

Rapid Communication**The “code red” for Balkan vineyards: occurrence of *Orientus ishidae* (Matsumura, 1902) (Hemiptera: Cicadellidae) in Serbia**

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Abstract

Orientus ishidae (Matsumura, 1902) (Hemiptera: Cicadellidae), known as the mosaic leafhopper, is an invasive alien species native to the Eastern Palaearctic, but also widespread and well established on the eastern territory of North America. Since its first detection in Europe in 1998, this polyphagous species has expanded its distribution area and rapidly spread through central and western European countries, inhabiting a wide range of broadleaf trees and shrubs (e.g. *Gleditsia triacanthos*, *Salix* spp., *Corylus* spp., *Acer* spp., *Betula* spp., *Populus* spp. and *Carpinus* spp.), which are usually present in the surrounding of vineyards. Over the last decade, *O. ishidae* was frequently found within European vineyards and was proven to be able to complete its life cycle on grapevine. Moreover, this leafhopper was shown to be naturally infected with the *Flavescence dorée* phytoplasma (FDp), a devastating disease which causes significant economic losses in the major vine-producing countries. This study provides data on the occurrence of *O. ishidae* in Serbia, clearly confirming that it has expanded its distribution range to the Balkan Peninsula which could lead to establishment of new FDp epidemiological cycles inside the local vineyard regions. As a consequence, there could be an increased negative impact on phytosanitary situation with a continual spread of this vector species and FDp epidemic outbreaks in Southeastern Europe.

Key words: the mosaic leafhopper, invasive alien species, *Flavescence dorée* phytoplasma, insect vector

Introduction

The mosaic leafhopper *Orientus ishidae* (Matsumura) (Hemiptera, Cicadellidae) is an invasive alien species which originates from the Eastern Palaearctic with a wide range of distribution, reported from Japan, Korea, Taiwan and the Philippines (EPPO 2015). Outside of its native range, *O. ishidae* was recorded for the first time on the territory of the USA in the early nineteenth century, where it was introduced probably via import of ornamental plants of *Aralia spinosa* (Sanders and DeLong 1919). It is a polyphagous species, inhabiting a wide range of broadleaf trees such as

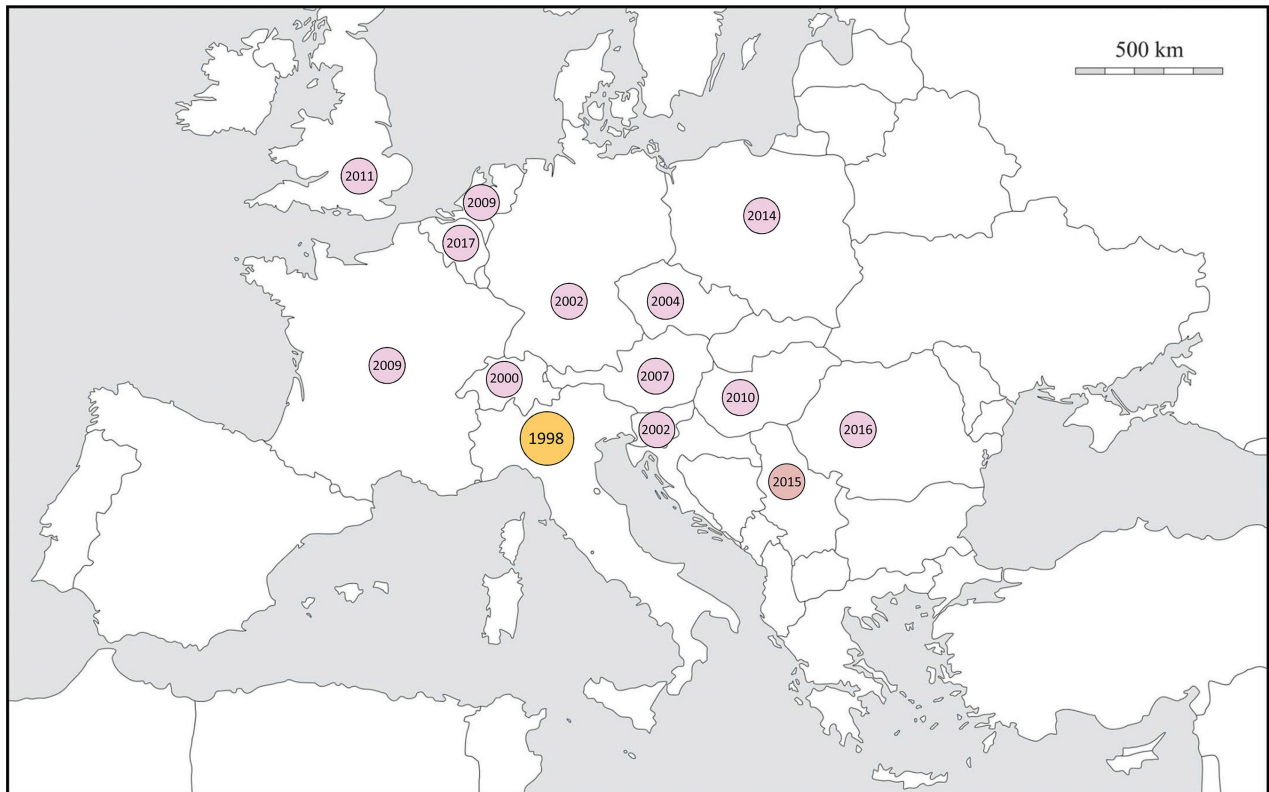


Figure 1. Current distribution of *Orientus ishidae* (Matsumura, 1902) in Europe. First records are indicated with year denoted in the circles. Map from d-maps.com (https://d-maps.com/carte.php?num_car=2232&lang=en).

Gleditsia triacanthos, *Salix* spp., *Corylus* spp., *Acer* spp., *Betula* spp., *Populus* spp. and *Carpinus* spp. (Valley and Wheeler 1985; Nickel 2010).

The first record from Europe dates back to 1998 from northern Italy (Guglielmino 2005). Since its first detection, *O. ishidae* has expanded its range and rapidly spread through central and western European countries: Switzerland (Günthart and Mühlethaler 2002), Germany (Nickel and Remane 2003), Slovenia (Seljak 2004), Austria (Holzinger 2009), Czech Republic (Malenovský and Lauterer 2010), France (Mifsud et al. 2010), Hungary (Koczor et al. 2013), the Netherlands (den Bieman and van Klink 2015), Great Britain (EPPO 2015), Poland (Klejdzysz et al. 2017), Romania (Chireceanu et al. 2017) and Belgium (Lock 2019) (Figure 1).

Due to its association with wild plants in vineyard surroundings in the areas severely affected by devastating diseases of grapevine yellows phytoplasmas, the mosaic leafhopper has gained special attention over the last decade and has become a research focus in viticultural regions across Europe (Mehle et al. 2010; Lessio et al. 2016, 2019; Casati et al. 2017). Placing focus on *O. ishidae* is justifiable, considering that alien invasive hemipteran species play an important role as phytoplasma vectors in new environments (D'Urso et al. 2019) and can even cause epidemic outbreaks (reviewed in Constable 2010). The best example of this is the epiphytotic outbreak of the *Flavescence dorée* (FD) in European vineyards (reviewed in Jeger et al. 2016).

The *Flavescence dorée* phytoplasma (FDp, taxonomic subgroups 16SrV-C and 16SrV-D) is a phloem-limited plant pathogenic bacteria, which causes the most serious phytoplasma quarantine disease of grapevine in European Union (EU) and EPPO region, with severe economic consequences in the major vine-producing countries (Jeger et al. 2016). The principal insect vector of FDp in European vineyards is a leafhopper of North American origin, *Scaphoideus titanus* (Hemiptera: Cicadellidae), which feeds primarily on grapevine, spreading the disease from plant to plant in an epidemic manner (reviewed in Constable 2010).

However, the finding of several wild plant taxa carrying FDp-related genotypes (*Alnus glutinosa*, *A. incana*, *Clematis vitalba*, *Ailanthus altissima*, *Salix* spp. and *Corylus avellana*) indicate an open transmission cycle of the disease (Angelini et al. 2004; Arnaud et al. 2007; Filippin et al. 2009, 2011; Casati et al. 2017; Malembic-Maher et al. 2020). In addition, several studies have revealed that other polyphagous planthoppers and/or leafhoppers are involved in the dispersal of FDp from wild reservoir plants into vineyards. The native planthopper, *Dictyophara europaea*, is able to transmit FDp from infectious *C. vitalba* to the grapevine (Filippin et al. 2009; Krstić et al. 2018), while native leafhoppers *Oncopsis alni*, *Allygus modestus* and *A. mixtus* could transmit FDp sourced from *Alnus* spp. (Maixner et al. 2000; Malembic-Maher et al. 2020).

Recently, alien *O. ishidae* has been identified as an additional serious risk for spreading epidemic genotypes of FDp in Slovenia, Italy, Switzerland and France (Mehle et al. 2010; Gaffuri et al. 2011; Trivellone et al. 2015; Malembic-Maher et al. 2020). Over the last decade, *O. ishidae* has been frequently found in European vineyards and their vicinity and was proven to complete its life cycle on the grapevine (Lessio et al. 2019). Moreover, it was shown to be naturally infected with FDp (Mehle et al. 2010) and to be able to transmit this phytoplasma to the grapevine after experimentally forced acquisition (Lessio et al. 2016) and among naturally infected European alders (Malembic-Maher et al. 2020).

In Serbian vineyards, the FDp epidemic outbreaks and occurrence of *S. titanus* were recorded for the first time in 2003 (Duduk et al. 2004; Magud and Toševski 2004). The role of *D. europaea* as a native vector and *C. vitalba* as an FDp reservoir plant were subsequently evidenced (Filippin et al. 2009), along with *Alnus* spp. as natural carriers of FDp-related genotypes (Cvrković et al. 2008). Despite all efforts to control the pathogen, FDp nowadays still spreads in Serbian vineyards and affects all grape-growing regions. For this reason and because of the documented importance of *O. ishidae* as an alternative vector in transmission routes of FDp, the occurrence of this leafhopper has been intensively monitored over the past decade across Serbia. This study clearly shows that *O. ishidae* has expanded its range to the Balkan Peninsula, indicating a potential negative impact on the phytosanitary situation as a consequence of further spread of this leafhopper and of FDp outbreaks in southeastern Europe.

Materials and methods

During a study of Auchenorrhyncha fauna in the wine-growing regions of Serbia, including Central Serbia (Belgrade, Topola), South-East Serbia (Niš, Pirot) and the Province of Vojvodina in North Serbia (Subotica, Fruška Gora, Vršac), extensive monitoring was undertaken in diverse natural ecosystems, in deciduous trees along river banks, in forest margins and ornamental trees in urban areas where trees and shrubs reported as preferred hosts for *O. ishidae* were present. The surveys were made every 15 days from July to September between 2013 and 2020. The insects were collected for 15 minutes at each location, with standard entomological sweep nets and mouth aspirators. Number of adults and nymphs were recorded on sites with noted *O. ishidae* occurrence.

Material identification

All collected specimens which, according to morphological characters, corresponded to *O. ishidae* were placed in 96% ethanol. Morphological characters of the adults and nymphs were examined under a Leica MS5 stereomicroscope. The genitalia of males were examined and compared with the descriptions provided by Biedermann and Niedringhaus (2004) and Guglielmino (2005). Nymphs were identified according to Valley and Wheeler (1985).

DNA extraction, PCR amplification and sequencing

Sixteen specimens, previously identified as *O. ishidae* based on morphology, were used for a DNA study. Total DNA from individual insects was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. The mitochondrial cytochrome *c* oxidase subunit I gene (mtCOI) was amplified and sequenced for its generally good resolution allowing a differentiation at species level (Hebert et al. 2003). PCR amplification for the barcode region (658 bp) of the mtCOI was performed using the LCO1490 and HCOd primers (Folmer et al. 1994; Chetverikov et al. 2015).

The polymerase chain reactions (PCR) contained High Yield Reaction Buffer A with MgCl₂ (1x), additional 2.25 mM MgCl₂, 0.6 mM of each dNTP, 0.5 μM of each primer and 1U of FastGene *Taq* DNA polymerase (NIPPON Genetics Europe) in a 20 μL final volume. PCR cycles were performed in a Mastercycler ep gradient S (Eppendorf) applying the following thermal steps: 95 °C for 5 min (initial denaturation), 35 cycles at 94 °C for 1 min, 54 °C for 1 min (annealing), 72 °C for 2 min and a final extension at 72 °C for 10 min.

PCR amplicons were purified using the QIAquick PCR purification Kit (QIAGEN) according to the manufacturer's instructions, and sequenced on an automated equipment by MacroGen Europe (Amsterdam, the Netherlands).

Table 1. Collection sites with records of *Orientus ishidae* (Matsumura, 1902) in Serbia.

Location	Date	GPS	Dominant plant species	No. of specimens (males/females/nymphs)
Topola	17.06.2015	44.286967N 20.693517E	<i>Salix purpurea</i>	1 (0/1/0)
New Belgrade 1	23.07.2020	44.791317N	<i>Vitis riparia</i> , <i>Populus alba</i> , <i>Malus</i> spp., <i>Prunus</i> spp.	20 (8/10/2)
	1.09.2020	20.376317E		10 (3/7/0)
New Belgrade 2	30.07.2020	44.777367N	<i>Vitis riparia</i> , <i>Populus alba</i> , <i>Salix alba</i> , <i>Amorpha fruticosa</i>	8 (4/3/1)
	1.09.2020	20.356383E		4 (2/2/0)
New Belgrade 3	30.07.2020	44.769517N	<i>Vitis riparia</i> , <i>Populus alba</i> , <i>Salix alba</i> , <i>Amorpha fruticosa</i>	15 (6/6/3)
	1.09.2020	20.348350E		5 (2/3/0)
New Belgrade 4	30.07.2020	44.741733N	<i>Vitis riparia</i> , <i>Populus alba</i> , <i>Salix alba</i> , <i>Amorpha fruticosa</i>	12 (8/3/1)
	1.09.2020	20.312983E		8 (3/5/0)


Figure 2. A typical site with an established population of *Orientus ishidae* in Serbia, New Belgrade 4, GPS: 44.741733N; 20.312983E with draperies of *Vitis riparia* on *Populus alba*, *Salix alba* and *Amorpha fruticosa*. Photograph by Ivo Toševski.

A comparison with sequences from the GenBank was carried out using the BLAST analysis (Basic Local Alignment Search Tool; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). In addition, sequences of the barcode portion of the mtCOI gene were compared with BOLD database (Barcode of Life Data Systems; <https://www.boldsystems.org/>), as well.

Results

A single specimen of *O. ishidae* was registered in 2015 on *Salix purpurea* in Topola, Central Serbia (leg. Valeria Trivellone). However, a further extensive survey revealed no new records of the species in this region. In 2020, between 23–30 July and on 1 September, a total of 82 individuals (36 males, 39 females and 7 nymphs) of *O. ishidae* were collected in four localities in a woodland area near the Sava River between the municipalities of New Belgrade and Ostružnica (Table 1). At all four collecting sites, *Vitis riparia* (Vitaceae) was a predominant food plant, forming characteristic draperies on silverleaf poplar (*Populus alba*) and white willow (*Salix alba*) (Figure 2).



Figure 3. *Orientus ishidae* (Matsumura, 1902) on *Vitis riparia*, Serbia, New Belgrade 1, 23.07.2020, GPS: 44.791317N; 20.376317E, 75 m, leg. Tatjana Cvrković.

Adults were also collected on wild apple (*Malus sylvestris*) and cherry plum (*Prunus cerasifera*) trees (Rosaceae), as well as on indigo bush (*Amorpha fruticosa*) (Fabaceae). At the collecting sites along the Sava River, the North American leafhopper *S. titanus* was also recorded on *V. riparia*.

The collected adult specimens were distinguished by their basic brownish grey color, with a characteristic mosaic-like pattern of white-, yellow- and black across the forewings, head, pronotum and scutellum, an orange pattern on the head between the eyes, dark legs with tarsus and tibia orange, except for tibia of the hind pair legs (Figure 3) and the structure of the male genitalia. In addition, the barcoding sequence of mtCOI of Serbian specimens (Acc. No. MT998287) subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) revealed a 100% identity with a 585 bp sequence of *O. ishidae* originating from Canada (Acc. No. KR584471).

Discussion

The unintentional introduction of alien species by anthropogenic activities is a growing worldwide phenomenon due to globalization, expanding markets and increasing amount of transported plant and food consignments (D'Urso et al. 2019). Phytophagous insects represent one of the most numerous groups of invertebrates introduced into Europe from other continents, mainly from Asia, North America and Africa and cause significant economic losses by feeding on plants or by transmitting diseases (Roques et al. 2009). To date, thirty non-native Auchenorrhyncha species have been accidentally introduced into Europe. Most of them belong to the family Cicadellidae which is the most numerous in species. Half of the introduced alien species was reported in the last 20 years, while twelve are well established in the continent (D'Urso et al. 2019).

The invasive mosaic leafhopper *O. ishidae* overwinters at the egg stage; eggs are inserted into the bark of woody plants and in this way the species was probably introduced into Europe through imported live plant material (Malenovský and Lauterer 2010; Mifsud et al. 2010).

The rapid range expansion through western and central European countries over the last 20 years indicates that *O. ishidae* is very adaptable and once established, it can rapidly spread. The main reason is probably its polyphagous feeding behavior, which includes woody plants (e.g. willow, hazelnut, hornbeam, walnut, beech, ash, etc.), as well as herbaceous plants (e.g. *Urtica dioica* and *Helianthus annuus*) which are widely spread in urban, suburban and ruderal habitats and agroecosystems.

After its first introduction into Europe, *O. ishidae* was recorded also in vineyards and their vicinity and the females were capable of laying eggs on grapevine canes and branches, especially in vineyards and wild rootstocks surrounded by hazelnut and hornbeam plants which are one of their preferred hosts (Lessio et al. 2019).

In 2015, during the survey on the diversity of potential grapevine yellows phytoplasma vectors in Serbia, a single specimen of *O. ishidae* was registered in Topola. Further extensive survey performed on the same locality over the following years revealed no additional specimens of the mosaic leafhopper in vineyards and their surroundings, suggesting that its population wasn't established in this region, or the population size was still very low and undetectable. This indicates that, if established, populations of *O. ishidae* are still scarce in the region and the species has not colonised Serbian vineyards yet. Based on the experience from other European countries (Lessio et al. 2016, 2019; Casati et al. 2017; Chireceanu et al. 2019), high population densities in localities where this leafhopper is present indicates that its naturalization and rapid range expansion is possible in the next few years.

The occurrence of *O. ishidae* in Serbia confirmed in 2020, as well as its association with wild *V. riparia* alongside with *S. titanus* as the main FDp vector, pose a real threat to the wine production in grape growing regions of Serbia and the Balkans. It is interesting to note that during our survey a number of collected specimens of *O. ishidae* exceeded the number of captured *S. titanus*, at all collecting sites (data not shown). The common occurrence of *O. ishidae* and *S. titanus* on wild or abandoned *Vitis* plants are of peculiar concern because of documented importance of these plants as an FDp source in new outbreaks at local scales (Rossi et al. 2019).

Besides *S. titanus*, other polyphagous Auchenorrhyncha species have been identified as FDp vectors, able to acquire and transmit FDp-related genotypes from reservoir plants in the areas surrounding vineyards. Based on genotyping of the epidemiologically informative house-keeping *map* gene, the FDp is comprised of variants within three genetic clusters namely Map-FD1 (16SrV-C, including strain FD70), FD2 (16SrV-D including

reference strain FD-D) and FD3 (16SrV-C, reference strain FD-C) (Arnaud et al. 2007). The climbing shrub *C. vitalba* has been proven as a natural reservoir of the primary FD3 genotype of FDp in Italy, Slovenia, Switzerland, Austria, Hungary and in the Balkans (Angelini et al. 2004; Filippin et al. 2009; Casati et al. 2017; Krstić et al. 2018; Reizenzein and Strauss 2019). Additionally, in France, Italy, Slovenia and the Balkans, alder trees (*Alnus glutinosa* and *A. incana*) have been confirmed as reservoir plants in which FD2 related genotypes are dominantly present, but in some plants FD1 and FD3 related genotypes were revealed (Arnaud et al. 2007; Cvrković et al. 2008; Mehle et al. 2011; Radonjić et al. 2013; Atanasova et al. 2014), while a vigorous invasive plant species, *A. altissima* was found infected with the FD3 genotype in Italy and Croatia and FD2 in Slovenia (Filippin et al. 2011; Plavec et al. 2019; Mehle et al. 2019). All these genotypes are transmitted by different vectors. Specifically, the native planthopper *D. europaea* transmits FD3 from *C. vitalba* to the grapevine, while the native leafhoppers *O. alni*, *A. modestus* and *A. mixtus* transmit FD1 and FD2 genotypes sourced by European alders (Malembic-Maher et al. 2020). *Orientus ishidae* has been identified as an additional potential risk for spreading the FD1 and FD2 genotypes in Slovenia, Italy, Switzerland and France (Mehle et al. 2010; Casati et al. 2017; Malembic-Maher et al. 2020). Recently, in the study from southern Switzerland *O. ishidae* was found infected with FD1 and FD3 related genotypes also found in asymptomatic willows (*Salix* spp.) and in asymptomatic uncultivated hazelnut shrubs sampled in a forest close to a FD infected vineyard, suggesting they could be additional hosts and reservoirs of FDp (Casati et al. 2017).

Moreover, recent studies have shown that symptoms of decline on cultivated hazelnut trees are associated with FD1 and FD2 related genotypes causing severe damage in hazelnut plantations in Slovenia (Mehle et al. 2019), while it is known that *O. ishidae* prefers wild hazelnut plants (Lessio et al. 2016, 2019; Casati et al. 2017) and widespread populations of this species infected with FDp were recorded (Mehle et al. 2010).

Epidemiological context of phylogenetic relatedness of the mosaic leafhopper to native FDp phytoplasma vectors, i.e., its potential to transmit the FDp epidemic strains (Malembic-Maher et al. 2020), as well as its feeding on diverse phytoplasma plant reservoirs in a new environment could potentially influence already complex FDp cycle and route(s) of transmission (e.g., Jeger et al. 2016; Casati et al. 2017; Malembic-Maher et al. 2020). Thus, the presence of this exotic leafhopper species in the local pathosystems and its potential spread across viticultural regions of the Balkan Peninsula could lead to new epidemiological cycles inside vineyards and cause new outbreaks, which would further complicate already complex epidemiological situation with FDp in vineyards in Serbia.

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