digestion, prey DNA molecules are broken into smaller fragments and thus, their detection in gut it is likely to enhanced by targeting short DNA fragments. Typically, primers targeting longer DNA sequences (more than 500 bps) have a higher probability of facilitating specieslevel identification, but their application to gut content analysis are in general unsuccessful (Clarke et al., 2014). Therefore, we hypothesized that the primer set T3F_T2R is suitable to detected *T. erytreae* in predator guts. However, further research should be undertaken to investigate this hypothesis.

Besides COI, 16S ribosomal RNA seems also to be useful for species-specific detection of predation of *T. erytreae*. The primer pair LSU_F2-LSU_R1 targets one region from 16S ribosomal RNA and generates a PCR product of 291 bp.

In conclusion, although preliminary, the findings suggest that the *T. erytreae* primers here designed and the optimized PCR-based diagnostic assay can provide an effective and sensitive method for detecting potential predators of the main vector of *Candidatus* Liberibacter spp. This PCR-based diagnostic assay can help in the implementation of more sustainable measures to limit the spread of this vector-borne pathogen. However, the effectiveness of this diagnostic tool must be first validated by screening for the presence of DNA of *T. erytreae* in the gut of a predator, using samples from a bioassay carried out under controlled conditions, i.e. by using predators that were seen to have fed on *T. erytreae*. The detection of *T. erytreae* in the gut of the predator must be also evaluated in field-collected samples.

5. Conclusion and future perspectives

5. Conclusion and future perspectives

To conclude, from the 15 primer sets developed in this study two showed to be highly specific and sensitive to detected *T. erytreae* DNA, inclusively when mixed at equal ratios with DNA of closely-related taxa of *Trioza* (such as *B. tremblayi*, *B. trigonica*, *B. nigricornis*, *C. pyri* and *E. olivina*). These two primers sets, targeting the COI gene (T3F - T2R) and the rrnL gene (LSU_F2-LSU_R1), generate a PCR product with 199 bp and 291 bp, respectively. Their high specificity and sensitivity was achieved with the following PCR cycling conditions: initial denaturation for 3 min at 94°C, followed by 30 cycles at 94°C for 40s, 61.4°C for 40 s, 72°C for 40s and a final extension at 72°C for 7 min. Because of the smaller size of the fragment that they amplify, there is more probability for their ability to detect *Trioza* DNA in the gut of potential predators. Despite these promising results, the suitability of both T3F - T2R and LSU_F2-LSU_R1 primer sets, to detected *T. erytreae* DNA must be tested in feeding assays and further evaluated in field-collected insects. We hope that this PCR-based diagnostic assay would be a contribution to identify potential predators of *T. erytreae*, and thus to limited the spread of the pathogen vectored by this insect, *Candidatus* Liberibacter spp., in Europe.

References

1. Agustí, N. U. R. I. A., Unruh, T. R., & Welter, S. C. (2003). Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. *Bulletin of entomological research*, *93*(3), 179-185.
2. Aidoo, O. F., Tanga, C. M., Khamis, F. M., Rasowo, B. A., Mohamed, S. A., Badii, B. K., ... & Borgemeister, C. (2019). Host suitability and feeding preference of the African citrus triozid *Trioza erytreae* Del Guercio (Hemiptera: Triozidae), natural vector of "Candidatus Liberibacter africanus". *Journal of Applied Entomology*, *143*(3), 262-270.
3. Albertini, A., Santos, S. A., Martins, F., Pereira, J. A., Lino-Neto, T., Petacchi, R., & Baptista, P. (2018). Detection of *Bactrocera oleae* (Diptera: Tephritidae) DNA in the gut of the soil species Pseudoophonus rufipes (Coleoptera: Carabidae). *Spanish journal of agricultural research*, *16*(3), 18.
4. Annecke, DP & Cilliers, C. J. (1963). The citrus psylla, *Trioza erytreae* (Del Guercio), and its parasites. *South African Journal of Agricultural Science*, *6*(2), 187-191.
5. Arenas-Arenas, F. J., Durán-Vila, N., Quinto, J., & Hervalejo, A. (2018). Is the presence of *Trioza erytreae*, vector of Huanglongbing disease, endangering the Mediterranean citrus industry? Survey of its population density and geographical spread over the last years. *Journal of Plant Pathology*, *100*(3), 567-574.
6. Arenas-Arenas, F. J., Durán-Vila, N., Quinto, J., & Hervalejo, A. (2019). Geographic spread and inter-annual evolution of populations of *Trioza erytreae* in the Iberian Peninsula. *Journal of Plant Pathology*, *101*(4), 1151-1157.
7. Ballantyne, K. N., Van Oorschot, R. A. H., & Mitchell, R. J. (2008). Locked nucleic acids in PCR primers increase sensitivity and performance. *Genomics*, *91*(3), 301-305.
8. Ballard, J. W. O. (2000). Comparative genomics of mitochondrial DNA in members of the Drosophila melanogaster subgroup. *Journal of Molecular Evolution*, *51*(1), 48-63.
9. Bové, J. M. (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of plant pathology*, 7-37.

10. Catling, H. D. (1970). The bionomics of the South African citrus psylla *Trioza erytreae* (Del Guercio)(Homoptera: Psyllidae) 4. The influence of predators. *Journal of the Entomological Society of Southern Africa*, *33*(2), 341-348.
11. Clarke, L. J., Soubrier, J., Weyrich, L. S., & Cooper, A. (2014). Environmental metabarcodes for insects: in silico PCR reveals potential for taxonomic bias. *Molecular ecology resources*, *14*(6), 1160-1170.
12. Cocuzza, G. E. M., Alberto, U., Hernández-Suárez, E., Siverio, F., Di Silvestro, S., Tena, A., & Carmelo, R. (2017). A review on *Trioza erytreae* (African citrus psyllid), now in mainland Europe, and its potential risk as vector of huanglongbing (HLB) in citrus. *Journal of pest science*, *90*(1), 1-17.
13. Del Guercio, G. (1918). Notes and Observations on Agricultural Entomology. Preliminary Notes. *Notes and Observations on Agricultural Entomology. Preliminary Notes.*
14. Dieffenbach, C. W., Lowe, T. M., & Dveksler, G. S. (1993). General concepts for PCR primer design. *PCR methods appl*, *3*(3), S30-S37.
15. Duran-Vila, N., & Bové, J. M. (2015). Citrus HLB is an emerging disease transmitted by psyllid vectors. Can it be prevented? If not, can it be managed?. *Int. Cent. Adv. Mediterr. Agron. Stud. Watch Lett*, *3*, 1-9.
16. Elbrecht, V., Braukmann, T. W., Ivanova, N. V., Prosser, S. W., Hajibabaei, M., Wright, M., ... & Steinke, D. (2019). Validation of COI metabarcoding primers for terrestrial arthropods. *PeerJ*, *7*, e7745.
17. Gottwald, T. R., Graça, J. V. D., & Bassanezi, R. B. (2007). Citrus huanglongbing: the pathogen and its impact. *Plant Health Progress*, *8*(1), 31.
18. Green, G. E., & Catling, H. D. (1971). Weather-induced mortality of the citrus psylla, Trioza erytreae (Del Guercio)(Homoptera: Psyllidae), a vector of greening virus, in some citrus producing areas of southern Africa. *Agricultural Meteorology*, *8*, 305317.
19. Grunenwald, H. (2003). Optimization of polymerase chain reactions. In *PCR protocols* (pp. 89-99). *Humana Press.*
20. Hernández, A. G. (2003). *Trioza erytreae* (Del Guercio 1918): nueva plaga de los citricos en Canarias. *Phytoma España: La revista profesional de sanidad vegetal*, (153), 112-118.

21. Hernández, A. G. (2003). *Trioza erytreae* (Del Guercio 1918): nueva plaga de los citricos en Canarias. *Phytoma España: La revista profesional de sanidad vegetal*, (153), 112-118.
22. Hollis, D., & Martin, J. H. (1997). Jumping plantlice (Hemiptera: Psylloidea) attacking avocado pear trees, *Persea americana*, in the New World, with a review of Lauraceae-feeding among psylloids. *Bulletin of Entomological Research*, *87*(5), 471-480.
23. Hoogendoorn, M., & Heimpel, G. E. (2001). PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. *Molecular Ecology*, *10*(8), 2059-2067.
24. Jerinić-Prodanović, D., & Protić, L. (2013). True bugs (Hemiptera, Heteroptera) as psyllid predators (Hemiptera, Psylloidea). *ZooKeys*, (319), 169.
25. King, R. A., Read, D. S., Traugott, M., & Symondson, W. O. C. (2008). INVITED REVIEW: Molecular analysis of predation: a review of best practice for DNAbased approaches. *Molecular ecology*, *17*(4), 947-963.
26. Klarica, J., Kloss-Brandstätter, A., Traugott, M., & Juen, A. (2012). Comparing four mitochondrial genes in earthworms–implications for identification, phylogenetics, and discovery of cryptic species. *Soil Biology and Biochemistry*, *45*, 23-30.
27. Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, *35*(6), 1547.
28. Lee, J. A., Halbert, S. E., Dawson, W. O., Robertson, C. J., Keesling, J. E., & Singer, B. H. (2015). Asymptomatic spread of Huanglongbing and implications for disease control. *Proceedings of the National Academy of Sciences*, *112*(24), 7605-7610
29. Magoga, G., Forni, G., Brunetti, M., Meral, A., Spada, A., De Biase, A., & Montagna, M. (2022). Curation of a reference database of COI sequences for insect identification through DNA metabarcoding: COins. *Database*, *2022*.
30. Molina, P., Martínez-Ferrer, M. T., Campos-Rivela, J. M., Riudavets, J., & Agustí, N. (2021). Development of a PCR-based method for the screening of potential predators of the African citrus psyllid Trioza erytreae (Del Guercio). *Biological Control*, *160*, 104661.

31. Otero, RP, Lorenzo, SP, & Vázquez, JPM (2016). The African citrus psylla (*Trioza erytreae* Del Guercio) and its current situation in Galicia. *Fruit growing magazine* , (48), 48-59.
32. Percy, D. M., Crampton-Platt, A., Sveinsson, S., Lemmon, A. R., Lemmon, E. M., Ouvrard, D., & Burckhardt, D. (2018). Resolving the psyllid tree of life: phylogenomic analyses of the superfamily Psylloidea (Hemiptera). *Systematic Entomology*, *43*(4), 762-776.
33. Pérez-Rodríguez, J., Krüger, K., Pérez-Hedo, M., Ruiz-Rivero, O., Urbaneja, A., & Tena, A. (2019). Classical biological control of the African citrus psyllid *Trioza erytreae,* a major threat to the European citrus industry. *Scientific reports*, *9*(1), 1-11.
34. Rejili, M., Fernandes, T., Dinis, A. M., Pereira, J. A., Baptista, P., Santos, S. A., & Lino Neto, T. (2016). A PCR-based diagnostic assay for detecting DNA of the olive fruit fly, *Bactrocera oleae*, in the gut of soil-living arthropods. *Bulletin of entomological research*, *106*(5), 695-699.
35. Rodrigues, I., Ramos, V., Benhadi-Marín, J., Moreno, A., Fereres, A., Pereira, J. A., & Baptista, P. (2022). A novel molecular diagnostic method for the gut content analysis of Philaenus DNA. *Scientific reports*, *12*(1), 1-9.
36. Schwarz, R. E., & Green, G. C. (1970). Das „Citrus-Greening" und der Citrusblattfloh *Trioza erytreae*—ein temperaturabhängiger Erreger-Überträgerkomplex. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz/Journal of Plant Diseases and Protection*, 490-493.
37. Sint, D., Raso, L., Kaufmann, R., & Traugott, M. (2011). Optimizing methods for PCR based analysis of predation. *Molecular ecology resources*, *11*(5), 795-801.
38. Symondson, W. O. C. (2002). Molecular identification of prey in predator diets. *Molecular ecology*, *11*(4), 627-64.
39. Timmer, L. W., Garnsey, S. M., & Broadbent, P. (2003). Diseases of citrus. *Diseases of tropical fruit crops*, 163-195.
40. Urbaneja-Bernat, P., Pérez-Rodríguez, J., Krüger, K., Catalán, J., Rizza, R., HernándezSuárez, E., ... & Tena, A. (2019). Host range testing of *Tamarixia dryi* (Hymenoptera: Eulophidae) sourced from South Africa for classical biological control of *Trioza erytreae* (Hemiptera: Psyllidae) in Europe. *Biological control*, *135,* 110-116.

41. Urbaneja-Bernat, P., Hernández-Suárez, E., Tena, A., & Urbaneja, A. (2020). Preventive measures to limit the spread of *Trioza erytreae* (Del Guercio) (Hemiptera: Psyllidae) in Europe. *Biological control*, *135*, 110-116.
42. Van den Berg, M. A. (1990). The citrus pyslla, *Trioza erytreae* (Del Guercio)(hemiptera: Triozidae): A review. *Agriculture, ecosystems & environment*, *30*(3-4), 171-194.
43. Van den Berg, M. A., and Cecelia D. Fletcher. "A bibliography of the citrus psylla, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), up to 1987." Phytoparasitica 16.1 (1988): 47-61.
44. Van den Berg, M. A., Deacon, V. E., & Jager, K. D. (1990). Ecology of the citrus psylla, *Trioza erytreae* (Hemiptera: Triozidae). 1. Daily activities and habits of adults. *Phytophylactica*, *22*(3), 323-328.
45. Wang, N. (2020). A perspective of citrus Huanglongbing in the context of the Mediterranean Basin. *Journal of Plant Pathology*, *102*(3), 635-640.
46. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics*, *13*(1), 1-11.