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FIRST RECORD OF Tinocallis zelkowae (Takahashi, 1919) (HEMIPTERA APHIDIDAE CALAPHIDINAE) IN THE IBERIAN PENINSULA

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Casiraghi A., Moreno-González V., Umaran A., Pérez Hidalgo N. – First record of Tinocallis zelkowae (Takahashi, 1919) (Hemiptera Aphididae Calaphidinae) in the Iberian Peninsula.

The East-Asian aphid Tinocallis (Tinocallis) zelkowae is recorded for the first time in the Iberian Peninsula on Zelkova serrata (Ulmaceae) in the Viveros Gardens of the city of Valencia (Spain). This is the second record of the species in Europe, after being cited on a Japanese zelkova bonsai in England in 1974. Alate viviparous females and sexuales (winged males and apterous oviparae) are here described, and we present the molecular sequence of the mitochondrial cytochrome oxidase I, which allows comparison with the recently described T. (T.) latifoliae on in Zelkova serrata var. latifolia. It is the fifth species of Tinocallis genus that is known in territories of Iberian Peninsula, three of which are also native to Southeast Asia.

KEY WORDS: aphids, biological invasion, Zelkova serrata, Spain

INTRODUCTION

The genus Tinocallis Matsumura 1919 (Hemiptera: Aphididae: Calaphidinae) groups in four subgenera, around 20 species associated mainly with plants from Ulmaceae family, although species have also been described from Lythraceae (Lagerstroemia, Duabanga), and other genera (Corylus, Dalbergia and Sapindus). Furthermore, woody Fabaceae seem to be recorded regularly as alternative or ‘casual’ hosts (Lee and Lee, 2017; Blackman and Eastop, 2021; Favel, 2021). The genus is of Asian origin but six of its species are considered exotic or invasive and have been regularly cited in Europe (Prior, 1971; Stroyan, 1979; Coeur d’acier et al., 2010; Kunturksi et al., 2018), American (Halbert and Pike, 1990; Quednau, 2001) and Australian (Zeck, 1933; Hales, 2020) territories.

All Tinocallis viviparous females are alate and usually present paired spinal and marginal tubercular processes; some of them also have conspicuous black pigmentation on dorsal body and/or forewings (Quednau, 2003; Kunturksi et al., 2018). However, the seasonal variation of winged forms in spring, as opposed to summer and autumn (more pigmented and with less sensilla) makes the definition of some species complex (Blackman and Eastop, 2021). The taxonomy of Tinocallis has been revised by Quednau (2003) establishing four subgenera besides the nominal (Eotinocallis Quednau, 2003, Orientinocallis Quednau, 2003 and Sappocallis Matsumura, 1919). Recently, a molecular study of sequences of cytochrome oxidase I (COI) of Calaphidinae revealed the presence of cryptic species (Lee and Lee, 2017).

In Europe, six species are recorded: Tinocallis nevskyi Remaudière, Quednau & Heie, 1988, T. platani (Kaltenbach, 1843), T. saltans (Nevsky, 1929), T. takachihoensis Higuchi, 1972, T. ulmiparvifoliae Matsumura, 1919, and T. zelkowae (Takahashi, 1919). At the end of the 1980s, only two of them (T. platani and T. saltans) were present in the Iberian Peninsula, but Nieto Nafria and Mier Durante (1998) indicated the probability of detecting the rest in a short period. Therefore, Mier Durante and Pérez Hidalgo (2002) cited T. takachihoensis in Ulmus pumila L. in Andorra and Pérez Hidalgo and Nieto Nafria (2005) found colonies of T. ulmiparvifoliae on Ulmus pumila in the city of León but acquired in a nursery in Barcelona. The latter was posteriorly recorded in an abandoned nursery in Valencia (Pérez Hidalgo and Molina, 2010), but since then it has not been cited, while T. takachihoensis has had a great expansion in European and Ibero-Balearic territories (Bella, 2013; Pérez Hidalgo et al., 2013; Kunturksi et al., 2018).

Ten species of Tinocallis are recorded living on different species of Zelkova (Blackman and Eastop, 2021): T. nevskyi, T. saltans, T. takachihoensis, T. ulmiparvifoliae, T. zelkowae, T. mushensis (Takahashi, 1925), T. sophorae Zhang, 1980, T. szukhowsiens Zhang, 1980, T. viridis (Takahashi, 1929), T. zelkowae Dzhibladze, 1957 (Holman, 2009; Blackman and Eastop, 2021).

The study of a colony of Tinocallis recorded on ornamental trees of Zelkova serrata in a public garden in the city of Valencia (southeast of Spain) allows us to record the Asiatic aphid T. zelkowae for the first time in the Iberian Peninsula.
MATERIALS AND METHODS

Aphid samples were collected for the first time in the Viveros Gardens in the city of Valencia from early September to middle of December of 2017, when the fall of the leaves ended and subsequently, the colonies were monitored during the years 2018 and 2019. In these two years and with a monthly periodicity, hundreds of winged females and dozens of sexuals (male and oviparous) were collected and studied in three trees of Zelkova serrata (coord. 39.478584, -0.368260) by the last of the authors (NPH) and finally, for this work, 20 specimens of each morph were measured. *Tinocallis takachihoensis* samples (used in the construction of the phylogenetic tree) were collected from Ulmus minor Mill. in Alcalá de la Selva (Santuario de la Virgen de la Vega), Teruel (18th July 2017). Samples were preserved in 70% and 100% ethanol for morphological and molecular studies, respectively. The aphids (sample references: 944 and 840) and the DNA material and sequences were deposited in the aphidological collection of the Institute for Integrative Systems Biology (Mixt Centre of University of Valencia and CSIC).

The microscopic preparations and measurements were taken following the methodology exposed by Nieto Nafría and Mier Durante (1998) using a Leica microscope and the illustrations using a clear camera adapted to it. Microphotographs were taken with a Leica DC digital camera with IM1000 version 1.10 software. Living specimen photos have been taken by Ángel Umaran with a Canon EOS 600D.

Abbreviations used for description, diagnosis and Table I are: Ant.I, Ant.II, Ant.III, Ant.IV, Ant.V and Ant. VIb, antennal segments I, II, III, IV, V and base of VI, respectively; PT, processus terminalis of Ant.VI; URS, ultimate rostral segment; HFM, hind femur; HTB, hind tibiae; 2HT, second segment of hind tarsi; SIPh, siphunculus; BDAnt.III, basal diameter of Ant.III.

Total DNA was extracted separately from three individuals of a sample of *T. zelkowae* that had been preserved in 100% ethanol since the collection date, following the HotSHOT (Hot Sodium Hidroxide and Tris) method (Truett et al., 2000) using 60 µl of both alkaline lysis and neutralizing reagents. A 710 bp fragment of the 5’ region of the mitochondrial gene coding the cytochrome c oxidase subunit 1 (COI) was amplified using primers LCO1490 and HCO2198 described by Folmer et al. (1994). PCR reaction and sequencing procedures are outlined in Pérez Hidalgo et al. (2012). As expected, a 710 bp DNA fragment containing a portion of the mitochondrial COI gene was amplified through PCR from the aphids analyzed. After removing sequences corresponding to primers used in the PCR reaction, the sequences obtained from each sample consisted of 658 nucleotides. Chromatograms were revised, primers trimmed and sequences corresponding to each individual aphid assembled using the Staden package v1.6.0 (Staden et al., 1998).

For the phylogenetic analysis, COI sequences identified as *Tinocallis* and *Sarucallis kahawaluokalani* were downloaded from NCBI nucleotide database (Nucleotide, 2020). Multiple sequence alignment was conducted with muscle v. 3.8.31 (Edgar, 2004). Phylogeny was then inferred using a neighbor joining algorithm (Saito and Nei, 1987) with the F8I model (Felsenstein, 1981) using the packages adegenet v. 2.1.3 (Jombart, 2008), ape v. 5.3 (Paradis and Schliep, 2018) and phangorn v. 2.5.5 (Schliep, 2011) under R v. 3.6.3 (R Core Team, 2020). *Sarucallis* sequences were used as outgroup and node support was computed with 1000 bootstrap replicates. The tree was represented with ggtree package v. 1.16.6 (Yu et al., 2018). The new identified sequences were deposited in GenBank (accession numbers: *T. zelkowae* MT511611.1 and *T. takachihoensis* MT511608.1).

RESULTS

According to Lee & Lee (2017) our winged viviparous females are identified as *T. zelkowae*, because morphological characteristics as the antennae are 0.65 to 0.75 times the body (while those of *T. (T.) latifoliae* described in Lee & Lee (2017) are 0.86 to 1.13) (Table I). Furthermore, the sequence of the cytochrome oxidase I gene from our samples groups (Fig. I) associated with the sequences of *Tinocallis* collected on *Z. serrata var. latifolia* of Lee et al. (2017, see Fig. 9), which is *T. zelkowae*.

**Description of Tinocallis (T.) zelkowae**

*Alate* viviparous females are small (1.075–1.75 mm), and alive are shining pale yellow or greenish yellow, with conspicuous pale eyes, banded antennae, a fine brown mid-dorsal line on head and pronotum, pale yellow legs and siphunculi and a pair of small brown dorsal spots on each of abdominal tergites 3-7 (Fig. II, 1 and 2). The antennae (Fig. III, 1) are shorter than the body with 14–18 secondary sensory sensilla on antennal segment III (Fig. IV, 1), and the terminal process is shorter (0.54–0.72) than the base of last antennal segment. The apical rostral segment is 0.070-0.077 mm and 1.55-1.81 times its basal width and 0.90-1.00 times the second joint of posterior tarsi and carries 2–5 complementary setae. The nymphs are pale yellow and only show pigmentation in the banding of the antennae and tarsi (Fig. II, 2). For other measurements and ratios, see Table I.

*Oviparae* (Fig. II, 3) measure 1.25-1.40 mm and in life are yellowish, with some areas of the antennae (Fig. II, 3; Fig. V, 3), a series of dorsal spots and part of the legs being black or smoked. Dorsal setae are claviform and very long. The antennae (Fig. IV, 3) are 0.54-0.56 times the body and the terminal process 0.54-0.78 times the base. The posterior tibiae are clearly swollen (the diameter of the tibia in the middle is 0.037-0.047 mm and have 18 to 34 pseudosensoria (Fig. V, 4). For other measurements and ratios, see Table I.

*Males* (Fig. II, 4; Fig. V, 1) are alatae and smaller (1-25-1.32 mm) than viviparous females. The antennae,
Fig. I - Neighbor joining phylogeny of *Tinocallis* sequences. New sequences are marked in red. Only nodes whose bootstrap value is greater than 0.5 are labelled.
head (with red eyes) and thorax are blackish, and abdominal pigmentation is more marked than on females (Fig. II, 4; Fig. V, 2). The antennae (Fig. IV, 2) have secondary sensoria in all flagellum segments (26-30, 8-11, 5-10 and 2-4 in the segments III to VI, respectively); the terminal process is shorter (0.055-0.065mm) than in viviparous female and 0.44-0.54 times the base of the antennal segment VI. For other measurements and ratios, see Table I.

**Biology and distribution**

*Tinocallis zelkowae* is a monoecious holocyclic species with sexual morphs (winged males and oviparae) present in October-November in Japan (INOUYE, 1968). It lives on undersides of leaves of *Z. serrata* and related species (*Z. acuminata*, *Z. formosana*, *Z. schneideriana*), although it has been collected frequently on Betulaceae (*Alnus japonica*, *Carpinus* sp.), Corylaceae (*Corylus sieboldiana*) and Fabaceae (*Robinia*, *Glycine max* and ‘cultivated beans’ (HOLMAN, 2009; BLACKMAN and EASTOP, 2021). According to BLACKMAN and EASTOP (2021) records on *Ulmus japonica* may be misidentifications of other species of *Tinocallis* or cryptic species (LEE and LEE, 2017). The colonies of the Iberian Peninsula are abundant on the three *Zelkova serrata* sampling and have sexed from the first of October until the fall of the leaves, although it should be noted that adult males appear before (about 15 days) than the adult oviparous females.

It is a species of Eastern-Asiatic origin. It is known from Japan, Korea and China (TAKAHASHI, 1919; HOLMAN, 2009; BLACKMAN and EASTOP, 2021); in addition, it has been recorded from California (USA) and England (STROYAN, 1979; KONO, 1983), so this is the first record, which seems stable year through year.
DISCUSSION

LEE & LEE (2017) have described a new species of *Tinocallis* living on *Z. serrata* var. *latifolia* according to the molecular differences in the COI sequences and to slight differences in body size (1.19-1.47 mm in *T. latifoliae*; 1.63-2.47 mm in *T. zelkowae*), antennae (0.86-1.13 times the body in *T. latifoliae*; 0.53-0.79 in *T. zelkowae*) and with spinal tubercles more pigmented than those of *T. zelkowae*. However, the dorsal tubercles of winged viviparous females captured in Valencia are always strongly pigmented (Fig. III, 1) at all times of the year in which the observations took place. For this reason, we consider this character must be used with due precautions in the identification keys.

On the other hand, the recent description of *T. latifoliae* based on molecular sequences and morphological characters (Lee and Lee, 2017) means that identifications and descriptions of *T. zelkowae* previously made on morphological characters can be doubtful, since they could be based on specimens of *T. latifoliae* or from mixed colonies or even hybrid individuals. Currently, it can be stated that the species described by TAKAHASHI (1919) and by HIGUCHI (1972) correspond to *T. zelkowae*. A separate question is to determine if under the name of *T. zelkowae* in his *Atlas of the Drepanosiphinae of the World* QUEDNAU (2003) has included different forms of various species of *Tinocallis* living in *Zelkova* species from different countries, so a review with current taxonomic criteria would be necessary. Because of this, we consider that it is necessary to know (and describe) the sexual morphs of *T. latifoliae* in order to establish the differences between both species and that the descriptions must be accompanied by molecular studies, since it is possible that “there are other species of *Tinocallis* still to be described from *Zelkova*” (BLACKMAN and EASTOP, 2021).

*Tinocallis zelkowae* has the ability to live in the southeast of the Iberian Peninsula on *Z. serrata* even if it is a deciduous plant because it develops sexuales. Furthermore, winters are mild in the abovementioned area. This implies that *T. zelkowae* may be living in other areas of Europe where its plant is present. It is interesting to highlight that the population in Valencia changed in density throughout the years, from apparently stable and very abundant colonies in 2017 to only few individuals recorded in 2019. This opens questions about the real identity of the species presence in Spain: *T. zelkowae* is considered non-native, but up to date it cannot be considered invasive, because it is not cause of concern. It will be interesting to follow the colony development in future years to confirm or deny its stability. Valencia presents hot summers, with maximal temperature peaks of more than 30°C. In other works, it is clear that another species of the same genus, *T. kahawaluokalani*, decrease in density with hot urban temperatures (PARSONS et al. 2019). The apparent slow disappearance of the colony could be linked to the same phenomenon.

It should also be noted that their presence has not been detected in *Zelkova carpinifolia*, which is located at very close distance (1.6 km) from the populations of *T. zelkowae* studied. There are no records on *Z. carpiniolata* of *T. zelkowae* but there are of other species of *Tinocallis* (*T. nevskyi*, *T. saltans*, *T. zelkowae*) (BLACKMAN and EASTOP, 2021). We do not know if the populations of *T. zelkowae* of England have been able to survive, but considering the above, it would not be possible to be present in England or in any other area of Europe living on some *Zelkova* used as an ornamental plant in parks and gardens.

Another species of the genus, *T. nevskyi*, is widely distributed in Europe (Germany, Austria, Belgium, Czech Republic, Denmark, Finland, France, Holland, Hungary, Italy, Poland, United Kingdom, Switzerland and Sweden) on different species of *Ulmus* and its presence in the Iberian Peninsula should not be ruled out.
**Table I - Measurements of the alate viviparous females (al), oviparae (ov) and males (m) of *Tinocallis zelkowae***

| Body parts                  | *T. (T.) latifoliae* Lee & Lee, 2017 | *T. (T.) zelkowae* (Matsumura) |
|-----------------------------|-------------------------------------|---------------------------------|
|                             | al. (n = 14)                        | al. (n = 20)                     | ov. (n = 20)                     |
| Length (mm)                 |                                     |                                 |
| Body length                 | 1.35 (1.19–1.47)                    | 1.29 (1.25–1.32)                | 1.34 (1.30–1.40)                |
| Whole antennae              | 1.32 (1.15–1.55)                    | 1.02 (1.00–1.06)                | 0.73 (0.73–0.75)                |
| Ant.I                      | 0.06 (0.05–0.07)                    | 0.052 (0.05–0.06)               | 0.061 (0.06–0.065)              |
| Ant.II                     | 0.05 (0.04–0.06)                    | 0.05 (0.05–0.05)                | 0.056 (0.055–0.06)              |
| Ant.III                    | 0.46 (0.35–0.58)                    | 0.33 (0.28–0.395)               | 0.38 (0.37–0.38)                |
| Ant.IV                     | 0.24 (0.20–0.30)                    | 0.18 (0.145–0.215)              | 0.18 (0.16–0.19)                |
| Ant.V                      | 0.22 (0.19–0.28)                    | 0.17 (0.15–0.195)               | 0.17 (0.17–0.18)                |
| Ant.Vib                    | 0.15 (0.12–0.19)                    | 0.11 (0.11–0.13)                | 0.12 (0.12–0.13)                |
| PT                         | 0.09 (0.07–0.11)                    | 0.074 (0.06–0.089)              | 0.061 (0.055–0.065)             |
| URS                        | 0.08 (0.07–0.08)                    | 0.072 (0.07–0.077)              | 0.071 (0.067–0.075)             |
| HFM                        | 0.37 (0.32–0.47)                    | 0.32 (0.28–0.38)                | 0.32 (0.32–0.33)                |
| HTB                        | 0.60 (0.47–0.77)                    | 0.50 (0.46–0.57)                | 0.53 (0.52–0.55)                |
| 2HT                        | 0.09 (0.07–0.10)                    | 0.075 (0.072–0.082)             | 0.075 (0.072–0.077)             |
| SIPH                       | 0.03 (0.02–0.03)                    | 0.026 (0.017–0.03)              | 0.03                            |
| Cauda                      | 0.10 (0.09–0.10)                    | 0.06 (0.06–0.075)               | 0.065                           |
| Longest setae on Ant.III   | 0.01                               | 0.0075                          | 0.01                            |
| No. of setae on URS (accessory setae) | 5 (4–5) | 4 (2–5) | 2–3 |
| 8th abdominal tergite      | 2                                  | 2 (2–2)                         | 2                               |
| Cauda knob                 | 8 (7–10)                           | 8 (8–9)                         | 8–9                             |
| Genital plate              | 12–13                              | -                               | 21–39                           |
| No. of rhinaria on Ant.III | 23 (16–26)                         | 16.41 (14–18)                   | 26–30                           |
| Ant. IV                    | 0                                  | 0                               | 9.12 (8–11)                     |
| Ant. V                     | 0                                  | 0                               | 7.87 (5–10)                     |
| Ant. VI                    | 0                                  | 0                               | 3 (2–4)                         |
| Pseudosensoria on HTB       | -                                  | -                               | 27.5 (18–34)                    |
| Ratio (times) Antennae/body length | 0.95 (0.86–1.13) | 0.69 (0.65–0.75) | 0.76 (0.75–0.77) |
| PT/Ant.Vib                 | 0.65 (0.55–0.83)                    | 0.62 (0.54–0.72)                | 0.48 (0.44–0.54)                |
| PT/Ant.III                 | 0.21 (0.16–0.24)                    | 0.22 (0.18–0.25)                | 0.27 (0.23–0.31)                |
| URS/2HT                    | 0.89 (0.78–1.00)                    | 0.95 (0.90–1.00)                | 0.95 (0.90–1.00)                |
| URS/Ant.Vib                | 0.53 (0.37–0.67)                    | 0.61 (0.58–0.63)                | 0.72 (0.66–0.78)                |
| URS(length)/URS(basal width) | 1.60 (1.55–1.81) | 1.48 (1.33–1.58) | 1.55 (1.33–1.66) |
| SIPH/body length           | 0.02 (0.01–0.03)                    | 0.016                           | 0.02                            |
| SIPH/Ant.III               | 0.06 (0.04–0.09)                    | 0.079 (0.062–0.092)             | 0.14 (0.12–0.15)                |
| SIPH/Cauda                 | 0.07 (0.06–0.09)                    | 0.08 (0.062–0.096)              | 0.12 (0.11–0.14)                |
| Setae on Ant.III/BDAnt.III | 0.28 (0.20–0.33)                    | 0.39 (0.29–0.48)                | 0.42 (0.34–0.50)                |

**Fig. V - *Tinocallis zelkowae*:** male, general view (1) and abdomen (2); ovipara, general view (3) and detail of the hind tibia (4).
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