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SHORT COMMUNICATION

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The ongoing dispersion of the Eucalyptus bronze bug (*Thaumastocoris peregrinus*) in Spain

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Abstract

Aim of study: Thaumastocoris peregrinus (Carpintero & Dellapé, 2006) is notable for its dispersion potential, and for its damage to a wide range of hosts of the genus *Eucalyptus*. The intense movement of people and cargo between continents contributes to the success of its geographical distribution on the globe and hinders the adoption of preventive measures. The celerity and precision in the identification of *T. peregrinus*, as well as its invasion and dispersion routes are fundamental for the implementation of measures to prevent new invasions.

Area of Study: Park of Retiro in the community of Madrid, Spain, where *T. peregrinus* is present.

Material and methods: We analyzed a fragment of COI mtDNA gene in *T. peregrinus* specimens, using samples collected at a public park in Madrid urban area, to study the possible pathways of incursion of this insect in Spain. The goal was achieved using molecular tools, with PCR amplification of partial mtDNA COI and sequencing the fragment, which is used as a barcode of life for identification at species level. Species identity was confirmed using the database in GenBank.

Results: The results confirms that the specimens found in Madrid are *T. peregrinus*, and all are from the same maternal lineage.

Research highlights: We present the first molecular information of *T. peregrinus* population present in Spain, and suggest and discuss possible routes of incursion of this pest.

Additional keywords: Invasive pest; DNA Barcoding; Invasion Routes; Forest Entomology.

Authors´ contributions: Conceived and designed the work: DNM, ECC, CRP, JAA, ACR. Performed the experiments and analyzed the data: DNM, ACR, CRP, JVL, MAS, GAU. Contributed materials/analysis tools: CRP, GAU, TLC. All authors assisted in writing and reviewed the article.

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Introduction

The genus *Eucalyptus* is widely used in the world for fast-growing tree plantations, covering large areas, which are increasingly attacked by a diverse array of invasive pests, most of them obviously originary from Australia (Hurley *et al.*, 2016). These insect pests produce significant reductions of the productivity of the plantations and widespread rapidly over many regions, as is the case of *Gonipterus scutellatus* complex, including *Gonipterus platensis* (Marelli, 1926) (Coleoptera: Curculionidae) and *Gonipterus pulverulentus* Lea, 1897 (Coleoptera: Curculionidae) (Mapondera *et al.*, 2012); as well as species of Hymenoptera order *Leptocybe invasa* Fisher & LaSalle, 2004 (Hymenoptera: Eulophidae) (Zheng *et*

al., 2014) and species of Hemiptera order, comprising *Glycaspis brimblecombei* (Moore, 1964) (Hemiptera: Aphalaridae) (Karaca *et al.*, 2017) and *Thaumastocoris peregrinus*) (Carpintero & Dellapé, 2006) (Hemiptera: Thaumastocoridae).

Thaumastocoris peregrinus stands out for causing severe damage to more than 50 species of *Eucalyptus*, including hybrids (Saavedra *et al.*, 2015b). Aspects such as its short life cycle and the high reproductive potential of females allow rapid population growth of the pest (Nadel *et al.*, 2015; Soliman *et al.*, 2012). When the infestation is severe, there is a significant reduction of leaves in the canopy and in some cases may lead to the death of the attacked trees, due to the feeding process being in chloroplasts and other cellular contents, leading to chlorosis (Santadino *et al.*, 2017). Thus, there is a reduction of the photosynthetic area of the plant and consequently its growth (Jacobs & Neser, 2005). The expansion of occurrence of *T. peregrinus* includes several countries: South Africa (Jacobs & Neser, 2005), Argentina (Carpintero & Dellapé, 2006), Zimbabwe (Chilima, 2007), Brazil (Wilcken, 2008), Uruguay (Martínez & Bianchi, 2010), Chile (Mayorga *et al.*, 2011), New Zealand (Sopow *et al.*, 2012), Paraguay (Díaz *et al.*, 2013), Italy (Laudonia & Sasso, 2012; Carapezza, 2014), Portugal (Garcia *et al.*, 2013), Spain (Vivas *et al.*, 2015), Reunion Island (Streito *et al.*, 2016), Israel (Novoselsky & Freideberg, 2016), Mexico (Jiménez-Quiroz *et al.*, 2016), United States of America (Hodel *et al.*, 2016), Albania (Heyden, 2017) and Greece (Petrakis, 2018). In Spain, *T. peregrinus* had been recorded at Barbaño, province of Badajoz in 2014 (Vivas *et al.*, 2015). The expansion of the species continues, and in this paper, we record it from the central area of Spain, at Madrid.

Based on the potential global distribution of the Bronze bug, European countries present localities with optimal climate conditions to its occurrence and dispersion (Saavedra *et al.*, 2015a). However, there is no information about the genetic constitution of populations of *T. peregrinus* in Spain, even if this information is extremely important to know the diversity and possible routes of invasion, which are determined by the genetic signatures of the populations present on site. In this context, this work aims to report the expansion of *T. peregrinus* occurrence area in Spain and to characterize, using molecular analysis, the genetic structure and possible invasion routes of the population found in Madrid, Spain.

Materials and Methods

Specimens sampling

Bronze bug specimens were sampled around Spain from November 2018 to April 2019, in Galiza region, including Vigo, Pontevedra, Santiago de Compostela and Baiona, and also in Barcelona and in Madrid. Galiza region have optimal climate conditions to a potential distribution of *T. peregrinus* in Europe as reported by Saavedra *et al.* (2015a), but we could not find any specimens in this area. Specimens of *T. peregrinus* were found in a city park "Parque de El Retiro" at Madrid, Spain (40°25'07.8"N - 3°41'13.2"W) (Fig. 1a) (40°24'54.4"N - 3°40'52.98"W) on April 12, 2019. We examined three *Eucalyptus globulus* and two *Eucalyptus camaldulensis* trees. However, only *E. camaldulensis* presented a moderate infestation of bronze-bug, since there were symptoms of tanning

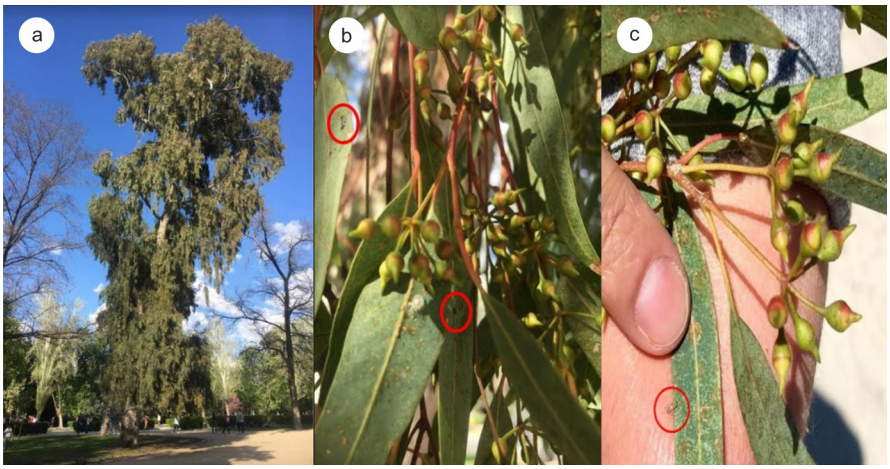

Figure 1. (a) *Eucalyptus* tree in the El Retiro Park of Madrid with attack of *T. peregrinus*; (b) Leaves with dark egg masses, aggregated as is typical of *T. peregrinus* and (c) adult of *T. peregrinus* (body length about 3 mm).

on leaves, many egg masses (Fig. 1b) and presence of nymphs and adults (Fig. 1c). *Eucalyptus* trees had a phytosanitary status of infestation by other insects, including *G. brimblecombei*. The weather during the day of sampling was between 5-19°C and had a clear sky (www.accuweather.com). We believe that the population of *T. peregrinus* sampled was in the beginning of infestation period, because we found many eggs and not many nymphs and adults on leaves.

Sampling procedure was performed on lower leaves of these trees collecting randomly the branches with bronze bug infestation. Insects were removed from leaves using a paint-brush and placed them in a plastic container (20 ml) with 96% alcohol, totalizing 10 specimens from each tree.

Samples collected in April were sent to the Laboratory of Forest Entomology - UFSM, Santa Maria, RS, Brazil, where the morphological characterization of the specimens was performed according to the morphological characters described by Carpintero & Dellapé (2006). The gDNA extraction and molecular characterization of the specimens were performed in the Laboratory of Integrated Pest Management (LabMIP-UFSM).

In August 5 of the same year with temperature around 35ºC the trees were rechecked for infestation of bronze-bug and adults were found abundant, but eggs and nymphs were rare. Specimens from August were not sampled and not genetically analyzed, because the high probability of these insects to be family related.

DNA extraction, amplification and sequencing

Five insects were selected randomly from the specimens collected from each tree, for the extraction of gDNA. We used the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) to gDNA extraction of individual insects, according to the manufacturer's protocol. First of all, each adult was exposed to dry air in a paper towel in order to allow the ethanol evaporation. Then, we used 180 µL of buffer ATL and 20 µL of proteinase K to macerate the entire insect body in a 1.5 ml tube and left incubating at 56°C for 12 hours. Afterwards, gDNA was purified in a silica-based matrix and eluted in 35 µL of buffer AE. gDNA concentration was assessed using a NanoDrop™ 1000 (Thermo Scientific, Wilmington, DE, USA).

Samples with a gDNA concentration over 5ng/µL were used to amplify a fragment of the mitochondrial COI gene (468 bp) by polymerase chain reaction (PCR). The primers Tp2390F (5'ACCCGAGCATACTTTAC-TTC) and Tp2937R (5' ATTGTGGCTCGTTTTGATA) (Nadel *et al.*, 2010) were used. The PCR reaction included: 1.25 µL of JumpStart™ 10X reaction buffer; 7,0 µL of ultra-pure water; 0.625 µL de dNTP mixture (10 nM of each); 1.0 µL of each primer (10 pM), 0.220 µL of JumpStart™ DNA Polymerase (2.5 U/ µL) (Sigma-Aldrich, St. Louis, MO, USA), and 1 µL of gDNA (05-100 ng/ µL).

The PCR reaction was performed following Nadel *et al.* (2010) protocol: initial denaturation at 98°C for 30 s, followed by 30 cycles at 95°C for 30 s, 48°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. The amplified products of mtDNA (COI fragment) were resolved on 1.0% agarose electrophoresis gel, pre-stained with Nancy-520 DNA gel stain (Sigma-Aldrich) and visualized with a gel documentation system. PCR products that were successfully amplified were sequenced by ACTGene genomics service provider (Alvorada, RS, BR), using an AB 3500 Genetic Analyzer.

Data analysis

The data from sequencing output were trimmed, edited and analyzed for each individual COI sequence, using the software Pregap and Gap4 within the Staden package (Staden & Bonfield, 2000). CLC Sequence Viewer (Version 7.8.1 - QIAGEN Aarhus A/S) was used to cut the ends, retrieve and align sequences of 468 bp long, based on a sequence model from GenBank (FJ623760). In addition, a pBlast analysis (amino acid homology confirmation) with *T. peregrinus* partial mtDNA COI genes on NCBI (National Center for Biotechnology Information, USA database) was performed. The sequence of *T. peregrinus* from Madrid was deposited in GenBank under accession number MN401749.

Results

From the morphological and molecular characterization of the adult insects found in the Parque de El Retiro of Madrid, we confirm that all the sequenced specimens belong to the species *Thaumastocoris peregrinus* (Carpintero & Dellapé, 2006). All the specimens belong to the haplotype A, described by Nadel *et al.* (2010).

Discussion

The expansion of the area of *T. peregrinus* in Spain was already expected, since the intense traffic of people and goods (Hurley *et al.*, 2016) and because the climatic suitable conditions predicted by CLIMEX, that reports between a low and moderate potential distribution of

this pest on Madrid region (Saavedra *et al.*, 2015a). Presently this species has been widely distributed in some countries, e.g. as Brazil (Wilcken *et al.*, 2010) and Portugal (Garcia *et al.*, 2013).

Currently, it is known that only four strains of *T. peregrinus* of the eight haplotypes found by Nadel *et al.* (2010) and the 44 haplotypes found by Lo *et al.* (2019) in Australia, are scattered in different countries, two in South Africa (D and G haplotypes), one in South America (samples from Argentina, Brazil, Uruguay) (haplotype A - FJ623760) (Nadel *et al.*, 2010) and one in Italy (IT haplotype - KF437485), not yet found in the center of origin of the pest (Nugnes *et al.*, 2014).

The *T. peregrinus* population found in Madrid belongs to haplotype A, and there is no genetic diversity among the insects collected. Generally, in their area of natural occurrence, the species possess greater genetic diversity in relation to populations introduced in new environments (Puillandre *et al.*, 2008).

When analyzing the first occurrence records of *T. peregrinus* in Europe, we verified that Italy (2011) (Laudonia & Sasso, 2012) and Portugal (2012) (Garcia *et al.*, 2013) reported the presence of the insect before its detection in Spain. Therefore, we can suggest some hypothesis of introduction of *T. peregrinus* in Spain (Fig. 2), based on the genetic information of the population of Madrid. The most likely route of introduction may have been through the border with Portugal by natural dispersion (flight, hitchhiking in birds), transit of people and plant material or unintentionally by the importation of *Eucalyptus* timber from Latin America.

According to Garcia *et al.* (2013), most of the introductions of invasive alien species are mainly due to human activity, and *T. peregrinus* was probably introduced in Portugal through the importation of eucalypt logs from a Latin American country. In fact, the populations of *T. peregrinus* from Brazil, Argentina and Uruguay belong to the same haplotype found in Spain (Nadel *et al.*, 2010). Thus, it is suggested that the haplotype present in Portugal is also haplotype A. Alternatively, it could have been introduced from Italy, however, the analysis of mtDNA from specimens of the population found in Italy have revealed the presence of a distinct haplotype, IT (Nugnes *et al.*, 2014).

An introduction from Israel would be improbable because the first record of *T. peregrinus* was dated one month earlier than in Spain (Novoselsky & Freideberg, 2016). Finally, a direct introduction from Australia would also be very unlikely. The possible routes of introduction in Spain, can be confirmed when DNA analysis of populations of Portugal became available, in order to determine which lineage(s) of *T. peregrinus* are in the country. In addition, specimens from the region of Badajoz, Spain, could confirm the presence of the same haplotype, due to the proximity to the border of Portugal, where the pest is widespread (Garcia *et al.*, 2013). We believe that an update of the genetic diversity of this species should be carried out in South America, Italy and South Africa, to find out if new introductory events with new haplotypes have occurred, thus increasing diversity in these places.

Thaumastocoris peregrinus is considered a prolific species, however, insects can go unnoticed until their abundance becomes critical. In Israel, it has been reported that these insects cause irritation and eruptions on the skin of some people who visited parks, possibly due to the direct contact of the specimens with the skin (Novoselsky & Freideberg, 2016). In this sense, the population of *T. peregrinus* found in a park in

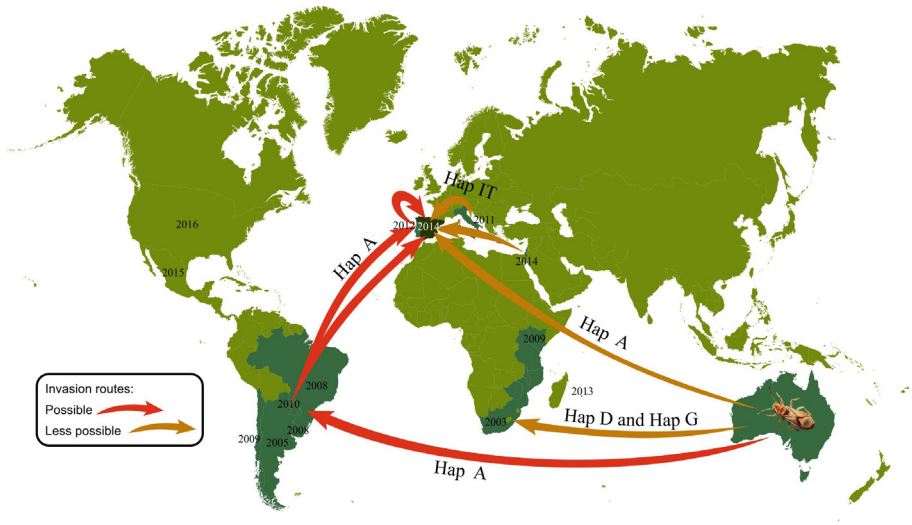

Figure 2. Possible routes of introduction of *T. peregrinus* in Spain.

Madrid, where the movement and the presence of people is intense, should be monitored. After detection, if preventive measures are not taken to control invasive species, their complete eradication will be more unlikely due to increasing population growth (Harvey & Mazzotti, 2014). It is important to highlight that eucalypt plantations in Spain comprise an area of approximately 500,000 ha (IFN, 2011; Santolamazza-Carbone *et al.*, 2019).

There are no effective strategies for controlling *T. peregrinus*. Using pesticides, however, there was a significant reduction in populations in trees treated with imidacloprid in Australia (Noack *et al.*, 2009). The results indicated that lambda-cyhalothrin + thiametoxam (1.2 + 25.8 g / ha) showed 100% control of insects after 24 hours of exposure (Machado *et al.*, 2016). On the other hand, biological control is being widely diffused for *T. peregrinus*. Two species of Mymaridae wasps, *Cleruchoides noackae* Lin et Huber and *Stethynium* sp. Enock were characterized as parasitoids of *T. peregrinus* eggs in Australia (Lin *et al.*, 2007). The species *C. noackae* was introduced in Brazil in 2012 and released in several states (Wilcken *et al.*, 2015), because the introduction and successful establishment of natural enemies is important to regulate pest populations (Gerard *et al.*, 2011; Thompson & Reddy, 2016). In addition, the species *Chrysoperla externa* (Hagen, 1861) (Neuroptera: Chrysopidae), was found preying nymphs of *T. peregrinus* (Wilcken *et al.*, 2010) and *Atopozelus opsimus* (Elkins, 1954) (Hemiptera: Reduviidae) (Dias *et al.*, 2014), adult insects of *T. peregrinus.*

Furthermore, there are studies with entomopathogenic fungi such as *Beauveria bassiana* Vuill and *Isaria* sp. (Lorencetti *et al.*, 2017). In addition, tests are in progress with isolates from the genera *Beauveria, Fusarium, Isaria, Lecanicillium, Paecilomyces, Pochonia, Purpureocillium* and *Simplicillium* (Corallo *et al.*, 2019) and *Metarhizium anisopliae* (Metsch.) (Soliman *et al.*, 2019), which demonstrated potential for control of *T. peregrinus*.

Biosecurity measures should be intensified globally in order to hamper the rapid spread of invasive pests and possible damage to plantations. In this sense, we report the expansion of *T. peregrinus* in Spain and confirm that the population found in Madrid belongs to haplotype A. This is the same maternal lineage found in South America, being one of the probable routes of introduction of this haplotype in Spain.

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