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Genetic identification of new alien pest species *Illinoia liriodendri* and its parasitoid *Areopraon silvestre* in Croatia

MILIVOJ FRANJEVIĆ[1]
MILAN GLAVAŠ[1]
BORIS HRAŠOVEC[1]
NIKOLA KOLETIĆ[2]
DAMJAN FRANJEVIĆ[3]

[1] Faculty of Forestry, University of Zagreb, Svetošimunska 25, 10 002 Zagreb, Croatia

[2] Institute for Research and Development of Sustainable Ecosystems, Jagodno 100a, 10410 Velika Gorica, Croatia

[3] Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10 000 Zagreb, Croatia

Correspondence:
Damjan Franjević
email: damianf@zg.biol.pmf.hr

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Abstract:

Background and Purpose: *During June 2015 in Zagreb city area (Croatia) samples of tulip tree (*Liriodendron tulipifera*) leaves were collected with symptoms of attack by some unknown aphid.*

Material and methods: *Aphids were collected form leaves of tulip trees on different locations in Zagreb during July 2015. Total genomic DNA was extracted from ethanol-preserved specimens. PCR analysis was carried out and PCR products were purified from 1% agarose gel for sequencing purposes. The obtained sequences were deposited in GenBank.*

Results: *The collected aphids were genetically determined as* Illinoia liriodendri *(Hemiptera, Aphididae), the North American invasive pest species. Although present in several neighboring countries on tulip trees in urban environment, this research presents the first record of* Illinoia liriodendri *for Croatia, confirmed on the genetic level. Interestingly enough, during genetic determination of tulip tree aphid pest, another DNA, one of parasitoid* Areopraon silvestre *(Hymenoptera, Braconidae) was also found in the collected samples.* A. silvestere *is a native European specialized solitary endoparasitoid of aphids.*

Conclusions: *The presence of alien pest species* Illinoia liriodendri *was successfully determined via genetic identification. Also, genetic identification of parasitoid species on* Illinoia liriodendri, Areopraon silvestre, *shows the quick establishment of natural regulation of new pest species in Croatia.*

INTRODUCTION

Liriodendron tulipifera L. (Magnoliaceae) is a North American species used in urban forestry in Zagreb as an ornamental and shade tree. Zagreb city area has 576 tulip trees, 380 of them being young trees under 10 cm of diameter and several trees being around 50 cm in diameter.

The genus Illinoia (Wilson 1910) includes about 45 North American species and one species from the Caucasus (*1,2*). Many species in the two subgenera, Illinoia and Masonaphis, are associated with Ericaceae in North America, but others feed on taxonomically diverse host plants (*3*). *Illinoia liriodendri* (Monell 1879) lives solely on the tulip tree, *Liriodendron tulipifera*, and is a monoecious holocyclic species.

I. liriodendri is a pest native to North America and it is found on *L. tulipifera* and *Magnolia grandiflora* L. (*4*). Today it is also present

throughout the European continent from France, 1998 (*5*); Italy, 2001 (*6*); UK, Germany and Slovenia, 2004 (*7*); to Luxembourg and Greece 2011 (*8*). *I. liriodendri* has also been present in Asia, since 1999 in Japan (*9*) and since 2008 in South Korea (*10*). In Croatia *I.liriodendri* was found for the first time in 2014 in Poreč (Istria) (*11*).

I. liriodendri overwinter as tiny eggs laid in the autumn, primarily in bark crevices near buds of *L. tulipifera*. In spring the eggs hatch when the leaves begin flushing. Aphids have predominantly parthenogenic reproduction, i.e. females are viviparous. Short generation time can lead to rapid build-ups in their populations during spring and summer. Alate males and egg-laying females occur in the late autumn usually during October (*12*). Symptoms of damages caused by aphids include mildly distorted buds, leaf discoloration, sometimes premature defoliation and heavy honeydew accompanied by sooty moulds, beginning about mid-June (*13*).

The aphids (Insecta: Hemiptera: Aphididae) and related families Adelgidae and Phylloxeridae are a group of approximately 5000 species of small, soft-bodied insects that feed on plant phloem using piercing/sucking mouthparts. Aphids have complex life cycles involving many morphologically distinct forms, and parthenogenetic generations alternating with a sexual generation. In about 10% of species, this is associated with host alternation (*14*).

Areopraon silvestre (Stary, 1971) (Hymenoptera, Braconidae) is a member of Aphidiidae family, which are known to be endoparasitoids on Aphids. *A. silvestre* inhabits parks, glades on chalky slopes and riversides as well as mixed forests and deciduous forests. (*15,16*). The distribution of *A. silvestre* has so far been found in Europe in Ukraine, France, Switzerland, Germany, Norway, Finland, Serbia, Bulgaria, Czech Republic and Slovakia (*15,16,17,18*). Natural hosts of *A. silvestre* are numerous species of *Periphyllus* genus and as such it represents good natural agent for controlling aphid attacks (*19*).

The aim of this research is to determine unequivocally, for the first time on the genetic level, the new alien pest species that occurred during early summer of 2015 on tulip trees in the urban area of Zagreb, Croatia.

MATERIALS AND METHODS

In order to determine this new pest species for Croatian urban forestry, samples from all locations mentioned below were collected, after which genetic identification was conducted.

Sampling

Aphids were collected from leaves of tulip trees on the following locations: Lanište 45°46'15.41"N, 15°56'16.49"E; Zrinjevac 45°48'38.10"N, 15°58'41.50"E; Babonićeva Street 45°49'4.09"N, 15°59'32.06"E; Josip Brunšmit Field 45°47'31.72"N, 15°57'0.12"E in Zagreb during July 2015. The collected samples were used for genetic identification of the unknown aphid pest species. The material was fixed and stored in 95% ethanol until the DNA extraction. DNA samples are stored at -80 °C at the University of Zagreb, Faculty of Science, Divison of Biology.

DNA barcoding

For the purpose of genetic identification of new alien pest species on *L. tulipifera* the DNA barcoding technique was used. DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify if it belongs to a particular species (*20*). DNA barcoding provides an efficient method for species-level identifications and contributes powerfully to taxonomic and biodiversity research (*21*). Although several loci have been suggested, the so called Folmer region (*22*) of the mitochondrial cytochrome c oxidase subunit I (COI) gene was proposed as a universal DNA barcode region for animals. In this research we used the COI gene fragment primers LCO 1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO 2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') in order to amplify Folmer region of COI gene and utilize it as DNA barcode for our samples.

DNA extraction, PCR, and sequencing

The total genomic DNA was extracted from ethanol-preserved specimens using the DNeasy Blood & Tissue kit (Qiagen, Germany), following the manufacturer protocol. PCR analysis was carried out in 50 µL reaction volumes containing 25 µL TopTaq Master Mix kit (Qiagen, Germany), 1 µL of DNA, 1 µL of 10 µmol/L of each primer, and 22 µL of distilled RNA-free water. PCR was performed using the following cycling settings: 5 min predenaturation at 95°C followed by 1 min at 94°C, 1 min at 54 °C and 1 min at 72°C, for 35 cycles, followed by 72°C final elongation for 10 min. All PCR products were tested for the presence of amplified products on 1 % agarose gel. Two distinctive bands that appeared on agarose gel pointed out two different species whose COI barcode regions were amplified during PCR. Both PCR products were cut out from gel and purified using QIAquick Gel Extraction Kit (Qiagen, Germany). For sequencing purposes, we used services of Macrogen Europe (Amsterdam, The Netherlands). The sequence chromatogram was viewed and edited manually by using Chromas Lite 2.0 (Technelysium Pty., South Brisbane, Australia). Forward and reverse sequences were checked for base ambiguity in BioEdit 7.2 (*23*). The obtained sequences were deposited in GenBank.

RESULTS

During June 2015, signs of aphid feeding on tulip trees were reported on several locations in private gardens and

Figure 1. *Honeydew on tulip tree leaves in Zagreb City area and subsequently drips due to aphid attack.*

Figure 3. *Apterous viviparous female of Illinoia liriodendri collected in July 2015 in Zagreb, Croatia.*

Figure 2. *Sooty mould on leaves of tulip tree from Zagreb city area.*

Figure 4. *Apterous females of I. liriodendri feeding on the underside of tulip tree leaves collected in Zagreb, Croatia.*

city park areas in Zagreb (Lanište, Zrinjevac, Babonićeva Street, and Josip Brunšmit Field). The attacked tulip trees showed heavy presence of honeydew (Figure 1.) accompanied by sooty moulds (Figure 2.).

Tulip trees were covered with colonies of aphids (Figure 3.) feeding on the underside of leaves (Figure 4.) and producing honeydew, which made them become a nuisance due to honeydew dripping on pavements and parked cars.

As DNA barcode marker for identifying unknown aphid pest from tulip trees in Croatia Folmer COI region was used. Effectiveness in the discrimination of over 300 species of aphids from more than 130 genera that was conducted by Foottit *et al*. in 2008 (*24*) showed that 96% of aphid species can be well differentiated with this barcoding marker. Sequence variation within aphid species was low, on average just 0.2%. The same authors concluded that despite the complex life cycles and parthenogenetic reproduction of aphids, DNA barcodes are an effective tool for identification. Genetic identification of morphologically determined *I. liriodendri* species was confirmed using highly similar sequences local alignment analysis. Results retrieved using the Megablast service and sequences obtained from this research showed 100% sequence similarity to sequences of *I. liriodendri* from Canada (British Columbia), accession number EU 701702, and 99% sequence similarity to sequences from USA (North Carolina), Canada (Ontario) and South Korea, deposited in GenBank under accession numbers EU 701704, KR 037400 and GU 978950 respectively. What was very interesting is the fact that we simultaneously amplified genomic DNA of *Aeropraon silvestre* (Stary, 1971) (Hymenoptera, Braconidae) from the same samples. Megablast analysis with GenBank nucleotide database using our sequence of *A. silvestre* as a query retrieved the results showing 100% similarity with the sample *of A. silvestre* from the United Kingdom accession number JX507446. Thus based on genetic identification we can conclude that samples of *I. liriodendri* from Croatia are

already infected with this aphid parasitoid. Our COI barcode sequences for *I. liriodendri* and *A. silvestre* are deposited in GenBank under accession numbers KT753299 for *Areopraon silvestre* and KT753300 for *Illinoia liriodendri.*

DISCUSSION

I. liriodendri is a pest well known from EPPO region (*7*). Transpacific and transatlantic introductions of this species are likely to have occurred almost simultaneously in Japan and France in the late 1990s, whereas the Korean introduction has occurred more than 10 years later (*10*). Alien species are species whose spread outside their natural distribution threatens biological diversity (*25*). Alien species introduction is usually vectored by human transportation and trade (*26*). The requirement for inhabiting new area is that a new habitat is similar enough to its native range so that alien species may survive and reproduce (*27*). For alien species to become invasive, it must successfully out-compete native organisms, spread through new environment, increase its population density and harm ecosystems in its introduced range (*28*). A good predictor of invasiveness is whether a species has successfully or unsuccessfully invaded elsewhere (*29*). One of the major problems regarding alien species is that ecosystems that have been invaded by alien species may not have their natural enemies present in its native range that would normally control their populations (*30*).

The primary concern about the presence of *I. liriodendri* in urban environment is the loss of ornamental value and nuisance to citizens through deposition of honeydew and littering of infested leaves, as well as the attraction of bees, wasps and hornets to honeydew. Black sooty mould associated with the honeydew is mostly an aesthetic problem, but if very severe it may limit photosynthesis and cause leaves to drop (*31*). In urban areas, the attraction of bees, wasps and hornets to the aphid honeydew can also cause potential health hazards for humans due to stings and consequent allergic reactions (*6,12*). To reduce this problem, some form of treatment is necessary (*31*). Three natural enemies of *I. liriodendri* were found in the eastern United States: *Praon silvestre*, *Ephedrus incompletus* and *Aphidius liriodendri* (Hymenoptera: Braconidae: Aphidiinae) (*32).* Aphidiinae is a family of ichneumonoid wasps, which are specialized solitary endoparasitoids of aphids (*33, 34*). Today near 700 species belonging to more than 60 genera have been identified worldwide (*17*). Aphidiids regulate aphids' population growth in natural ecosystems and in agricultural landscapes. Some Aphidiid species are successful agents of biological pest control (*35*). *A. silvestre* represents a specialized parasitoid of some *Periphyllus* (van der Hoeven 1863) aphids. *Periphyllus* aphids are associated with *Acer* trees abundant in Croatia and in Central and Southeast Europe (*19, 35*). In this respect, there is a difference between *Areopraon* and *Praon* too, as the latter is also capable of parasitizing the phylogenetically derived aphid groups (*36*). Because of the limited use of insecticides in urban areas, the occurrence of parasitoid *A. silvestre* is valuable in biological control of this invasive species. The results of this research confirm that *I. liriodendri* as new pest species in Croatia is present. In two years the species has been recorded in Istria (11) and now in Zagreb. The widening of the area where *I. liriodendri* is present in Croatia is most likely due to infection via plants from nurseries. Our research that was primarily based on confident genetic identification of new alien species for Croatia requires further sampling and analysis of the severity of infection with this pest in Croatia. In contrast to the West Coast of the United States of America, where it was necessary to release natural enemies in order to prevent spreading of *I. liriodendri* (37), our research indicates that in Croatia some regulating factors of I. liriodendry are present since the samples from Zagreb area contain *A. silvestre* DNA. This indicates that many if not all *I. liriodendri* aphids are infected with this endoparasitoid.

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