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The mosaic leafhopper *Orientus ishidae*: host plants, spatial distribution, infectivity, and transmission of 16SrV phytoplasmas to vines

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Abstract

Orientus ishidae (Matsumura) is an Asian species introduced into Europe and recently associated with 16SrV phytoplasmas, related to grapevine "flavescence dorée". Its life cycle, host plants, spatial distribution, infection and vector capability have been investigated in vine-growing areas of Piedmont, NW Italy. The spatial distribution of adults in vineyards was studied by applying interpolation methods to trap capture data. Insects were subject to molecular analyses to verify phytoplasma presence and identity. DNA extraction and PCR were made to detect 16SrV phytoplasmas. Transmission experiments were set up, using different sources for phytoplasma acquisition, and two plant species and an artificial diet for inoculation. Whole mount *in situ* hybridization was made to detect phytoplasmas in the salivary glands of adults. In the vineyard agro-ecosystem, 19 plant species (11 families), mainly broadleaf trees and shrubs, were recognized as host plants of the insect. Adults were more abundant on putative host plants than on grapevines, with a clear clustering at the edges of vineyards, and without a massive intrusion into the vineyard from outside. 16SrV phytoplasmas were detected only in adults captured with yellow sticky traps (20 out of 188 tested). The transmission of 16SrV phytoplasmas was successful after phytoplasma acquisition from infected broad bean and inoculation on grapevine: this is the first evidence of *O. ishidae* as a vector for this prokaryote.

Key words: mosaic leafhopper, host range, spatial interpolation, transmission, fluorescence *in situ* hybridization.

Introduction

Plant diseases associated to the presence of phytoplasmas are one of the major threats to agriculture worldwide. Within this frame, insect vectors play a key epidemiological role (Weintraub and Beanland, 2006; Wilson and Weintraub, 2007; Alma *et al*., 2015). Grapevine's "flavescence dorée" (FD) is associated with the presence of phytoplasmas belonging to the 16SrV ribosomal group, in the subgroups 16SrV-C and 16SrV-D (Lee *et al*., 1998; 2000; Bertaccini and Duduk, 2009). In Europe, 16SrV phytoplasmas infecting grapevine have been grouped in three different phylogenetic clusters: FD1, FD2 and FD3 (Arnaud *et al*., 2007). Cluster FD1 includes the strain FD70 isolated in France (Caudwell *et al*., 1970); cluster FD2 includes two identical reference strains FD92 and FD-D (Angelini *et al*., 2003); and cluster FD3 includes the reference strain FD-C (Angelini *et al*., 2003). FD is one of the main threats to viticulture, as no prophylaxis can be applied to vines after plantation. To date, the main FD insect vector is *Scaphoideus titanus* Ball (Hemiptera Cicadellidae Deltocephalinae), a Nearctic species introduced into Europe and monophagous on grapevines (Chuche and Thiery, 2014). For limiting the disease's spread, insecticidal sprays against *S. titanus* are mandatory in many European countries. However, other insects may play a role in transmitting FD. It is known that *Dictyophara europaea* (L.) (Hemiptera Dictyopharidae), a Palearctic species, transmits phytoplasmas from *Clematis vitalba* L. to grapevines under experimental conditions (Filippin *et al*., 2009), and that *Oncopsis alni* (Schrank) (Cicadellidae Macropsinae) can transmit 16SrV-C '*Candidatus* Phytoplasma ulmi' (Arnaud *et al*., 2007) from alder to alder causing alder yellow (AldY) (Maixner and Reinert, 1999), and from alder to grapevine, causing palatinate grapevine yellow (PGY) (Maixner *et al*., 2000). The "search for vector" strategy is focused often either on alien species, or on species that are particularly abundant in vineyards. Recently, another alien species has been associated with 16SrV phytoplasmas in Europe: the mosaic leafhopper *Orientus ishidae* (Matsumura) (Cicadellidae Deltocephalinae). After the first report in Slovenia, where many specimens were found to be positive to 16SrV-C and -D phytoplasmas (Mehle *et al*., 2010; 2011); there was a second report from Italy, where only 16SrV-D phytoplasmas were detected (Gaffuri *et al*., 2011); and a third is from Switzerland, where *O. ishidae* resulted positive to 16SrV-C phytoplasmas (Trivellone *et al*., 2015).

O. ishidae, formerly known as *Phlepsius tinctorius* (Sanders and DeLong) is considered an Asian species, and was introduced first into the USA probably along with *Aralia* sp. ornamental plants (Felt and Bromley, 1941). Its first introduction into Europe is supposed to have happened in 1998 in Switzerland and Northern Italy (Guglielmino, 2005). It was found also in Piedmont, Northern Italy (Alma *et al*., 2009), Veneto, Northern Italy (Forte *et al*., 2013), Tuscany, Central Italy (Mazzoni, 2005), Slovenia (Seljak, 2004), Germany (Nickel, 2010), France (Misfud *et al*., 2010), Czech Republic (Malenovsky and Lauterer, 2010) and recently also in The Netherlands (den Bieman and Klink, 2015), Great Britain, Austria (EPPO, 2015) and Hungary (Koczor *et al*., 2013). Another species, known as *Orientus amurensis* Guglielmino, probably confined to the Amur region of Russia and in China, in the Liaoning Province, was

also described, whereas the Nearctic and European populations should be referred to *O. ishidae* (Guglielmino, 2005).

This leafhopper is thought to be monovoltine, and to overwinter in the egg stage (probably laid close to buds) (Valley and Wheeler Jr, 1985; Nickel, 2010). It is polyphagous, feeding mainly on broadleaf trees such as *Gleditsia triacanthos* L. (Valley and Wheeler Jr, 1985), *Quercus palustris* L. (Johnson and Freytag, 2001), *Prunus virginiana* L. (Rosenberger and Jones, 1978), *Betula* spp. (Nickel, 2010; Koczor *et al*., 2013), *Viburnum* spp. (Koczor *et al*., 2013), *Salix* spp. (Guglielmino, 2005; Mazzoni, 2005; Nickel, 2010), *Corylus* spp., *Acer* spp., and *Carpinus* spp. (Nickel, 2010). Both nymphs and adults cause yellowing and stunting on leaflets of their host plants (Felt and Bromley, 1941; Valley and Wheeler Jr, 1985). Its vectoring ability was documented by Rosenberger and Jones (1978) who proved the transmission to celery test plants of peach-X disease, subsequently classified as '*Ca.* P. pruni' (Davis *et al*., 2013). The recent detection of its harbouring 16SrV phytoplasmas suggests its possible role in the transmission of phytoplasmas to grapevine. Moreover, little is known about the ecology and dispersal abilities of the mosaic leafhopper.

This research was aimed to investigate on the seasonal presence, the putative host plants for nymphs and adults, the spatial distribution, and the presence in nymphs and adults of *O. ishidae* of 16SrV phytoplasmas in the vineyard agroecosystem, as well as to verify its ability as a vector.

Materials and methods

Study area and field collections

Field surveys were made during 2011-2012 in Piedmont in several grapevine growing areas, in the Provinces of Asti and Cuneo. According to the Plant Protection Service of Piedmont Region, all the districts included in the present research are defined as "FD settlement areas", where the eradication is considered impossible to achieve. The incidence of FD in the single vineyards sampled is given in table 1 (data were collected on a symptomatic basis, and random molecular analyses confirmed the presence of 16SrV phytoplasmas).

Nymphs and adults of *O. ishidae* were sampled on putative host plants, which included broadleaf trees (e.g. willow, hazelnut, walnut, beech, ash, etc.), weeds and grasses. Samplings were made from the middle of May to the end of September. As this leafhopper causes evident leaf yellowing followed by necrosis by feeding (Felt and Bromley, 1941; Valley and Wheeler Jr, 1985), nymphs were quite easy to detect and they were collected directly on the leaves with a mouth aspirator. On the other hand, adults were captured with a sweep net. Both nymphs and adults were placed in glass tubes and brought alive to the laboratory, where they were killed by freezing, and preserved inside glass tubes at −20 °C before analyses. Genitalia were extracted from 10 males randomly chosen from different locations for species identification according to Guglielmino (2005).

Yellow sticky traps (cm 20 × 30) were used in vine-growing areas to monitor flight activity and spatial distribution of adults. Traps were placed both inside the vineyards directly on the grapevine's foliage, and outside on putative host plants, in 10 vineyards (table 1). In 7 sites, 3 traps were placed inside the vineyard and 3 outside on putative host plants for *O. ishidae* (trees and shrubs). Moreover, in 3 vineyards (plots 1, 3, and 5), the number of traps was increased to 20-24 in order to study the spatial distribution of the mosaic leafhopper and to detect its hot spots. In this case, they were placed at a distance of 20-25 m from each other on the vineyard rows, and 9-12 m between rows (figure 1). All traps were changed every two weeks, from the beginning of July to the end of October, wrapped into a plastic film, labelled, and stored inside a freezer (−20 °C). Subsequently, they were observed in the lab under a stereomicroscope to detect and count *O. ishidae* adults. A subsample of adult leafhoppers was removed from the traps using a drop of β-limonene (BioClear®), placed into Eppendorf tubes, and stored at −20 °C. All vineyards were subject to insecticidal sprays for controlling *S. titanus*: one spray with thiamethoxam at the end of June, and one with organophosphates at the end of July.

In order to test the possibility that *O. ishidae* is capable of laying eggs on grapevines, pruned grapevine wood was collected during winter from each of the vineyards. Canes were placed in a cool chamber (5 °C),

Table 1. Main features of experimental plots. * FD percentage was calculated on a symptom basis; molecular analyses made at random confirmed the presence of 16SrV phytoplasmas.

Plot	District	GPS coordinates (N, E)	Size (m^2)	Variety	FD %*	Uprooting of plants
1	Cocconato	45.08296; 8.05631	21000	Barbera	2.8	No
2	Portacomaro	44.97029; 8.25216	2800	Freisa	17.5	No
3	Vezza d'Alba	44.77528; 7.98204	8900	Arneis, Nebbiolo	5.0	Yes
4	Canale	44.78675; 8.00498	7200	Pinot Nero	15.0	Yes
5	Mongardino	44.84185; 8.21657	9600	Barbera	36.0	Yes
6	Dogliani	44.55236; 7.93580	2700	Dolcetto	3.0	No
7	Scurzolengo	44.97011; 8.25209	8000	Barbera	20.4	No
8	Canale	44.81713; 7.98625	5500	Barbera	20.4	Yes
9	Neviglie	44.69509; 8.13237	17400	Arneis, Chardonnay, Moscato	3.0	No
10	Coazzolo	44.72009; 8.15535	7400	Moscato	1.5	No

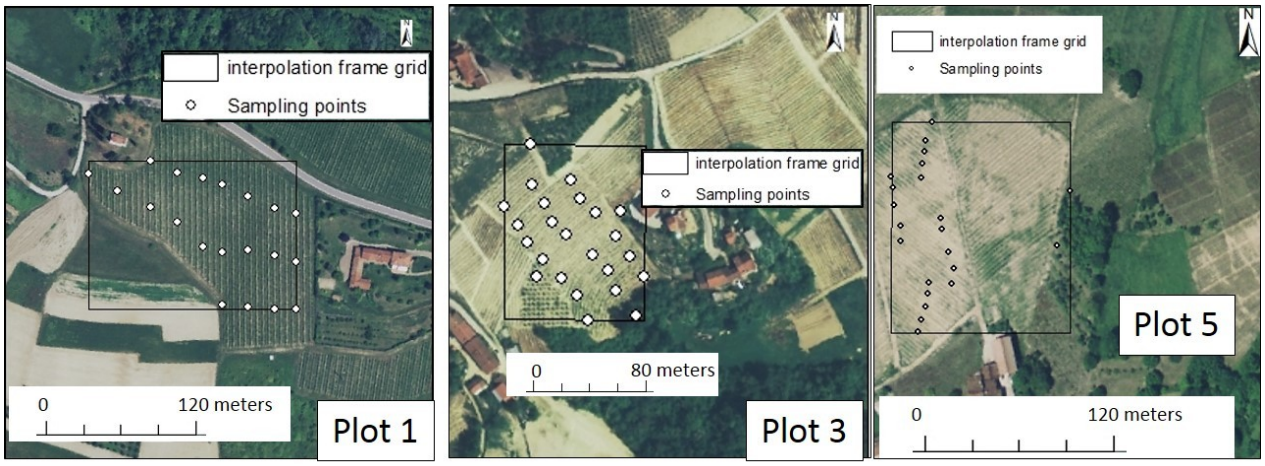

Figure 1. Maps of the experimental plots used for spatial interpolation analyses.

and during spring they were put inside rearing cages containing potted grapevine and broad bean plants to observe the emergence of nymphs following the method described in Lessio *et al.* (2009).

Data analyses

Data of captures on traps placed inside and outside the vineyards in plots 2, 4, 6, 7, 8, 9, and 10 were subject to one-way ANOVA to test for differences, both in 2011 and 2012. Each trap was considered as a single replication, without taking into account the "plot" factor. Data were square root transformed for normalization and homogeneity of variances. Analyses were run with SPSS 20.0® statistical software.

Spatial interpolations were performed with ArcGIS desktop 10.1 (www.esri.com) software package, using Ordinary Kriging (OK) and Inverse Distance Weighted (IDW), for each of the plots where a high number of traps was placed (1, 3, and 5) (figure 1). OK is a stochastic method based on spatial correlation and variograms, and minimizes the variance of the expected error (Liebhold *et al*., 1993), whereas IDW is a deterministic method based on the Euclidean distances between sampling points (Bartier and Keller, 1996). Concerning OK, experimental variograms were produced using a lag value of 12 m, with a number of lags N = 10, assuming isotropy, and data were fitted to theoretical variogram models (exponential, Gaussian, and spherical) (Liebhold *et al*., 1993) by means of the nonlinear regression function available in SPSS 20.0®. No trend removal was applied. In order to take into account the different crops or spontaneous vegetation on the edges of the vineyards, the neighbourhood search was performed as a standard type with a 4-sector with 45° offset: by doing this, the sampling points compared with each other were supposed to be influenced by the same external sources of *O. ishidae.* The minimum and maximum number of neighbours was chosen equal to 2 and 5, respectively (ArcGIS default value). IDW was performed choosing an exponent equal to 2, 3 and 4; the neighbourhood search was also standard type with a 4-sector with 45° offset, but the minimum and maximum number of neighbours was 10 and 15, respectively (ArcGIS default value).

Cross-validation, where single observations are removed in turn from the data set, was performed to test the significance of experimental spatial interpolation maps for both OK and IDW methods, calculating the mean prediction error:

$$ME = \sum_{i=1}^{n} \frac{(\bar{x}_i - x_i)}{n}$$

and the root mean square error:

$$RMSE = \sqrt{\sum_{i=1}^{n} \frac{(\bar{x}_i - x_i)^2}{n}}$$

where $\bar{x}_i$ is the predicted value, x_i the observed value, and n the sample size. Both ME and RMSE are given in the same units of measure of the data: an ideal model should have a ME equal to 0, and a RMSE as small as possible. While RMSE gives an estimate of the error as a whole, ME mainly provides an estimate of the bias: that is, positive and negative ME values indicate that the model over or underestimates the data, respectively (Rhodes *et al*., 2011).

Interpolation maps were realized using the Geostatistical Analyst extension available in the ArcToolbox of ArcGIS desktop 10.1.

Transmission experiments

Transmission experiments were set up using different plant sources for acquisition of FD -phytoplasmas, and two different plant species and an artificial diet as recipients for inoculation. Sources for phytoplasma acquisition included broad bean (*Vicia faba* L.), and grapevine; moreover, some adults collected in the field were used directly for inoculation. Broad beans were infected with FD-C phytoplasma (Miliordos *et al*., 2016) using the model species *Euscelidius variegatus* (Kirschbaum) (Hemiptera Cicadellidae Deltocephalinae) (Salar *et al*., 2013), whereas acquisition on infected grapevines was made directly in the field on symptomatic plants, after having confirmed the infection by molecular diagnosis (see specific section).

Field-collected nymphs (I-III instar) were kept for a 3-5 days long acquisition access period (AAP) on different phytoplasma sources. When using broad bean, nymphs were placed in batches of 30-40 in an insect-proof cage made of nylon mesh and polyethylene film (40 × 40 × 30 cm) with 2 potted infected plants inside, and kept outside under a shading canopy. For grapevine, nymphs were isolated in batches of 50-70 individuals on a branch with symptomatic leaves by means of a sleeve of insect-proof nylon mesh (mesh size 0.5 mm, length 25 cm) and left for a 72 h AAP; then the sleeve was collected by cutting the branch, and brought to the laboratory. Acquisitions were performed on two FD-C infected grapevines, the first one of cv Barbera (23-27 June) and the second one of cv Dolcetto, (30 June-3 July). Afterwards, insects which performed an AAP from different sources were transferred onto potted hazelnut trees (latency access period, LAP) using the same type of cage described for AAP on broad beans, and kept outside under a shading canopy. Hazelnut plants were obtained from seedlings and previously maintained under an insect-proof screen in order to be phytoplasma-free. LAP lasted from a minimum of 21 days until insects reached the adult stage (maximum 28 days).

For inoculation, we used adult leafhoppers either originated from nymphs that performed AAP and LAP in the lab, or collected directly in the field. Adults were placed onto the following: seedlings of broad bean, grapevines of cv Barbera obtained by *in vitro* culture (Gribaudo *et al*., 2007), or artificial sugar diet (Pajoro *et al*., 2008). In all cases, the inoculation access period (IAP) lasted 5 days. Concerning the inoculation trials on plants, single potted broad beans (2-3 fully developed leaves) and vines (6-10 fully developed leaves) were isolated under cylindrical Plexiglas cages (height cm 30, diameter cm 12) with a nylon mesh on the top, and adults of *O. ishidae* were put in batches of 3-5 specimens per plant. The cages were kept outside under a shading canopy. On the whole, we used 33 and 18 batches for inoculation on single plants of broad bean and grapevine, respectively. The details on the number of single insects and batches used to inoculate different plants after having performed AAP on different sources are given in the results (table 7). The inoculation with the artificial feeding system was made instead placing singular adults on artificial diets, according to Pajoro *et al*. (2008), in a climatic chamber (25 °C, RH 70%). In this experiment, 43 field-collected adults, and 6 adults originated from nymphs which performed AAP on infected broad beans were used. After the conclusion of the experiments both insects and diets were stored at −20 °C for further molecular analyses.

Plants and vials were checked daily, and dead specimens were periodically collected. At the end of IAP (or previously if dead), adult *O. ishidae* were placed into Eppendorf tubes and preserved at −20 °C before analyses. Inoculated plants were treated with an insecticide (Dichlorvos, 0.5 g/L) to avoid infestation by aphids, mealybugs, mites, etc., and transferred into an insect-proof chamber (25 °C, RH 70%) up to 45 (broad beans) and 60 (grapevines) days after IAP. Hazelnut plants used for LAP were kept outside, closed into insect-proof cages.

Molecular analyses

The presence of FD phytoplasmas was recorded in *O. ishidae* nymphs and adults, both collected in the field and used for transmission experiments, in order to evaluate FD infection rates. Moreover, inoculated plants and sugar diets too were tested for the presence of the FD phytoplasma, as well as field-infected grapevines used for AAP and potted hazelnuts provided to leafhoppers for LAP. Broad beans and grapevines were tested 21 and 60 days after the beginning of IAP, respectively. In addition, hazelnuts were tested twice during the following year, in June and September. Total DNA was extracted from insects, following a procedure previously described for leafhoppers (Marzachì *et al*., 1998), from single individuals. Plant DNA was extracted according to DNeasy Plant Mini Kit protocol (Qiagen, Italy) instructions. DNA from artificial diets was extracted as previously reported by Gonella *et al*. (2012). FD phytoplasma presence was diagnosed on all samples by performing a direct PCR using the phytoplasma universal primers P1/P7, followed by a nested PCR with 16r758f/M23Sr primers (Angelini *et al*., 2001). Moreover, phytoplasma detection was confirmed by means of a PCR targeting non-ribosomal DNA. All samples were subjected to amplification with the FD9f2/r primers, followed by a nested PCR with the FD9f2/r3 primer pair, according to Angelini *et al*. (2001). Amplicons from both nested PCRs were digested with the endonuclease *Taq*I (Promega, Italy) for the identification of the FD strain based on the RFLP profile (Angelini *et al*., 2001).

Whole mount *in situ* hybridization

Whole mount fluorescent *in situ* hybridization (FISH) analyses were performed on salivary glands of *O. ishidae* at the end of transmission experiment number 3 (AAP and IAP on broad bean, table 7). All experiments were carried out using specific (group 16SrV phytoplasmas) and universal (Mollicutes) fluorescent probes. The newly-designed, 16SrV phytoplasma-specific, probe ph1298 5'-TCAATCCGTACTGAGACTAC-3', labelled with Texas Red (absorption and emission at 595 nm and 620 nm, respectively) was used along with the universal Mollicute probe MCP52, labelled with fluorescein isothiocyanate (FITC, absorption/emission at 494/520 nm), as described by Gonella *et al.* (2011).

Salivary glands were dissected in a sterile saline solution, then fixed for 2 min at 4 °C in 4% paraformaldehyde and washed in PBS. All hybridization experiment steps were performed following Gonella *et al.* (2011). FISH experiments included negative controls consisting in hybridization step performed in the absence of probes. After hybridization, the samples were mounted in antifading medium and then observed in a laser scanning confocal microscope SP2- AOBS (Leica).

After the dissection, the remaining body parts were subject to DNA extraction and molecular analyses as previously described.

Results

Host plants and flight activity

During field surveys 19 plant species, mainly broadleaf trees, belonging to 11 families were identified as putative host plants for *O. ishidae*; the majority of species hosted both nymphs and adults (table 2). High numbers were observed in particular on hazelnut (*Corylus avellana* L.), in untreated orchards and/or bushes, willow (*Salix* spp.), hornbeam (*Carpinus* spp.), and walnut (*Juglans regia* L., *Juglans nigra* L.). On the other hand, *O. ishidae* was not found on grasses or weeds, nor on *Ailanthus altissima* (Mill.) and *C. vitalba.* The first nymphs (1st instar) were observed at the end of May, whereas the last ones (5th instar) were observed at the middle of July, regardless of the host plants examined. From the beginning of August on, no more nymphs were found. Nymphs were usually seen on the lower leaf blade. First instars nymphs colonized at first the leaves of twigs close to the trunk and branches of the different host plants. The feeding activity of nymphs was always associated with yellowing and stunting of leaves, independently from the host plant species. Sweep net captures of adults were successful from the middle of July to the end of August, whereas after that period very few adults were captured. The inspection of male genitalia, performed on 10 specimens randomly chosen from different locations, confirmed that the species was *O. ishidae*.

Concerning traps, 462 and 596 specimens were collected in 2011 and 2012, respectively (table 3). Adults were captured from the beginning of July until the middle of October, and the flight peaks occurred at the middle of August and at the middle of July in 2011 and 2012, respectively (figure 2). Captures were more abundant on traps placed outside the vineyards on putative host plants (that is, plants where either nymphs were collected, or reported as hosts for *O. ishidae* in the literature) rather than inside the vineyards. In plots 2, 4, 6, 7, 8, 9 and 10, captures outside the vineyards ranged

Table 2. Plant species that harboured *O. ishidae* nymphs and adults, resulting from sweep-net, beating trays or direct samplings.

Family	Species	Nymphs	Adults
Corylaceae	*Corylus avellana* L.	X	X
Corylaceae	*Ostrya carpinifolia* Scopoli	X	X
Corylaceae	*Carpinus betulus* L.	X	X
Rosaceae	*Malus domestica* Borkhausen	X	X
Rosaceae	*Prunus domestica* L.	X	X
Rosaceae	*Rubus fruticosus* L.		X
Rosaceae	*Rosa canina* L.	X	X
Rosaceae	*Crataegus oxyacantha* L.		X
Juglandaceae	*Juglans regia* L.	X	X
Juglandaceae	*Juglans nigra* L.	X	X
Berberidaceae	*Berberis* spp.	X	X
Berberidaceae	*Buxus sempervirens* L.	X	X
Papaveraceae	*Chelidonium majus* L.		X
Vitaceae	*Vitis vinifera* L.		X
Caprifoliaceae	*Viburnum tinus* L.		X
Ulmaceae	*Ulmus minor* L.	X	X
Salicaceae	*Salix* spp.	X	X
Araliaceae	*Hedera helix* L.	X	X
Cornaceae	*Cornus sanguinea* L.	X	X

Table 3. Captures of *O. ishidae* adults with yellow sticky traps in the different experimental sites during 2011 and 2012.

Plot	2011			2012		
	Total captures	Traps	Mean ± s.e.	Total captures	Traps	Mean ± s.e.
1	78	20	3.90 ± 1.05	56	20	2.80 ± 0.57
2	42	6	7.00 ± 3.08	100	6	16.66 ± 8.04
3	25	24	1.04 ± 0.43	34	24	1.42 ± 0.59
4	4	6	0.67 ± 0.37	4	6	0.67 ± 0.80
5	6	22	0.27 ± 0.18	26	22	1.18 ± 0.81
6	65	6	10.83 ± 8.21	59	6	9.83 ± 7.43
7	45	6	7.50 ± 2.45	38	6	6.33 ± 1.40
8	1	6	0.17 ± 0.17	10	6	1.67 ± 0.84
9	82	6	13.67 ± 8.40	57	6	9.50 ± 4.58
10	114	6	19.00 ± 17.27	212	6	35.33 ± 20.20
Total	462			596		

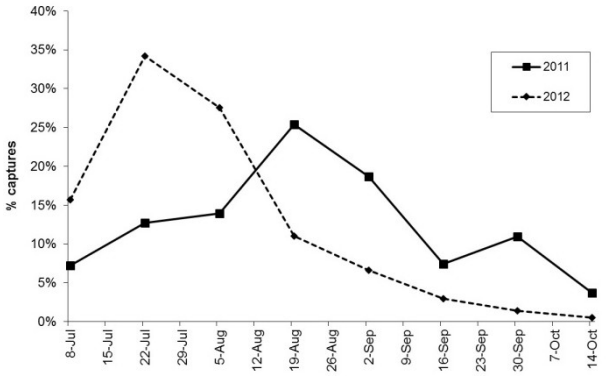

Figure 2. Seasonal abundance of *O. ishidae* adults captured on yellow sticky traps.

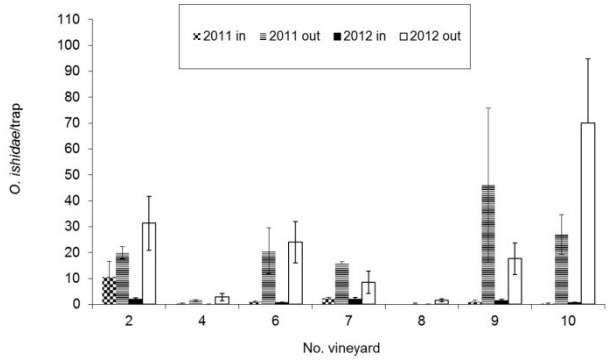

Figure 3. Captures of *O. ishidae* adults (mean ± s.e.) on yellow sticky traps placed inside the vineyards (in) and outside, on putative host plants (out).

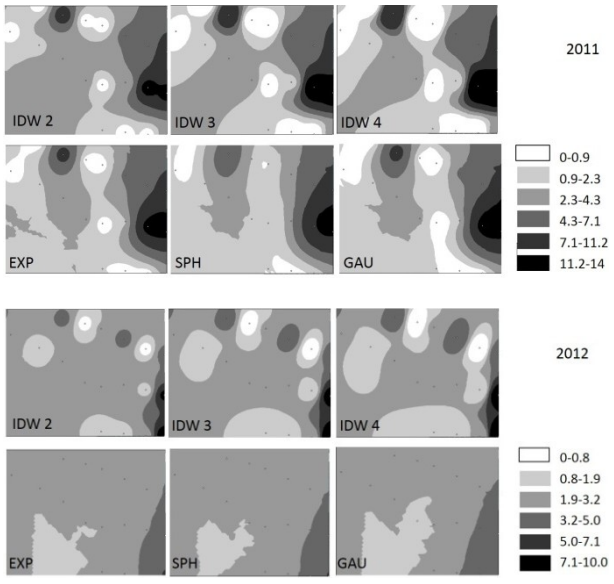

Figure 4. Spatial interpolation of the captures of adult *O. ishidae* in plot 1. IDW 2, 3, 4: Inverse Distance Weighted interpolation with an exponent of 2, 3, 4. EXP, SPH, GAU: Ordinary Kriging following an exponential, spherical, and Gaussian model, respectively.

from 1.5 to 46 adults per trap during 2011, and from 3 to 70 during 2012. Captures inside the vineyard were always lower, ranging from 0 to 10 in 2011, and from 0 to 4 in 2012 (figure 3). Collectively, significant differences between captures of traps placed inside and outside the vineyard were detected both in 2011 ($F = 22.98$; $df = 1, 40$; $P < 0.001$) and in 2012 ($F = 29.13$; $df = 1, 40$; $P < 0.001$).

Concerning grapevine canes collected in the vineyards and incubated in the lab for egg-hatching, nymphs of *O. ishidae* were obtained only from the vineyard of Coazzolo (Plot 10), both in 2012 (40 specimens), and 2013 (3 specimens).

Spatial distribution

Adults of *O. ishidae* were clustered mainly at the edges of the vineyards, in correspondence to putative host plants (figures 4-6). In plot 1, the density was higher particularly at the South-East corner, in correspondence with a garden with ornamental trees including willows (figure 4). In plot 3, the hot spots were set at the North-West corner (apple trees, cherry trees, and many shrubs) and in the Southern part (walnut trees), whereas in the western corner (plum trees) fewer adults

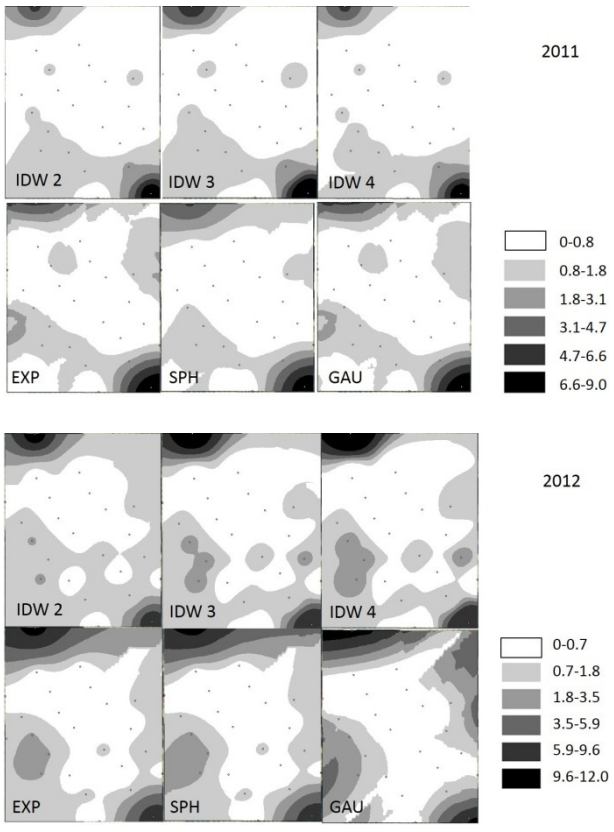

Figure 5. Spatial interpolation of the captures of adult *O. ishidae* in plot 3. IDW 2, 3, 4: Inverse Distance Weighted interpolation with an exponent of 2, 3, 4. EXP, SPH, GAU: Ordinary Kriging following an exponential, spherical, and Gaussian model, respectively.

Table 4. Experimental variogram values and result of cross-validation analyses of Ordinary Kriging spatial interpolation of adult *O. ishidae* captured on yellow sticky traps placed inside and outside the vineyard. Var.: variogram; EXP: exponential; SPH: spherical; GAU: Gaussian; *: significant R^2 ($P < 0.05$); ME: mean error; RMSE: root mean square error.

Year	Plot	Var	Nugget	Sill	Range	R^2	ME	RMSE
2011	1	EXP	0.00	26.56	83.60	0.27	−0.06	3.72
2011	1	SPH	0.75	23.37	58.95	0.25	−0.15	3.72
2011	1	GAU	7.67	17.10	58.94	0.16	−0.11	3.75
2011	3	EXP	0.00	4.01	64.17	0.15	−0.43	3.58
2011	3	SPH	0.00	3.98	85.59	0.22	−0.22	2.11
2011	3	GAU	0.00	3.88	60.65	0.15	−0.42	2.18
2012	1	EXP	4.39	3.50	215.6	0.55*	−0.15	2.69
2012	1	SPH	4.42	4.19	215.6	0.64*	−0.11	2.70
2012	1	GAU	4.83	4.98	215.6	0.71*	−0.07	2.65
2012	3	EXP	0.00	12.78	167.11	0.72*	−0.37	3.33
2012	3	SPH	0.00	16.03	167.11	0.33*	−0.36	3.42
2012	3	GAU	0.02	16.59	113.71	0.80*	−0.52	3.80
2012	5	EXP	0.00	15.72	120.00	0.58*	−0.21	2.83
2012	5	SPH	0.00	15.72	120.00	0.58*	−0.14	3.15
2012	5	GAU	0.02	19.44	120.00	0.80*	−0.14	3.15

were captured (figure 5). In these two plots, no substantial displacement differences were detected between years. No spatial interpolation was performed in plot 5 in 2011 due to the low captures obtained, whereas in 2012 no captures were detected in the vineyard but only externally at the Western corner (hazelnut trees) (figure 6). Experimental variograms showed a range between 59 and 215 m, indicating a fairly strong spatial correlation sometimes covering the whole plot size. ME and RMSE calculated via cross-validation were similar either within IDW changing the exponent, or within OK changing the variogram model. As well, within the same plot they were comparable either using OK or IDW (tables 4, 5). ME values were negative, indicating an underestimation of the observed values (tables 4, 5). The fits of variogram models were not significant during 2011, whereas they were significant during 2012 (table 4).

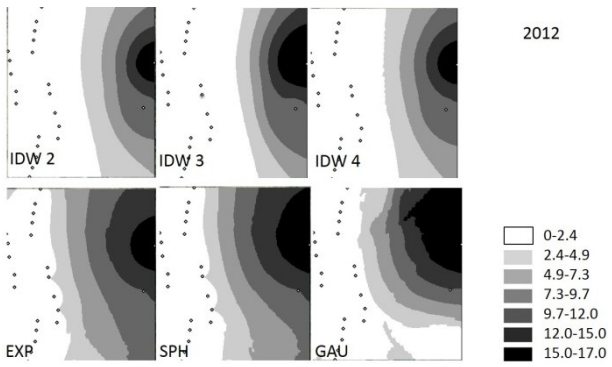

Figure 6. Spatial interpolation of the captures of adult *O. ishidae* in plot 5. IDW 2, 3, 4: Inverse Distance Weighted interpolation with an exponent of 2, 3, 4. EXP, SPH, GAU: Ordinary Kriging following an exponential, spherical, and Gaussian model, respectively.

Table 5. Results of cross-validation analyses for Inverse Distance Weighted (IDW) spatial interpolation of adult *O. ishidae* captured on yellow sticky traps placed inside and outside the vineyard. ME: mean error; RMSE: root mean square error.

Year	Plot	IDW exponent	ME	RMSE
2011	1	2	−0.06	3.93
2011	1	3	−0.14	3.76
2011	1	4	−0.18	3.83
2011	3	2	−0.30	1.99
2011	3	3	−0.36	1.94
2011	3	4	−0.48	1.99
2012	1	2	−0.26	2.87
2012	1	3	−0.34	3.10
2012	1	4	−0.40	3.30
2012	3	2	−0.47	3.03
2012	3	3	−0.60	3.02
2012	3	4	−0.64	3.02
2012	5	2	−0.67	3.08
2012	5	3	−0.50	2.74
2012	5	4	−0.72	3.05

Transmission experiments, molecular analyses and FISH

On the whole, PCR analysis for the presence of FD phytoplasmas was performed on 70 nymphs collected on putative host plants (*Corylus avellana* L.: 53; *Juglans regia* L.: 11; *Cornus sanguinea* L.: 3; *Juglans nigra* L. 2; *Malus domestica* Borkhausen: 1), and 43 adults (*C. avellana* 26; *Salix alba* L.: 5; *Prunus domestica* L.: 5; *C. sanguinea*: 3; *Crataegus oxyacanhta* L.: 2; *M. domestica*: 2). Positive PCR results were neither obtained in case of nymphs nor in case of adults. On the other hand, many adults collected with sticky traps showed a positive response to 16SrV phytoplasmas after PCR. Collectively, 5 adults out of 71 (7.04%) and 15

Table 6. Detection of FD-related phytoplasmas in *O. ishidae* adults captured on yellow sticky traps.

Year	Month of collection	Total tested	16SrV positive	%	FD-C	FD-D
2011	July	0	-	-	-	-
2011	August	17	1	5.88	1	0
2011	September	54	4	7.41	4	0
2011	Total	71	5	7.04	5	0
2012	July	16	6	37.5	3	3
2012	August	66	3	4.54	3	0
2012	September	35	6	17.14	4	2
2012	Total	117	15	12.82	10	5

Table 7. Transmission experiments of 16SrV-C phytoplasmas by means of *O. ishidae*. No.: number of transmission experiment; AAP: acquisition access period; HZ LAP: hazelnut plants used for latency access period; IAP: inoculation access period; MPG: micropropagated grapevine; BB: broad bean; AFM: artificial feeding medium (=diet); FG: infected grapevine in the field; FCA: field collected adults. Adults were placed in batches of 3-6 for IAP on broad beans and vines, whereas for AFM we had 1 adult=1 diet. *groups placed on a single plant where at least 1 insect resulted positive.

No.	Phytoplasma source for AAP	Plants AAP	Insects in AAP	HZ LAP	Substrate used for IAP	Insects in IAP	Positive insects	Batches used/plant or diet inoculated	Positive batches*	Positive plants/diets
1	FCA	-	-	-	BB	39	0	12	0	0
2	FCA	-	-	-	AFM	43	0	43	-	0
3	BB	8	400	5	BB	63	15	16	9	0
4	BB	2	30	1	AFM	6	0	6	-	0
5	BB	6	250	4	MPG	47	8	13	7	2
6	FG	1	70	1	BB	18	6	5	3	0
7	FG	1	50	1	MPG	11	0	3	0	0

out of 117 (12.82%) were positive in 2011 and 2012, respectively. No substantial differences were found in patterns of infected leafhoppers in different time of the season. After RFLP analyses, both subgroups 16SrV-C (strain FD-C) and 16SrV-D (strain FD-D) were detected. During 2011, all positive insects resulted infected with subgroup C, whereas in 2012 the ratio C:D was 2:1 (table 6).

Concerning transmission experiments, 16SrV phytoplasmas were identified in *O. ishidae* specimens that performed AAP on both infected broad beans in the lab (23 positive insects out of 116), and field-infected grapevines (6 out of 29). On the other hand, none of the 82 adults collected in the field with sweep net and used for direct inoculation on either broad bean or artificial diets resulted positive after PCR. All of the 12 hazelnut plants used for LAP resulted negative. Inoculation on broad bean and artificial diet was always unsuccessful, whatever the insects feeding on them (field-collected or artificially-infected). Whereas, after inoculation by leafhoppers that had previously acquired phytoplasmas on infected broad bean, two *in vitro* vines resulted positive. A high mortality of nymphs was observed during AAP, both on broad beans (about 83%) and field grapevine (76%), as well as many adults placed on the artificial feeding medium died after 2-3 days. Data of transmission experiments are shown in table 7.

FISH analysis on *O. ishidae* salivary glands after transmission to micropropagated vines showed a massive presence of 16SrV phytoplasmas, confirming their capability to colonize these organs in the mosaic leafhopper (figures 7D-E, G-H). Conversely, FISH experiments carried out in the absence of probe as a negative control did not show any hybridization signal (figures 7F, I).

Discussion

The mosaic leafhopper *O. ishidae* is widespread in grapevine-growing areas of Piedmont. It seems that its spread has been quite quick since its first discovery in 2007 (Alma *et al*., 2009); moreover, it was already detected in Tuscany (Mazzoni, 2005), and Switzerland (Guglielmino, 2005). At present, the data collected are in accordance with Gaffuri *et al.* (2011) reporting it in the surroundings of vineyards in Lombardy, NW Italy. Adults were found also on traps placed on *A. altissima* hedgerows close to vineyards in Veneto region (Forte *et al*., 2013). On the other hand, in other European regions, *O. ishidae* was found in ecosystems other than vineyards, mainly in forests (Nickel, 2010; Mehle *et al*., 2011), and urban or suburban areas (Koczor *et al*., 2013). In Hungary, it was specifically but unsuccessfully searched in vine-growing areas (Koczor *et al*., 2013). In the US, this species was found on ornamental landscape trees (Valley and Wheeler Jr, 1985; Johnson and Freytag, 2001), and in stone fruit orchards (Rosenberger and Jones, 1978), but to date it was not detected in vineyards.

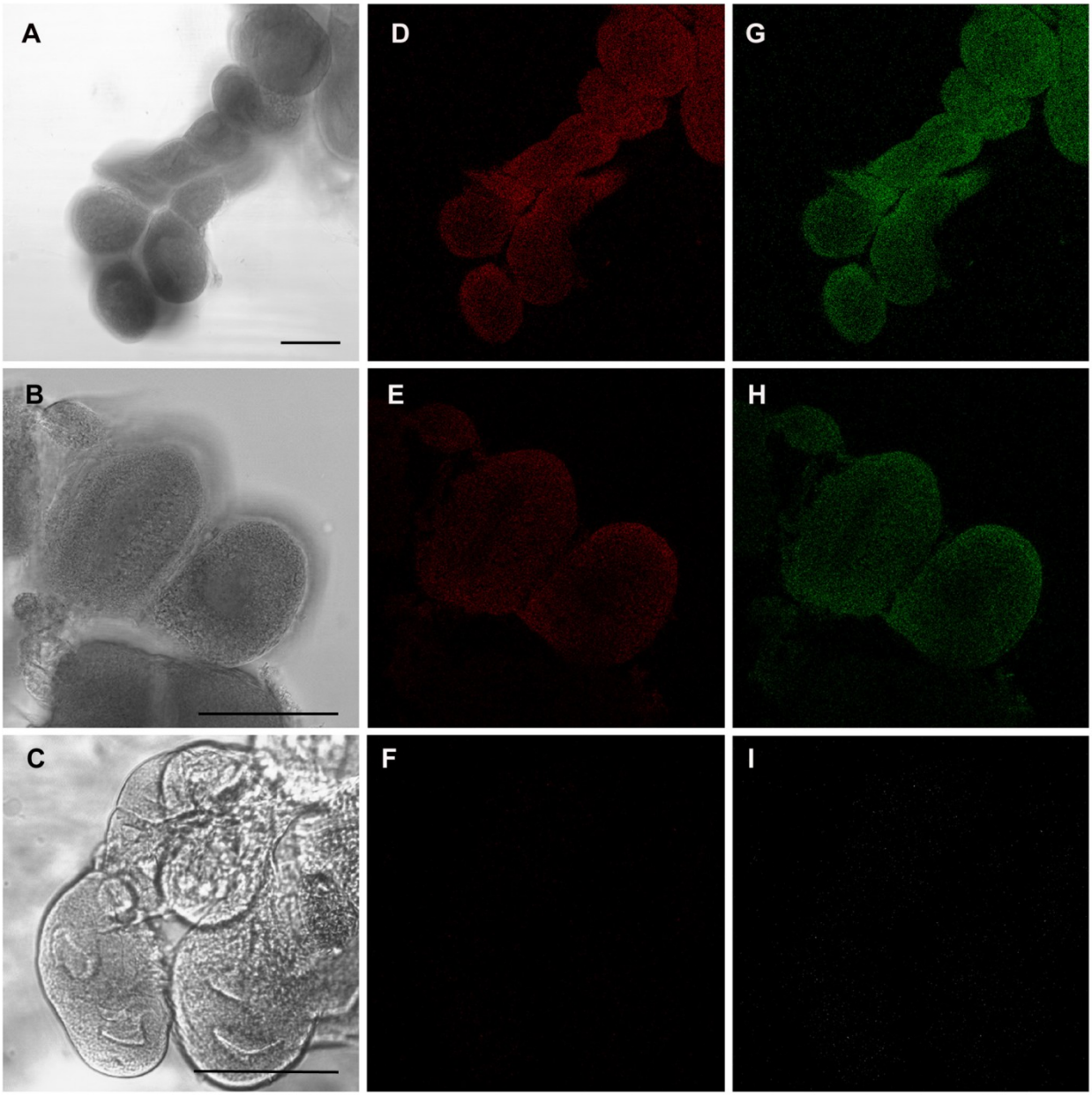

Figure 7. Whole mount *in situ* hybridization of *O. ishidae* salivary glands (A-C) at interferential contrast. Confocal laser scanning microscopy images of salivary glands after hybridization with group 16SrV phytoplasma-specific probe labelled with Texas Red (D-F). FISH of the same salivary glands with the FITC-labelled Mollicute-specific probe (G-I). Panels C, F and I represent the negative controls. Bars = 120 μm.

The widespread presence of *O. ishidae* adults in NW Italian vineyards may be due to habitat fragmentation, with many potential host plants on the edges. The species is polyphagous, and can complete its life cycle on many wild or cultivated trees and shrubs within the vineyard agro-ecosystem. However, the data collected suggest that the movement of adults from the edges into the vineyards does not occur massively. Spatial analyses showed that adults are aggregated at the edges, particularly in presence of suitable host plants (e.g. hazelnut, willow, bramble). Other Auchenorrhyncha that only occasionally feed on grapevines, such as *Hyalesthes obsoletus* Signoret and *Neoaliturus fenestratus* (Heirrich-Schaffer) have a similar distribution pattern (Orenstein *et al*., 2003; Mori *et al*., 2012; Minuz *et al*., 2013), which is probably due to the distribution of their own host plants. In contrast, a grapevine specialist, such as *S. titanus*, shows a different pattern of colonization of cultivated vineyards from wild grapevine on the edges, resulting in a very clear gradient of infestation (Lessio *et al*., 2014). Another reason of the little movement of *O. ishidae* in vineyards may be the mandatory insecticidal treatments against *S. titanus* (Chuche and Thiery, 2014), that target other leafhoppers feeding on grapevines. This aspect is supported by data from plot 3, where populations of *O. ishidae* are scarce on plum trees which were targeted by insecticidal sprays. Besides that, the range values of experimental variograms

were quite high (in some cases > 200 m), indicating a strong spatial correlation. It may be concluded that a few individuals are likely to move a long way from reservoirs, but the main part of the population remains in the same site.

The seasonal occurrence of nymphs and adults of *O. ishidae* in Piedmont matched the data from previous researches conducted in the US on honey locust (Valley and Wheeler Jr, 1985), and in Germany, on various host plants (Nickel, 2010). In our research, nymphs appear at the end of May and are present until the end of July, whereas adults are present from the beginning of July to the end of August. The life cycle is therefore very similar to the one of *S. titanus* (Chuche and Thiery, 2014), which belongs to the same subfamily (Deltocephalinae) that includes the majority of phytoplasma vector species (Weintraub and Beanland, 2006). Since we found adults inside vineyards, it is possible that *O. ishidae* lays eggs on grapevine too. This aspect has been confirmed in the present research under laboratory conditions, particularly during 2012 when up to 40 nymphs were obtained from pruned grapevine wood. On the other hand, during extensive monitoring of *S. titanus* nymphs made in Piedmont, observing the lower grapevine's leaf blade, no nymphs of *O. ishidae* were found (Lessio *et al*., 2011). Another species, *Anoplotettix fuscovenosus* (Ferrari), lays eggs in grapevine's wood but after hatching nymphs move immediately onto weeds in the inter-row (Alma, 1995). However, no nymphs of *O. ishidae* were captured on weeds in the inter-row during sweep net samplings in the present research. Therefore, egg-laying of *O. ishidae* on grapes should be considered to occur very rarely, without an important influence on epidemiology.

Concerning phytoplasmas, the rate of infected individuals ranged from 7 to 12%, which is more or less in accordance with the data from Lombardy (Gaffuri *et al*., 2011), and Switzerland (Trivellone *et al*., 2015), whereas higher percentages of infection (25-50%) were observed in Slovenia (Mehle *et al*., 2011). These values are quite low if compared to data concerning *S. titanus* (Bressan *et al*., 2006), logically because the latter is a grapevine specialist and the main vector for FD. The 16SrV group phytoplasmas identified in *O. ishidae* adults from Piedmont in the present research belonged to the strains FD-D (cluster FD2) and FD-C (cluster FD3) according to Angelini *et al*., 2003, with a prevalence of the latter. In Piedmont, FD-C is more widespread, but FD-D is also present (Morone *et al*., 2007; Roggia *et al*., 2014). In Slovenia, *O. ishidae* resulted positive to all of the clusters FD1, FD2 and FD3, whereas FD2 and FD3 were detected in grapevine, and only FD3 in *C. vitalba*; moreover, all clusters (and also AldY) were identified in *Alnus* spp. (Mehle *et al*., 2011); since in this case *O. ishidae* specimens were captured only outside vine growing areas, it is possible that alder has served as a reservoir for phytoplasmas. In Switzerland, *O. ishidae* resulted associated with FD-C, FD-D and FD70 (Trivellone *et al*., 2016); in this country, FD is confined to the canton of Ticino (Schaerer *et al*., 2007), but no information is available concerning the strains. In Lombardy, *O. ishidae* resulted associated to 16SrV-D phytoplasmas (Gaffuri *et al*., 2011), which is widespread in grapevines whereas subgroup -C is limited to the Oltrepò Pavese region (Belli *et al*., 2010). There is therefore a certain correspondence between phytoplasma types identified in *O. ishidae* and grapevines. Moreover, nymphs and adults collected on host plants during this research were never found to be positive, therefore these plants are unlikely to be sources for phytoplasma infection for the mosaic leafhopper, whereas it is possible that adults have acquired phytoplasmas by feeding directly on infected vines. To date, in Italy FD-related phytoplasmas have been found, besides grapevine, in *C. vitalba* (Filippin *et al*., 2009) and *A. altissima* (Filippin *et al*., 2011). However, neither nymphs nor adults of *O. ishidae* were found on these host plants during our surveys. In the Veneto region, adults were recruited on traps placed on hedgerows of *A. altissima* (Forte *et al*., 2013), but there is no evidence that they were actually feeding on it. Phytoplasmas in the 16SrV-C ribosomal subgroup are found also on many broadleaf plants such as *Alnus glutinosa* (L.) Gaertn., *Ulmus* spp., *Rubus fruticosus* L., but without being FD-related (Arnaud *et al*., 2007). Concerning other host plants of *O. ishidae*, other ribosomal groups were identified in many species of willow: 16SrI (Hiruki and Wang, 1999; Wei *et al*., 2009; Mou *et al*., 2014), 16SrVI (Zhang *et al*., 2012) and 16SrXII (Alfaro-Fernandez *et al*., 2011). Hazelnut trees were infected with 16SrI in Poland (Cielinska and Kowalik, 2011), with 16SrIII in Oregon (Jomantiene *et al*., 2000), which may be in agreement with the fact that *O. ishidae*, in the Nearctic region, had been associated to the X-disease (Rosenberger and Jones, 1978); group 16SrX phytoplasmas were also identified in hazelnut trees in southern Italy (Marcone *et al*., 1996). However, to date there are no reports of *C. avellana* hosting 16SrV phytoplasmas; moreover, in the present research, all the hazelnut plants used for LAP of *O. ishidae* resulted negative after PCR, which reinforces the hypothesis that this botanical species is not a source for 16SrV phytoplasmas.

The feeding behaviour of the mosaic leafhopper may explain its low vectoring efficiency. Leafhoppers are generally divided into three groups depending on their feeding *habitus*: salivary sheath feeders, that produce a sheath of gel saliva when probing into the phloem and then inject water saliva containing digestive enzymes and feed directly into phloem sieves (this group includes the most efficient vectors); stipple feeders, that probe into the mesophyll tissues causing the removal of cell content and cause light spots on leaves without damaging sieve tubes; and cell rupture feeders, that make multiple probes in a destructive manner causing the typical symptoms called "hopperburning" (Backus *et al*., 2005; Jarausch and Weintraub, 2013). The evident leaf yellowing and necrosis left by *O. ishidae* while feeding may support the hypothesis that this species uses the cell rupture strategy without forming salivary sheaths, which makes it only an occasional vector. The same aspect has been theorized about *Empoasca decipiens* Paoli as a vector of 16SrV phytoplasmas under laboratory conditions (Galetto *et al*., 2011). However, to date no members of the subfamily Deltocephalinae are known as hopperburners: in fact, cell rupture feeders belong

mostly to the subfamily Typhlocybinae, and to a second extent to the family Delphacidae, e.g. *Nilaparvata lugens* Stal and *Sogatella furcifera* Horvath (Backus *et al*., 2005). Further studies, e.g by means of electro-penetration graphics (EPGs) should be carried out to shade light on this matter.

Another important aspect of the vectoring ability is the presence of the pathogens in the salivary glands. The FISH conducted after the whole transmission process (AAP/LAP/IAP) has proved that 16SrV phytoplasmas were acquired and reached the salivary glands of *O. ishidae*. The salivary gland barrier is one of the most selective sites of recognition between vectors and pathogens (Bosco and D'Amelio, 2010). Their detection in salivary glands therefore is evidence that 16SrV phytoplasmas are able to complete a persistent-propagative cycle inside this leafhopper species.

In conclusion, it seems that *O. ishidae*, despite being widespread in the vineyard agroecosystem, may not move frequently inside vineyards. Its ability of transmitting 16SrV phytoplasmas to grapevine under laboratory conditions has been confirmed, although with a low efficiency; however, to date the sources of inoculum in nature are not known. All these aspects concur to acknowledge the mosaic leafhopper as a very occasional vector of 16SrV group phytoplasmas.

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