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First record of *Ceroplastes japonicus* (Gray) (Hemiptera: Coccidae) in Greece and a combined approach of morphological identification and DNA barcoding

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High levels of infestation of a wax soft scale insect (Hemiptera: Coccidae) have been observed on *Laurus nobilis* L. (Lauraceae) and *Diospyros kaki* Thunb. (Ebanaceae) in Greece in recent years. Specimens of *Ceroplastes* were collected from regions of Thessaloniki, Naousa and Kavala (northern Greece) and Attica (southern Greece) in 2012–2017. The specimens were identified based on DNA barcoding and female morphological characteristics as *Ceroplastes japonicus* Green (Hemiptera: Coccidae).

Introduction

The family Coccidae (Hemiptera: Coccomorpha) is the third largest within the Coccoidea with about 1200 described species (García Morales *et al.* 2016). Hodgson (1994) recognized 12 subfamilies of which the wax scale insect (Ceroplastinae) is one of the largest. Ceroplastinae can be characterized by the thick waxy layer (test) that covers the whole of the dorsum of the live insect (Gimpel *et al.*, 1974; Qin & Gullan, 1994) and includes many important pest species of agricultural and ornamental plants (García Morales *et al.,* 2016; Qin & Gullan, 1994). *Ceroplastes* Gray genus contains 144 species in total worldwide, of which 73 appear to be endemic to the Neotropics, 58 to the Ethiopian Region (Hodgson & Peronti, 2012), and only 4 or 5 to other zoogeographical regions. In the Mediterranean part of the Palaearctic and from the Nearctic regions *Ceroplates* species are mostly introduced (Kozár & Ben-Dov, 1997; García-Morales *et al.,* 2016.

Sixteen *Ceroplastes* species have been recorded in the Palaearctic (Fetyko & Kozar, 2012). In recent years, *Ceroplastes japonicus* has been introduced into European countries such as Bulgaria, Croatia, France, Italy, Slovenia and Turkey (Pellizzari & Camporese, 1994; Masten-Milek et al., 2007; Kaydan & Kondo, 2008; Trencheva *et al.*, 2010).

Pellizzari *et al.* (2015) recorded 207 species of which 35 species belong to the Coccidae family on the Greek mainland. *Ceroplastes* species recorded on the Greek mainland are *C. rusci* (Linnaeus) (Bodenheimer, 1928; Ayoutantis, 1940), *C. sinensis* (Del Guercio) (Argyriou *et al.*, 1976), *C. floridensis* (Comstock) (Argyriou, 1979; Argyriou & Kourmadas, 1980) and *C. cirripediformis* (Comstock) (Argyriou, 1983).

Identification of the insects to species level is necessary to understand the diversity of the species and evolutionary relationships. Identifications based on morphological characteristics are often difficult and time-consuming (Barrett & Hebert, 2005). Molecular methods are used widely by taxonomists due to the complexity associated with traditional morphological methods (Navajas & Fenton, 2000). Among these methods, DNA barcoding is the easiest and most frequently used (Papadopoulou, 2013).

In Greece, the first record of *C. japonicus* (Gray) (Hemiptera: Coccidae) was presented in a poster at the Panhellenic Entomological Congress by the first three authors of this paper based on morphological characteristics (Papadopoulou, Kaydan, Manganaris, 2017).

The aim of the present work was to study the *Ceroplastes* species in the Greek mainland and to construct an identification key to those known in the country. DNA barcoding methodology was used for the first time for European scale for this species, as a tool for quick and reliable identification. This paper provides the first report in the scientific literature of *C. japonicus* in Greece.

Materials and methods

During 2012−2017 insect pest surveys were carried out on different host plants. Serious scale insect infestations were observed *on Laurus nobilis* and *Diospyros kaki* (specifically on fruits stems and foliage) in Greece, in the prefectures of Thessaloniki and Kavala (northern Greece) by the first and third authors, and in the prefecture of Attica (southern Greece) by the last author (C. Chrysochoidis). The samples of *L. nobilis* came from trees that are cultivated as ornamental and greenhouse plants.

Plants infested with adults of wax scale insects were collected and brought to the Entomological Laboratory of International Hellenic University for identification.

Specimens were slide-mounted for light microscopy using the method of Kosztarab & Kozár (1988). The morphological terminology of Hodgson (1994) was used and identification was made by using the keys by Pellizari and Camporese (1994).

Specimens were deposited in the Coccoidea Collection in Çukurova University, Biotechnology Application and Research Centre, Balcalı, Adana, Turkey and in Laboratory of Entomology, in International Hellenic University; School of Agriculture, Greece.

Sampling and DNA extraction

Five individuals collected from the above regions were used for DNA barcoding. Genomic DNA was extracted from whole samples using a PureLink™ Genomic DNA Mini kit (Invitrogen, Carlsbad, California, USA). The DNA concentration was determined using a ND-2000 NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and was adjusted to approximately 30 ng μL^{-1} through dilution with Tris-EDTA (TE) buffer.

PCR and sequencing analysis

The mitochondrial cytochrome c oxidase I (COI) gene was chosen as the target for the analysis of each sample. The primers for the amplification of a fragment of the selected gene were C1-1554F: 5'-CAGGAATAATAGGAACATCAATAAG-3' and C1-2342R: 5'-ATCAATGTCTAATCCGATAGTAAATA-3' (Deng *et al*, 2012). PCR was performed in 10 μL volumes containing 8 μL (1×) of *Taq* 2X Master Mix (New England Biolabs, Ipswich, Massachusetts, USA), 0.5 μL of each forward and reverse primer (0.5 μM each) and 1 μL of template DNA (~30 ng). Cycling conditions consisted of an initial denaturation step of 95°C for 3 min followed by 35 cycles of 94°C for 30 s, 52°C for 50 s and 72°C for 1 min, with a final extension at 72°C for 10 min. A volume of 3 μL of each PCR product was electrophoresed on a 1.5% agarose gel stained with Midori Green DNA stain (NIPPON Genetics Europe, Dueren, Germany). The quality and quantity of the fragments were checked against a FastGene 100 bp DNA Ladder (NIPPON Genetics Europe). Finally, the amplified DNA fragments were purified with microCLEAN DNA cleanup reagent (Gel Company, San Francisco, California, USA) according to the manufacturer's protocol.

The purified PCR products were sequenced using BigDye Terminator v3.1 (Life Technologies, Carlsbad, California, USA) cycle sequencing methodology on an ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The resulting sequences, after manual checking and editing in BioEdit v7.2.6 (Hall, 1999), were submitted to the BLAST program (blastn) in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and to The Barcode of Life Data System (BOLD, http://www.boldsystems.org/index.php) to compare them with those deposited in public databases. The identity threshold for species identification was set at 99%.

Results and discussion

Key to *Ceroplastes* species in Greece (adapted from Pellizari and Camporese, 1994)

1. Tubular ducts with enlarged inner filament; stigmatic setae lanceolate with pointed apices 3
-. Tubular ducts with narrow inner filament; stigmatic setae conical or bullet-shaped, with blunt apices 4

2. Stigmatic setae of anterior and posterior cleft usually forming an uninterrupted row on the body margin (average 111 stigmatic setae on each side) ..... *C. japonicus* Green.
-. Stigmatic setae of anterior and posterior rows of stigmatic setae on body margin separated by 7–12 marginal setae, bristle shaped (average 60 stigmatic setae on each side) ..... *C. floridensis* Comstock.

3. Antennae 6 segmented ..... *C. rusci* (Linneaeus)
-. Antennae 7 segmented 4

4. Filamentous ducts present on ventral submargin; without multilocular pores on abdominal segments II and III and near coxae of meso- and metathoracic legs ..... *C. sinensis* Del Guercio.
-. Filamentous ducts absent; multilocular pores present on abdominal segments II and III and near coxae of meso-and metathoracic legs ..... *C. cirripediformis* Comstock.

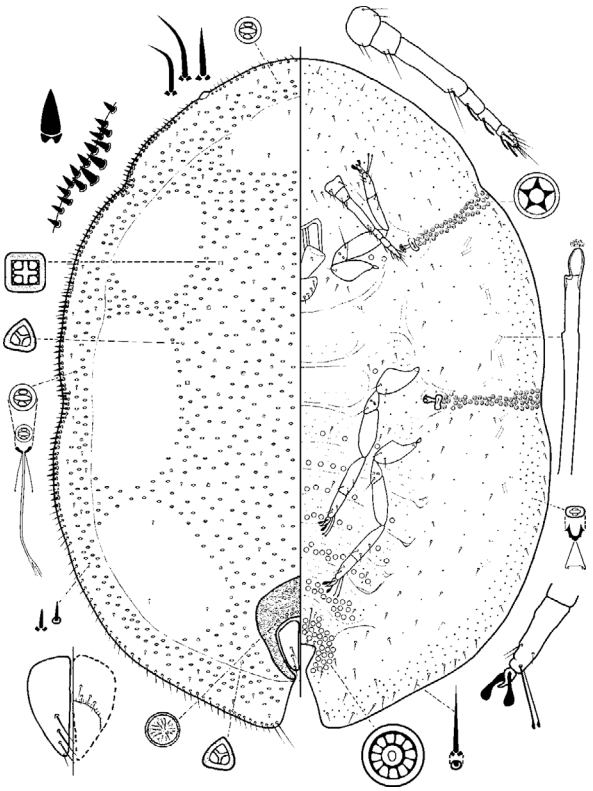

Fig. 1 *Ceroplastes japonicus* (Gray), Kaydan & Kondo 2008, with changes.

Ceroplastes japonicus (Gray) (Figs 1 and 2)

Synonym: *Ceroplastes floridensis japonicus*, Green; *Cerostegia japonica*, De Lotto, *Paracerostegia japonica*, Tang 1991.

Material examined: Greece: 2 ♀♀, Thessaloniki (Lagadas), *Laurus nobilis*, 14 September 2012, Sm. Papadopoulou; Kavala, *Laurus nobilis*, 3 October 2012, Sm. Papadopoulou; 2 ♀♀, Naousa, *Diospyros kaki,* 4 October 2012, Ath. Manganaris, Thessaloniki (Sindos) *Diospyros kaki,* 9 October 2012, Ath. Manganaris, 2 ♀♀, Attica (Kifisia) *Laurus nobilis*, 8 September 2016, C. Chrysochoidis; Attica (Drosia) *Laurus nobilis*, 14 October 2017, C. Chrysochoidis.

Mounted female oval in shape, 6 segmented antennae, legs well developed.

Stigmatic setae lanceolate with mounted female oval in shape, 6 segmented antennae, legs well developed.

Stigmatic setae lanceolate with pointed apex, placed in two rows, each row with 3–4 enlarged setae extending onto dorsum; others in marginal row. The row is not separated along the body margin. Dorsum, membranous in young adult females, with 1 cephalic and 6 lateral clear areas. Dorsal setae short. Dorsal pores scattered, mostly with oval trilocular pores, some quadrilocular pores present in medio dorsal region. Anal plates with 3 or 4 dorsal setae. Preopercular pores on caudal area. Ventral tubular ducts with enlarged inner filament, quinquelocular pores in stigmatic furrows forming an irregular band from stigmatic atrium to stigmatic setae. Multilocular disc pores numerous around vulva and on last six abdominal segments and next to each coxa.

Sequence alignment and comparisons

Amplification and sequencing were successful for all DNA samples. The query length in the BLAST program was 572 bp for each of the five specimens after alignment and trimming of their respective sequences in BioEdit software. All submitted sequences were identified as belonging to the *C. japonicus* species, with an identity percentage of 99–100% and a query coverage of 100%. The second best match among possible species was *C. floridensis*, with a substantially lower identity percentage (92%). These results were verified when submitting the sequences to the BOLD database.

Biological notes*:* The above plants were heavily infested by *C. japonicus* (Hemiptera: Coccidae) in Greece. Infestations of *C. japonicus* were recorded on stems of *L. nobilis* and on stems and foliage of *D. kaki* (Fig. 2).

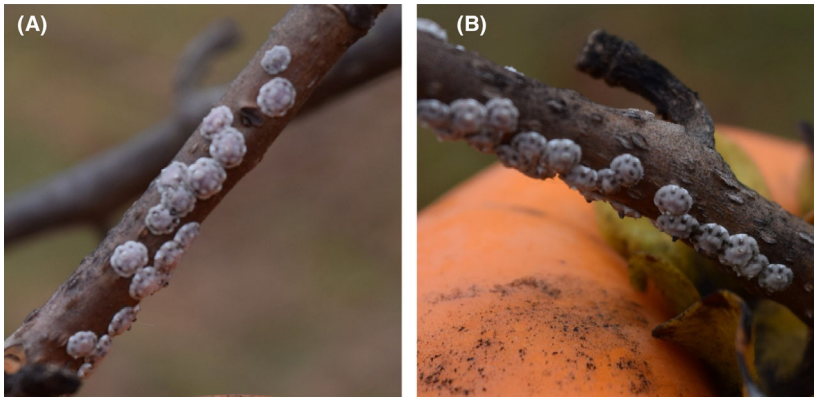

Fig. 2 *Ceroplastes japonicus* on *Laurus nobilis* (A) and *Diospyros kaki* (B) in Greece.

Conclusions

C. japonicus is a new insect record in Greece. It was observed for the first time on stems of *L. nobilis* and on stems and foliage of *D. kaki* (Fig. 2). Both plants were heavily infested by *C. japonicus.* Morphological characteristics and DNA barcoding were used for identification of *C. japonicus*. The information provided in this paper demonstrates that the combination of traditional taxonomy with molecular methods gives accurate and reliable results to enable the identification of this pest.

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Premier signalement de *Ceroplastes japonicus* (Gray) (Hemiptera: Coccidae) en Grèce et approche combinée d'identification morphologique et par barcoding moléculaire

De fortes infestations de cochenille à bouclier cireux mou (Hemiptera : Coccidae) ont été observées sur *Laurus nobilis* L. (Lauraceae) et *Diospyros kaki* Thunb. (Ebanaceae) en Grèce au cours des dernières années. Des spécimens de ces cochenilles ont été collectés dans les régions du Thessalonique, de Náoussa et de Kavala (Grèce du nord) et dans l'Attique (Grèce du sud) entre 2012 et 2017. Les spécimens ont été identifiés comme étant *Ceroplastes japonicus* Green (Hemiptera : Coccidae) par barcoding moléculaire et sur la base des caractéristiques morphologiques des femelles.

Первое сообщение о *Ceroplastes japonicus* (Gray) (Hemiptera: Coccidae) в Греции и комбинированный подход к морфологической идентификации и штрихкодированию ДНК

В последние годы в Греции наблюдался высокий уровень заражения восковой ложнощитовкой (Hemiptera: Coccidae) на *Laurus nobilis* L. (Lauraceae) и *Diospyros kaki* Thunb. (Ebanaceae). В 2012-2017 гг. особи *Ceroplastes* были собраны в районах Салоники, Науса и Кавала (северная Греция) и Аттика (южная Греция). На основе штрихкодирования ДНК и морфологических характеристик самок, особи были идентифицированы как *Ceroplastes japonicus* Green (Hemiptera: Coccidae).

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