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Presence of the invasive whitefly *Aleurocanthus spiniferus* (Hemiptera: Aleyrodidae) in Greece

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Aleurocanthus spiniferus (Quaintance) (Hemiptera: Aleyrodidae), commonly known as the citrus (or orange) spiny whitefly, is an important pest of various economic crops such as citrus and tea and causes severe economic losses. It is reported for the first time in Greece, in the island of Corfu (North-West Greece) on sweet orange trees (*Citrus sinensis*). Morphological and molecular data has been used for species identification. Sequences of the mitochondrial cytochrome oxidase I (*COI*) gene from Greek populations have been compared with sequences of the species from Italy and Montenegro, as well as with *COI* sequences retrieved from GenBank, to examine the genetic diversity of the species. Based on the preliminary results, it appears that the population from Corfu probably arose through several independent introductions.

Introduction

The citrus (or orange) spiny whitefly, *Aleurocanthus spiniferus* (Quaintance) (Hemiptera: Aleyrodidae), originating from South-East Asia (China) (Uesugi *et al.*, 2016), has extended its distribution to tropical and subtropical Asia (Kuwana, 1934). Since the early 20th century, the whitefly has been introduced into new areas (Van den Berg *et al.*, 1990; Nguyen *et al.*, 1993; EPPO/CABI, 1997; Muniappan *et al.*, 2006; Gyeltshen *et al.*, 2010). In Europe, it was detected for the first time in 2008, on orange trees in the Apulia region of Southern Italy (Porcelli, 2008), and since then it became acclimatized and spread northward up to the Brindisi district (Cioffi *et al.*, 2013). Four years later, it was recorded in Croatia, on the coast of the Adriatic Sea, on orange seedlings (*Citrus aurantium* L.) from a greenhouse in Split (Šimala & Masten Milek, 2013). One year later, in October of 2013, it was found again on the Adriatic coast in Montenegro, on citrus orchards (Radonjić *et al.*, 2014).

Aleurocanthus spiniferus is a highly polyphagous pest infesting more than 90 plant species belonging to 38 families (Cioffi *et al.*, 2013). These include several highly important crop plants, such as citrus (Kuwana, 1928; Weems, 1974; Byrne *et al.*, 1990), tea plants (Han & Cui, 2003), grapes (*Vitis vinifera*), guava (*Psidium guajava*), pear (*Pyrus* spp.), persimmon (*Diospyros kaki*) and rose (*Rosa* spp.) (Nakao & Funasaki, 1979; Evans, 2008). In tropical Asia, *A. spiniferus* is considered to be one of the most destructive aleyrodid pests of citrus and one of the most important pests in Japan (Anonymous, 1975), Australia (Gillespie, 2012) and the USA (Cioffi *et al.*, 2013).

Aleurocanthus spiniferus is recorded in the lists of quarantine pests of East and Southern Africa, Argentina, Brazil, the USA, Uruguay, Bahrain, Israel, Jordan, Russia, Turkey and Ukraine (EPPO, 2018). It is listed in the EPPO A2 List of pests recommended for regulation as a quarantine pest and regulated by the EU Annex II/A1 Council Directive 2000/29/EC as a quarantine pest, and its introduction and distribution in all member states of European Union (EU) is prohibited.

Both larvae and adults cause severe damage to host plants. Due to piercing and sucking of plant phloem sap, leaf growth is damaged and fruit production is reduced. Under intense infestations, the nitrogen level in leaves is decreased and plants are weakened with a deformed appearance. Moreover, excreted honeydew that covers the leaves, branches and fruit surfaces, induces the development of sooty mould fungus which reduces photosynthesis (EPPO/CABI, 1997; Muniappan *et al.*, 2006; Cioffi *et al.*, 2013).

Knowledge of the genetic composition of invading populations is important for understanding the invasion process (Kreiser *et al.*, 2000; Cristescu *et al.*, 2001; Patti & Gambi, 2001), as substantial genetic variability is expected to favour adaptation, and thus the successful establishment of the introduced populations (Lee, 2002; Kolbe *et al.*, 2004; Facon *et al.*, 2006; Lavergne & Molofsky, 2007). Despite the importance of DNA-based methods for evaluating genetic diversity, limited studies focusing only on Asian populations of the species are available regarding the genetic diversity of *A. spiniferus* (Fu & Han, 2007; Kanmiya *et al.*, 2011; Tang *et al.*, 2014, 2015; Chen *et al.*, 2016; Uesugi *et al.*, 2016).

The aim of this study is to report the first data of the presence of *A. spiniferus* in Greece and to address the genetic variability among and between the Greek populations and those from neighbouring countries (Italy and Montenegro), providing a first insight into its genetic variability.

Materials and methods

Official surveys for the presence of quarantine insect pests harmful to plants, including the citrus spiny whitefly, are performed annually in Greece under the auspices of the National Plant Protection Organization of Greece. Based on the methodology of the official surveys, phytosanitary inspectors from Greek regional units perform visual inspection in certain crops including citrus.

In the case of *A. spiniferus*, whenever suspicious symptoms are observed, samples must be collected and sent to the Benaki Phytopathological Institute (BPI) for further examination. Laboratory examination of suspicious samples is conducted according to the EPPO Diagnostic Protocol for regulated pests (EPPO, 2002). Briefly, male puparia are slide-mounted as permanent microscopic slides and identified on the basis of morphological characters using the identification keys provided by EPPO (EPPO, 2002). If, based on the morphological keys, insects appear to be *A. spiniferus* then molecular analysis of a partial nucleotide sequence of the cytochrome oxidase I (*COI*) gene of the mitochondrial DNA (mtDNA) is conducted to confirm the results.

In the present study, samples from various plants (mainly *Citrus* spp.) with suspicious symptoms from the island of Corfu were examined in the BPI's Laboratory of Agricultural Entomology in September 2016. Insect specimens were removed from the leaves and prepared for morphological examination (EPPO, 2002). Additional samples were received in November and December 2016 and in August 2017.

Moreover, 36 individuals from the aforementioned samples, from three different locations on the island (Perama, 39.5777182/19.91020790; Gimari, 39.73342516/19.92260811; Kassiopi, 39.78938695/19.92286028) were selected for molecular species identification and nucleotide variation. In addition, 22 individuals in total, coming from two foreign populations (Italy, Gagliano del Capo 39.841881/18.372622 and Montenegro) were also analysed to investigate the intraspecific sequence variation of the species between the populations found in Greece and in neighbouring countries.

Total genomic DNA (gDNA) was extracted from single whitefly adults or pupae using the cetyltrimethyl ammonium bromide (CTAB) DNA isolation method following the protocol as described by Milligan (1998). The extracted DNA was used as the DNA source for the polymerase chain reaction (PCR). The primers Alspin-REP-FOR (5′-GTGTCCCATTTAATTAGTAGAGA-3′) and Alspin-REP-REV (5′-GAGCCATAATAAAAGACTCCATC-3′) were used to amplify a 636 bp fragment of the mitochondrial *COI* gene (Uesugi *et al.*, 2016). Two microlitres of the gDNA extract was used as the template in 20 μL reactions containing 0.2 mM dNTPs, 1.0 mM of each oligonucleotide primer, 1 μL Kapa-Taq DNA polymerase (Kapa Biosystems) and 1× Enzyme Buffer. A negative control (de-ionized water instead of the DNA template) was added to each reaction to confirm there was no contamination. PCR tests were performed under the following conditions: one step of initial denaturation at 95°C for 3 min; 35 cycles at 95°C for 30 s, 53°C for 1 min and 72°C for 1 min; and one step of final extension at 72°C for 2 min. Amplification products were visualized on a 1.2% agarose gel electrophoresis containing Midori Dye, Green Staining. The PCR products were purified using the Nucleo-Fast PCR Clean-up Kit (Macherey-Nagel) according to the manufacturer's instructions and commercially sequenced in both directions, using the primers mentioned above, by the Macrogen sequencing service (Macrogen Inc., Europe). Sequences obtained in the present study were analysed and examined for variation using BioEdit v.7.0 software (Hall, 1999) and were compared with the corresponding ones available in GenBank using the BLAST algorithm of NCBI.

Results and discussion

The presence of *A. spiniferus* in Greece was recorded for first time in the north-east part of the island of Corfu in September 2016. A branch from an orange orchard (*Citrus sinensis*) was sent to the Entomology and Agricultural Zoology Department of BPI in Athens for laboratory examination. The infested leaves of the branch were covered by black nymphs that were surrounded by a white fringe of waxy secretion and by adults having a metallic grey-blue appearance with white patterns on their wings. The pest was identified to the species level as *A. spiniferus* on the basis of morphological examination and molecular analysis. Additional samples of infested host plants (*Citrus* spp., *Vitis vinifera*, *Rosa* sp.) that were received in November and December of 2016 and in August of 2017 were also found to be infested by *A. spiniferus*.

Four different haplotypes were observed within the Greek populations examined. The authors designated them using the numbers 1–4 (H1–H4) (haplotype designation was not consistent with any previous literature). All individuals collected from *C. sinensis* plants from Corfu were genetically similar to each other, presenting a single haplotype (H1). The aforementioned sequence had only one point mutation compared with two identical sequences from China (accession nos AB786721 and AB786723) (Uesugi *et al.*, 2016). The H2 haplotype which was found to comprise some of the specimens from *Citrus limon* in Corfu, the majority of the Italian population on *C. sinensis* and all the specimens of the Montenegro population on *Citrus reticulata*, was identical to one from China deposited in the NCBI (accession no. AB786720) (Uesugi *et al.*, 2016). Haplotype H3 was detected for the first time and comprised some of the individuals collected from both *Rosa* sp. and *C. limon* plants from Corfu and a few specimens of the Italian population on *C. sinensis*. The H4 haplotype, which was also a novel one, was found in a few specimens collected from Corfu from *C. limon* and *Rosa* sp. (Table 1). The nucleotide sequences of the new haplotypes were deposited to GenBank under the accession numbers MH700443 to MH700446.

Table 1. Percentages of the detected haplotypes (H1–H4) in all populations examined

Region	Host	Number of insect specimens analysed	Haplotypes			
			H1 (%)	H2 (%)	H3 (%)	H4 (%)
Greece (Corfu)	*Citrus sinensis*	20	100			
Greece (Corfu)	*Citrus limon*	7		28.6	42.8	28.6
Greece (Corfu)	*Rosa* sp.	9			77.8	22.2
Italy	*Citrus sinensis*	15		80	20	
Montenegro	*Citrus reticulata*	7		100		

High levels of genetic diversity and the creation of novel genetic combinations over a short period of time are commonly associated with multiple introductions in invasions from different source regions (Ellstrand & Schierenbeck, 2000; Bossdorf *et al.*, 2005; Novak & Mack, 2005; Wares *et al.*, 2005; Roman & Darling, 2007; Dlugosch & Parker, 2008). The origin of the species in Greece is still unknown, but according to the molecular data presented in this paper and the genetic diversity identified in this study, the authors suggest that the pest is likely to have had several independent introduction events (Porcelli, 2008; Radonjić *et al.*, 2014).

Genetic diversity within populations of *A. spiniferus* in introduced areas may favour its settlement and spread to regions with suitable conditions. In Corfu, *A. spiniferus* host plants are abundant and climatic conditions are also favourable for its development. Its introduction in Corfu is expected to pose a serious threat for the citrus production of the island and eventually of the whole country, as the possibility of the insect spreading to the Greek mainland is high. Whiteflies can reduce citrus production by over 40–50%, if they are not properly managed (Cioffi *et al.*, 2013). The use of insecticides is expensive and generally ineffective (Gyeltshen *et al.*, 2010). An alternative solution for the reduction of pest damage in citrus plants could be the use of natural enemies. *Aleurocanthus spiniferus* appears to be successfully controlled by natural enemies in its native range (Kuwana & Ishii, 1927; Peterson, 1955; Van den Berg & Greenland, 1997). Various parasitoid species such as *Ablerus connectans*, *Amitus hesperidum*, *Encarsia* spp. and *Eretmocerus* spp. (Silvestri, 1928; Clausen *et al.*, 1978; Muniappan *et al.*, 1992; Evans, 2008) have been used as biological control agents worldwide (Japan, Guam, Ponape, Micronesia) and have resulted in an effective management of the pest (Smith, 1945; Ohgushi, 1969; Quezada, 1974; Clausen *et al.*, 1978). Thus, the release of natural enemies in the island of Corfu originating from the native range of *A. spiniferus* might be a promising option for its control.

Conclusion

Currently, *A. spiniferus* has a limited distribution in Europe and in Greece is restricted to a single geographical area, in the north part of the island of Corfu. Its genetic diversity suggests that there have been multiple introductions of the pest in Corfu. Intense surveillance monitoring is required to estimate the extent of its distribution in the specific area. Plant health authorities and farmers should cooperate to tackle the invasion of this quarantine insect.

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Présence de l'aleurode envahissant *Aleurocanthus spiniferus* (Hemiptera : Aleyrodidae) en Grèce

Aleurocanthus spiniferus (Quaintance) (Hemiptera : Aleyrodidae), communément appelé aleurode épineux du citronnier, est un organisme nuisible de diverses cultures d'intérêt économique telles que les agrumes et le thé, et causant d'importantes pertes économiques. Il est signalé pour la première fois en Grèce, sur l'île de Corfou (Nord-Ouest de la Grèce) sur des orangers doux (*Citrus sinensis*). Des données morphologiques et moléculaires ont été utilisées pour l'identification de l'espèce. Les séquences du gène mitochondrial de la cytochrome oxydase de type I provenant des populations grecques ont été comparées à aux séquences de populations de l'espèce provenant d'Italie et du Monténégro, ainsi qu'à des séquences de COI extraites de Genbank, pour examiner la diversité génétique de l'espèce. Sur la base de résultats préliminaires, il est probable que plusieurs introductions indépendantes soient à l'origine de la population de Corfou.

Присутствие инвазивной белокрылки *Aleurocanthus spiniferus* (Hemiptera: Aleyrodidae) в Греции

Aleurocanthus spiniferus (Quaintance) (Hemiptera: Aleyrodidae), обычно известная как цитрусовая (или апельсиновая) колючая белокрылка, является серьезным вредным организмом для различных экономических культур, таких как цитрусовые и чай, и вызывает

серьёзные экономические потери. Впервые сообщается о её присутствии в Греции, на острове Корфу (северо-западная Греция) на деревьях сладкого апельсина (*Citrus sinensis*). Для идентификации вида использовались морфологические и молекулярные данные. Для изучения генетического разнообразия вида митохондриальная цитохромоксидаза гена I греческих популяций сравнивались с последовательностями вида из Италии и Черногории, а также с последовательностями COI, полученными из Genbank. Исходя из наших предварительных результатов, похоже, что популяции Корфу возникли путём нескольких независимых интродукций.

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