Identification and ecology of alternative insect vectors of ‘Candidatus Phytoplasma solani’ to grapevine

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Bois noir, a disease of the grapevine yellows complex, is associated with ‘Candidatus Phytoplasma solani’ and transmitted to grapevines in open fields by the cixiids Hyalesthes obsoletus and Reptalus panzeri. In vine-growing areas where the population density of these vectors is low within the vineyard, the occurrence of bois noir implies the existence of alternative vectors. The aim of this study was to identify alternative vectors through screening of the Auchenorrhyncha community, phytoplasma typing by stamp gene sequence analyses, and transmission trials. During field activities, conducted in Northern Italy in a vineyard where the bois noir incidence was extremely high, nine potential alternative insect vectors were identified according to high abundance in the vineyard agro-ecosystem, high infection rate, and harbouring phytoplasma strains characterized by stamp gene sequence variants found also in symptomatic grapevines. Transmission trials coupled with molecular analyses showed that at least eight species (Aphrodes makarovi, Dicranotropis hamata, Dictyophara europaea, Euscelis incisus, Euscelidius variegatus, Laodelphax striatella, Philaenus spumarius, and Psammotettix alienus/confinis) are alternative vectors of ‘Candidatus Phytoplasma solani’ to grapevines. These novel findings highlight that bois noir epidemiology in vineyard agro-ecosystems is more complex than previously known, opening up new perspectives in the disease management.

Bois noir (BN), a disease of the grapevine yellows (GY) complex, causes serious crop losses in wine-making grape varieties in the Euro-Mediterranean area and in other vine-growing countries. BN is associated with strains of ‘Candidatus Phytoplasma solani’ (CaPsol) (subgroup 16SrXII-A), a phloem-limited cell wall-less bacterium of the Mollicutes class 1–5. Hyalesthes obsoletus Signoret (Hemiptera, Cixiidae), a polyphagous insect feeding mainly on bindweed (Convolvulus arvensis L.), nettle (Urtica dioica L.), chaste tree (Vitex agnus-castus L.), and stinking hawk’s beard (Crepis foetida L.), is the principal vector of CaPsol strains to grapevine6–8. In the last years, the spreading of CaPsol in vineyards where H. obsoletus was absent suggested the existence of additional vectors. Recently, a study conducted in Serbia demonstrated the capability of Reptalus panzeri Löw to transmit CaPsol to grapevine9. On the other hand, Macrosteles quadripunctulatus (Kirschbaum), Anaceratagallia ribauti Ossiannilsson, and Reptalus quinquecostatus (Dufour) were found able to transmit CaPsol in experimental conditions but no evidence of transmission of the pathogen to grapevines is available yet10–12. Furthermore, numerous CaPsol-harbouring planthopper (Cixiidae) and leafhopper (Cicadellidae) species were found within or around BN-affected vineyards13,14. Considering such evidences, the identification of which insect species can effectively transmit the phytoplasma to grapevine is of paramount importance to formulate effective control strategies to reduce the BN incidence.

Based on tufB gene sequence analysis, three CaPsol tuf-types, identified in both BN-affected grapevines and non-crop host plants, were associated with two distinct CaPsol ecological cycles related to bindweed (tuf-type b) and nettle (tuf-type a and ab (formerly known as b2))15,16. A study conducted in Eastern Europe reported the direct epidemiological role of chaste trees as CaPsol source in the H. obsoletus-mediated transmission to grapevines17,18. This novel finding highlights that bois noir epidemiology in vineyard agro-ecosystems is more complex than previously known, opening up new perspectives in the disease management.
grapevine. Moreover, in the Balkan region, it was recently highlighted that *H. obsoletus* population related to stinking hawk’s beard can acquire CaPsol *tuf*-type b from this source plant and transmit it to grapevine. A larger genetic diversity among CaPsol strains was described by molecular characterization of less conserved genes (i.e., *secY*, *vmp1*, and *stamp*) Interestingly, studies focused on *stamp* gene molecular markers improved the knowledge on CaPsol strain population structure and dynamics, revealing the phytoplasma transmission ways in vineyard agro-ecosystems. In detail, it was found that CaPsol strains grouped in diverse *stamp*-based phylogenetic clusters are associated with bindweed- and nettle-related ecological cycles. Moreover, recent findings highlighted that in Tuscany (central Italy) BN is prevalently associated with a CaPsol strain never found before in grapevine but detected exclusively in other host plants. This reinforced the evidence of the existence of alternative BN epidemiological cycles that could include other insect vectors able to transmit CaPsol to grapevine.

Thus, in the present study, we investigated the composition of Auchenorrhyncha community in vineyards where CaPsol is significantly spreading, identified putative insect vectors and conducted transmission trials, along with molecular analyses to investigate the capability of prevalent species of Cixiidae and Cicadellidae to transmit CaPsol to grapevine.

**Results**

**Auchenorrhyncha community description.** During field activities, 1018 and 896 adult insects were collected in 2013 and 2014, respectively. Stereomicroscope analysis, based on observation of morphological characters, allowed the identification of 48 distinct taxonomic groups, 44 defined at species level and four at genus level, belonging to a total of nine different families (Table 1). The most represented family was Cicadellidae, as 30 out of 48 taxonomic groups belong to this family, adding up to 377 individuals out of the 1018 captured in 2013 (37%) and 377 out of the 896 captured in 2014 (42%). The other most relevant families were Delphacidae (representing 17.7% and 18.5% of insects captured in 2013 and 2014, respectively), Cixiidae (representing 11% and 13.5% of insects captured in 2013 and 2014, respectively) (around the borders), and Aphrophiidae (representing 9.1% and 10.1% of insects captured in 2013 and 2014, respectively), mostly represented by the species *Laodelphax striatellus*, *H. obsoletus*, *Philaeus spumarius*, respectively. Cercopidae (most of which belong to the *Cercopis vulnerata* species), Dictyopharidae (with *Dictyophara europaea* species) and Flatidae (with *Metcalflia pruinosa* species) families were highly represented with 24% and 15% of captured insects in 2013 and 2014, respectively. The remaining two families, Caliscelidae and Membracidae, had the lowest abundance (less than 1%) among captured insects. The grapevine leaffoppers *Empoasca vitis* (Göthe) and *Zygina rhamni* Ferrari were not considered, as their population density was very low.

**Identification and molecular characterization of ‘Ca. P. solani’ in insects and grapevines.** PCR-based amplification of *stamp* gene revealed the presence of CaPsol in 63 out of 621 analyzed insect pools from 2013 and 82 out of 529 pools from 2014, belonging to 19 species in 2013 and 18 species in 2014, 12 of which are common to both years (Table 1). The amplification of *stamp* gene also revealed the presence of CaPsol in 54 grapevine leaf samples (29 out of 30 in 2013, and 25 out of 30 in 2014). The 112 *stamp* fragments (StampF1/StampR1) of the expected size, amplified in 2013 and 2014 (58 from insects and 54 from grapevines) were sequenced (Table 2). Based on the nucleotide sequence identity, six *stamp* sequence variants (here named GuSt1 to GuSt6) were identified. CaPsol strains characterized by *stamp* sequence variants GuSt1, GuSt2, GuSt3 and GuSt5 were identified in both insects and grapevines, while GuSt4 and GuSt6 were found only in insects (Table 2). Comparison of such sequence variants (GuSt1 to GuSt6) with the previously published dataset revealed that they were identical to the previously described sequence variants St5 [representative strain GGY, GenBank Accession Number (Acc. No.) FN813256], St11 (representative strain 19–25, Acc. No. FN813267), St19 (representative strain CRI10_1183, Acc. No. KJ469719), St21 (representative strain AA21, Acc. No. KJ145380), St30 (representative strain Vv24, Acc. No. KC703022) and St36 (representative strain Carv2, Acc. No. KT184880), respectively (Table 2). In detail, in 2013 and 2014, insects contained CaPsol strains characterized by *stamp* sequence variants St5 (63.8%), St11 (3.5%), St19 (6.9%), St21 (1.7%), St30 (15.5%) and St36 (8.6%). Grapevines harboured CaPsol strains characterized by *stamp* sequence variants St5 (50%), St11 (22.2%), St19 (14.8%) and St30 (13%) (Table 2).

The alignment of *stamp* nucleotide sequences of CaPsol strains representative of the GuSt sequence variants identified in Gussago (GuSt1 to GuSt6) and those previously described (St1 to St58) was used for generating a phylogenetic tree in which three main clusters (I–III) and two subclusters (a1 and a2) were observed. CaPsol strains identified in the present study are found in four of these groups, as no strain clusters within the I–III cluster. CaPsol strains sharing the *stamp* sequence variant St19 (12 strains) grouped in the nettle-related subcluster a2; those with sequence variant St11 (14 strains) grouped in the nettle-related subcluster a1; those with phenotype variants St5, St21 and St30 (81 strains) grouped in the bindweed-related cluster b-II; those with sequence variant St36 (five strains) in the bindweed-related cluster b-III (Fig. 1, S1).

**Identification of ‘Ca. P. solani’ insect vectors by transmission trials.** Based on the criteria applied to select potential CaPsol insect vectors to be tested, transmission trials were conducted in 2015 and/or 2016 on the insect species *Aphrodes makarovi* Zachvatkin, *Cicadella viridis* L., *Dircanotropis hamata* Boheman, *Dictyophara europaea* Spinola, *Euscelis incisus* Kirschbaum, *Euscelidius variegatus* Kirschbaum, *Hyalethes obsoletus*, *Laodelphax striatellus*, *Philaeus spumarius* and *Psmamnettix alienus/confinis* (Table 3). Due to the fact that dead specimens of both *P. alienus* and *P. confinis* were found in the cage after the transmission period, it is not possible to distinguish the species transmission ability. In the 2015 transmission trials, *stamp* gene amplification and SYBR Green real-time amplification assay allowed the detection of CaPsol in *D. hamata, E. incisus* and *H. obsoletus* specimens, and in leaf samples (collected one year after the transmission trials, July 2016) of the grapevines on which each of these insect species was forced to feed. Only grapevines hosting *H. obsoletus* were found infected by CaPsol as early as October 2015, the end
| Family            | Subfamily | Insect                                     | 2013 | Number of captured specimens | Number of pools | Number of CaPsol infected pools | Proportion of infected pools | 2014 | Number of captured specimens | Number of pools | Number of CaPsol infected pools | Proportion of infected pools |
|-------------------|-----------|--------------------------------------------|------|------------------------------|----------------|-------------------------------|-----------------------------|------|------------------------------|----------------|-------------------------------|-----------------------------|
| Cixiidae          | Cixiinae  | *Cixius* spp. Latreille, 1804             | 2    | 2                           | —              | —                             | —                           | —    | 2                           | —              | —                             | —                           |
| Cixiidae          | Cixiinae  | *Hyalodes obsoletus* Sign., 1865           | 100  | 44                          | 13             | 30                            | 113                         | 43   | 10                          | 23             | —                             | —                           |
| Cixiidae          | Cixiinae  | *Hyalodes scotti* Ferrari, 1882           | 8    | 6                           | —              | 4                             | 2                           | —    | —                           | —              | —                             | —                           |
| Cixiidae          | Cixiinae  | *Reptialis* spp. Emeljanov, 1971          | 2    | 2                           | —              | 4                             | 4                           | —    | —                           | —              | —                             | —                           |
| Delphacidae       | Astracinae| *Astraca clavicornis* (F., 1794)          | 6    | 6                           | 2              | 33                            | —                           | —    | —                           | —              | —                             | —                           |
| Delphacidae       | Delphacinae| *Drosneotropis hamata* (Boh., 1847)        | 31   | 15                          | 2              | 13                            | 12                          | 7    | 1                           | 14             | —                             | —                           |
| Delphacidae       | Delphacinae| *Laudophila striatella* (Fall., 1826)      | 121  | 56                          | 6              | 11                            | 71                          | 30   | 2                           | 7              | —                             | —                           |
| Delphacidae       | Delphacinae| *Toya propinqua* (Fieb., 1866)            | 20   | 9                           | —              | 81                            | 24                          | 2    | 8                           | —              | —                             | —                           |
| Delphacidae       | Kelisia spp. |                             | 8    | 6                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |
| Delphacidae       | Kelisia spp. |                             | 2    | 2                           | —              | 2                             | 2                           | —    | 2                           | 2              | —                             | —                           |
| Dictyopharidae    | Dictyopharinae|                             | 47   | 47                          | —              | 59                            | 59                          | 12   | 20                          | —              | —                             | —                           |
| Flatidae          | Flatinae  | *Metalliction pruinosa* (Say, 1830)       | 68   | 35                          | 9              | 26                            | 79                          | 41   | 1                           | 2              | —                             | —                           |
| Cercopidae        | Cercopinae| *Cercopis vulnerata* Rossi, 1807          | 120  | 120                         | 2              | 2                             | —                           | —    | —                           | —              | —                             | —                           |
| Cercopidae        | Cercopinae| *Haematoloma dorsatum* (Ahr., 1812)       | 12   | 7                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |
| Aphrophoridae     | Aphrophorinae|                             | 93   | 28                          | 2              | 7                             | 91                          | 53   | 14                          | 26             | —                             | —                           |
| Membracidae       | Centrotinae|                             | 1    | 1                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |
| Membracidae       | Smilinae  | *Stictocephula bisasia* Kopp & Yon., 1977 | 1    | 1                           | —              | 3                             | 3                           | 1    | 33                          | —              | —                             | —                           |
| Cicadellidae      | Agallinae | *Anacatagallia rubra* (Oss., 1938)        | —    | —                           | —              | 5                             | 4                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Aphrodesinae|                             | 29   | 21                          | 5              | 24                            | 2                           | 2    | —                           | —              | —                             | —                           |
| Cicadellidae      | Cicadellinae|                             | 43   | 35                          | 6              | 17                            | 142                         | 123  | 21                          | 17             | —                             | —                           |
| Cicadellidae      | Cicadellinae| *Evainustus acuminatus* (F., 1794)        | 3    | 3                           | 2              | 67                            | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 13   | 12                          | 1              | 8                             | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 2    | 1                           | —              | 2                             | 1                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 1    | 1                           | —              | 6                             | 3                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 25   | 24                          | 2              | 8                             | 33                          | 9    | 1                           | 10             | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 30   | 15                          | 2              | 13                            | 11                          | 10   | 1                           | 10             | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 58   | 31                          | 3              | 10                            | 21                          | 20   | 1                           | 5              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 5    | 5                           | 1              | 20                            | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 5    | 5                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 4    | 4                           | —              | 13                            | 13                          | 1    | 8                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 2    | 2                           | —              | 10                            | 5                           | 1    | 20                          | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 5    | 4                           | —              | 1                             | 1                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 4    | 2                           | —              | 5                             | 3                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 2    | 2                           | —              | 1                             | 1                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 5    | 2                           | 1              | 50                            | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | —    | —                           | —              | 1                             | 1                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | —    | —                           | —              | 8                             | 8                           | 6    | 75                          | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 30   | 12                          | —              | 81                            | 34                          | 4    | 12                          | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 4    | 1                           | 1              | 100                           | 8                            | 8    | 2                          | 25             | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 5    | 5                           | 1              | 20                            | 9                           | 5    | 1                           | 20             | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 5    | 5                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 70   | 25                          | —              | 1                             | 1                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 13   | 6                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 4    | 4                           | 2              | 50                            | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 1    | 1                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Deltocephalinae|                             | 9    | 5                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |
| Cicadellidae      | Caliscelinae| *Caliscelis boellii* (Latreille, 1807)    | 7    | 7                           | —              | —                             | —                           | —    | —                           | —              | —                             | —                           |

**Table 1.** Captures and infection rate of CaPsol potential insect vectors in the examined vineyard in 2013 and 2014.
of the growing season in which transmission trials were conducted. No amplification was obtained from insect specimens of *C. viridis*, *E. variegatus*, *L. striatella*, or *P. alienus/confinis* nor from the leaf samples of the grapevines on which each of these insect species was forced to feed (Table 3).

In the 2016 transmission trials, *stamp* gene amplification and SYBR Green real-time amplification allowed the detection of CaPsol in *A. makarovi*, *D. europaea*, *E. incisus*, *E. variegatus*, *H. obsoletus*, *L. striatella*, *P. spumarius* and *P. alienus/confinis* specimens, and in leaf samples (collected one year after the transmission trials, July 2017) of the grapevines on which each of these insect species was forced to feed. Only one grapevine plant hosting *E. variegatus* was found to be infected by CaPsol as early as October 2016, the end of the growing season in which transmission trials were conducted. No amplification was obtained from insect specimens of *D. hamata*; despite this, the grapevine plant on which this insect was forced to feed was found to be infected. On the contrary, CaPsol was detected in *C. viridis* specimens but no amplification was obtained from the grapevine plant on which this insect was forced to feed. Furthermore, in both years no amplification was obtained from control plants, kept in controlled conditions without insects (Table 3). Moreover, *stamp* gene nucleotide sequences were obtained from 75 representative CaPsol-harbouring insect specimens forced to feed on the 24 grapevine plants that were found to be CaPsol-infected after the transmission trials. Sequence identity analysis evidenced that (i) all 24 infected grapevines harboured CaPsol strains characterized by the same *stamp* sequence variant (St5); (ii) in each of the

| Species                         | Year | CaPsol infected pools | Sequenced | CaPsol strain (stomp gene sequence variant) |
|---------------------------------|------|-----------------------|-----------|---------------------------------------------|
| *Vitis vinifera L.*, 1753       | 2013 | 29                    | 15        | GuSt1 (St5)                                 |
|                                 | 2014 | 25                    | 12        | GuSt2 (St11)                                |
| *Hyalocnemus abietis Sign.*, 1865 | 2013 | 13                    | 4         | GuSt3 (St19)                                |
|                                 | 2014 | 10                    | 1         | GuSt4 (St21)                                |
| *Astrupia clavicornis* (F., 1794) | 2013 | 2                     | 1         | GuSt5 (St30)                                |
|                                 | 2014 | 1                     | 1         | GuSt6 (St36)                                |
| *Dicranotropis humata* (Bob., 1847) | 2013 | 2                     | 1         | GuSt1 (St5)                                 |
|                                 | 2014 | 1                     | 1         | GuSt2 (St11)                                |
| *Laodelphax striatellae* (Fall., 1826) | 2013 | 6                     | 3         | GuSt3 (St19)                                |
|                                 | 2014 | 2                     | 1         | GuSt4 (St21)                                |
| *Tobia propinqua* (Fieb., 1866)  | 2014 | 2                     | 1         | GuSt5 (St30)                                |
| *Dictyophora europaea* (L., 1767) | 2014 | 12                    | 2         | GuSt1 (St5)                                 |
| *Metcalia pruinosa* (Say, 1830)  | 2013 | 9                     | 1         | GuSt2 (St11)                                |
|                                 | 2014 | 1                     | 1         | GuSt3 (St19)                                |
| *Cercospora vulnerata* Rossi, 1807 | 2013 | 2                     | 2         | GuSt1 (St5)                                 |
|                               | 2014 | 2                     | 1         | GuSt2 (St11)                                |
| *Philaenus spumarius* (L., 1758) | 2013 | 2                     | 2         | GuSt1 (St5)                                 |
|                                 | 2014 | 14                    | 4         | GuSt1 (St5)                                 |
| *Stictocephala bisonia* Kopp & Yon, 1977 | 2014 | 1                     | 1         | GuSt1 (St5)                                 |
| *Aphrodes makarovi* Zachv., 1948 | 2013 | 3                     | 2         | GuSt1 (St5)                                 |
| *Cicadella viridis* (L., 1758)  | 2013 | 6                     | 2         | GuSt1 (St5)                                 |
|                                 | 2014 | 12                    | 5         | GuSt1 (St5)                                 |
| *Evacanthus acuminatus* (F., 1794) | 2013 | 2                     | 1         | GuSt1 (St5)                                 |
| *Allygdias furcatus* (Ferr., 1882) | 2013 | 2                     | 1         | GuSt1 (St5)                                 |
|                                 | 2014 | 1                     | 1         | GuSt1 (St5)                                 |
| *Anaplotettix fusconervosus* (Ferr., 1882) | 2013 | 1                     | 1         | GuSt1 (St5)                                 |
| *Euclidis incisus* (Kbm., 1858) | 2013 | 2                     | 1         | GuSt1 (St5)                                 |
|                                 | 2014 | 1                     | 1         | GuSt1 (St5)                                 |
| *Euclidis variegatus* (Kbm., 1858) | 2013 | 3                     | 2         | GuSt1 (St5)                                 |
|                                 | 2014 | 1                     | 1         | GuSt1 (St5)                                 |
| *Fieberiella florii* (Stål, 1864) | 2013 | 1                     | 1         | GuSt1 (St5)                                 |
| *Hishimonus hamata* Koeh, 1976   | 2014 | 1                     | 1         | GuSt1 (St5)                                 |
| *Japananus hyalinus* (Osb., 1900) | 2014 | 1                     | 1         | GuSt1 (St5)                                 |
| *Nasoilurus fenestratus* (H.-S., 1834) | 2013 | 1                     | 1         | GuSt1 (St5)                                 |
| *Orientus ishidae* (Mats., 1902) | 2014 | 6                     | 2         | GuSt1 (St5)                                 |
| *Psammotettix alienus/confinis* (Dhlb, 1850) | 2014 | 4                     | 3         | GuSt1 (St5)                                 |
| *Scaphoideus titanus* Ball, 1932 | 2013 | 1                     | 1         | GuSt1 (St5)                                 |
|                                 | 2014 | 2                     | 1         | GuSt1 (St5)                                 |
| *Thamnotettix zelleri* (Kbm., 1868) | 2013 | 1                     | 1         | GuSt1 (St5)                                 |
|                                 | 2014 | 1                     | 1         | GuSt1 (St5)                                 |
| *Megophthalmus scanicus* (Fall. 1806) | 2013 | 2                     | 1         | GuSt1 (St5)                                 |

Table 2. *Stamp* sequence variants of CaPsol identified in symptomatic grapevines and potential insect vectors.
nine insect species forced to feed on these 24 grapevines (all species used in the transmission bioassay except C. viridis) the prevalent CaPsol strains were characterized by the same stamp sequence variant (St5, 68 out of 75 specimens); CaPsol strains characterized by the stamp sequence variant St19 and St11 were identified in five and two out of 75 insect specimens, respectively; (iii) at least one insect specimen that was forced to feed on each of the 24 infected grapevines harbouring CaPsol strain characterized by the stamp sequence variant St5 (Table 3). Yellowing and downwards rolling of leaves and lack of cane lignification were observed in September 2018 in one and two grapevines where H. obsoletus and E. incisus were confined, respectively.

**Plants eaten by alternative insect vectors.** rbcL gene was amplified in 59 out of 154 insects of eight species. No amplification was obtained for L. striatella and P. alienus/confinis. BLAST analysis of rbcL nucleotide sequences showed that, with the exception of A. makarovi, grapevine was the most prevalent plant on which all the insects have had their last meal. Moreover, specimens of the species D. hamata, D. europaea, E. incisus, and P. spumarius had their last meal on nettle and/or Crepis spp. (Table 4).

**Discussion**

In Euro-Mediterranean regions the main insect vector of 'Ca. P. solani' (CaPsol) is *Hyalesthes obsoletus*[^8]-[^10]. Management of its host plants in the vineyards and surrounding areas is therefore considered crucial for BN control[^11]-[^12]. The existence of additional vectors has been theorized from the observations that BN incidence is not always correlated to high densities or presence of *H. obsoletus* populations[^12]-[^13]. Thus, several studies, conducted to discover alternative insect vectors, detected more than 35 insect species harbouring CaPsol, 16 of which were identified according to high abundance in the vineyard agro-ecosystem, high CaPsol-infection rate, and recognition mechanism[^14]-[^15], which involves the binding of insect cytoskeleton proteins with the antigenic membrane protein encoded by the CaPsol *stamp* gene[^16]. Thus, *stamp*-based molecular typing of CaPsol strains has been employed to identify its insect vectors and transmission routes in this and previous studies[^17]-[^19].

In order to optimize the experimental conditions allowing identification of CaPsol insect vectors, this study was conducted in a vineyard where BN incidence was extremely high without any correlation with *H. obsoletus* density and distribution, the ground cover had many CaPsol host plants, and the agro-ecosystem included a high plant biodiversity (presence of forest, grass and broadleaf species). In fact, probably due to this high biodiversity, many more insect species were found in the target area compared to a previous study conducted in Lombardy Region[^20]. The differences in insect population presence and density observed in 2013 and 2014 should be explained by diverse climate conditions (dry and cold in 2013, wet and hot in 2014) and consequently by grass cover management within and around the vineyard, such as cutting and/or mowing, that affect the Auchenorrhyncha population density as previously demonstrated[^21].

Within the investigated Auchenorrhyncha community, nine potential insect vectors, besides *H. obsoletus*, were identified according to high abundance in the vineyard agro-ecosystem, high CaPsol-infection rate, and harbouring CaPsol strains characterized by *stamp* gene sequence variants undistinguishable from those found in symptomatic grapevines.

Interestingly, except *D. hamata*, an extraordinary infection rate was found in the tested insects, in agreement with data obtained in previous study conducted on other phytoplasma insect vectors[^21]. The different temperatures registered in the two investigated years could also explain the diverse CaPsol-infection rates observed, as reported for *Candidatus* Phytoplasma asteris (CYP) in *Chrysanthemum carinatum* and its vector *Macrosteles quadripunctatus*[^22]-[^23]. In this CYP epidemics the mean latency period in the insect and in the host plant was faster at high

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[^8]: Fieber have been proven as vectors of CaPsol to grapevine plants in Serbia and Spain[^9]. The small number of effective vectors compared to CaPsol-infected insects is related to the specific phytoplasma-vector recognition mechanism[^27]-[^28], which involves the binding of insect cytoskeleton proteins with the antigenic membrane protein encoded by the CaPsol *stamp* gene[^28]. Thus, *stamp*-based molecular typing of CaPsol strains has been employed to identify its insect vectors and transmission routes in this and previous studies[^27]-[^29].

[^9]: In this CYP epidemics the mean latency period in the insect and in the host plant was faster at high
in vineyards (Table 5), it is reasonable to hypothesize that Northern Italian vineyards, known host plants, and feeding preference towards grapevine and/or common weeds vine is strictly related to their ecology. Considering their abundance, development cycle and adult presence in to grapevine in these experimental conditions, further investigation is necessary.

As no insects were able to transmit such phytoplasma strains, grouping in the nettle-related subclusters a1 and a2, respectively, were found in 37% of the examined tive insect vectors could be involved in CaPsol spreading also in worldwide. Furthermore, St11 and St19 CaPsol sion to grapevine of at least St5 CaPsol strain in Europe. Due to their wide geographical distribution, the alterna-
is reasonable to hypothesize that alternative insect vectors, identified in this study, can play a role in the transmis-

temperatures than low ones as consequence of faster phytoplasma multiplication in the host plants and higher frequency of feeding bouts of vectors at higher temperatures (Maggi et al., 2004). The difference in the infection rate between the two investigated years was probably due to the diverse insect collection period.

Transmission trials proved that eight insect species are vectors of CaPsol to grapevine. Among these, Euscelidius variegatus and Euscelis incisus were previously reported as able to transmit the pathogen to in vitro grapevine plantlets and to solanaceous plants, respectively; the other six insect species (Dicranotropis hamata, Laodelphax striatella, Dicyophara europea, Philaenus spumarius, Aphrodes makarovi, Psammotettix alienus/ confinis) were found harbouring CaPsol in vineyards but there was no evidence of transmission to grapevine (Table 5). Moreover, D. hamata and L. striatella are related to the species Javesella discolor (Boheman 1847) (Delphacidae, Delphacinae) proved to be able to transmit CaPsol to artificial diet medium. Psammotettix alienus/confinis is related to the species Psammotettix striatius, previously proved to be able to transmit CaPsol to artificial diet medium. A. makarovi is related to the species A. bicincta proved to be able to transmit CaPsol to various plants in Europe.

The eight alternative insect vectors were found largely infected by St5 CaPsol strain, prevalent in sympto-
matic grapevines in the examined vineyard. Even if St11 and St19 CaPsol strains were present in individuals used in transmission trials, the insects were able to vector exclusively the St5 CaPsol strain to adult grapevines. Interestingly, St5 CaPsol strain grouped in the bindweed-related stamp phylogenetic cluster b-II, and was exten-
sively reported in vineyard agro-ecosystems in Italy, Austria, Germany, Macedonia, Serbia and Slovenia. It is reasonable to hypothesize that alternative insect vectors, identified in this study, can play a role in the transmission to grapevine of at least St5 CaPsol strain in Europe. Due to their wide geographical distribution, the alterna-
tive insect vectors could be involved in CaPsol spreading also in worldwide. Furthermore, St11 and St19 CaPsol strains, grouping in the nettle-related subclusters a1 and a2, respectively, were found in 37% of the examined grapevines but in only 10% of the analyzed insects. As no insects were able to transmit such phytoplasma strains to grapevine in these experimental conditions, further investigation is necessary.

The potential significance in BN epidemiology of the insect species identified as new CaPsol vectors to grape-
vine is strictly related to their ecology. Considering their abundance, development cycle and adult presence in Northern Italian vineyards, known host plants, and feeding preference towards grapevine and/or common weeds in vineyards (Table 5), it is reasonable to hypothesize that D. hamata, D. europaea, P. spumarius, E. incisus, and E. variegatus could play a role in the transmission of CaPsol to grapevine at least in the examined area. In fact, even some of them are known as oligophagous on Poaceae, in the present study these insects were found feeding on grapevine (Table 4). Moreover, as previously reported, these insects can survive on grapevine for at least two days (Table 5). Regarding P. spumarius, a detailed study on its feeding habit on olive revealed no indication of any puncturing of phloem tissue. Nevertheless, since P. spumarius has been reported as a vector of the elm yellows phytoplasma, in certain conditions and on certain hosts, the insect might divert its behavior from xylem to phloem feeding as some phloem feeding leafhoppers feed occasionally on xylem.

| Species                  | Number of insects | Number of grapevines | Infected (CaPsol strain) |
|--------------------------|-------------------|----------------------|--------------------------|
|                          | Total             | Infected             | Sequenced                | Total     |
| Hydastes oboletus Sign., 1865 | 2015 15 | 8 | 8 | St5 (8) | 2 | 2 (St5) |
|                          | 2016 24 | 22 | 4 | St5 (3); St19 (1) | 2 | 1 (St5) Sympt |
| Dicranotropis hamata (Boh., 1847) | 2015 120 | 5 | 3 | St5 (3) | 3 | 2 (St5) |
|                          | 2016 4 | — | — | — | 1 | 1 |
| Laodelphax striatella (Fall., 1826) | 2015 46 | — | — | — | 2 |
|                          | 2016 16 | 12 | 6 | St5 (4); St11 (1); St19 (1) | 2 | 1 (St5) |
| Dicyophara europea (L., 1767) | 2016 26 | 25 | 5 | St5 (5) | 3 | 1 (St5) |
| Philaenus spumarius (L., 1758) | 2016 20 | 18 | 3 | St5 (3) | 4 | 2 (St5) |
| Aphrodes makarovi Zachv., 1948 | 2016 5 | 4 | 2 | St5 (2) | 2 | 1 (St5) |
| Cicadella viridis (L., 1758) | 2015 16 | — | — | — | 1 |
|                          | 2016 15 | 13 | — | — | 1 |
| Euscelis incisus (Kbm., 1858) | 2015 12 | 4 | 4 | St5 (3); St19 (1) | 3 | 3 (St5) |
|                          | 2016 20 | 18 | 12 | St5 (11); St19 (1) | 3 | 3 (St5) Sympt |
| Euscelidius variegatus (Kbm., 1858) | 2015 10 | — | — | — | 3 |
|                          | 2016 52 | 51 | 12 | St5 (12) | 5 | 2 (St5) |
| Psammotettix alienus/confinis (Dhhlb, 1856) | 2015 100 | — | — | — | 3 |
|                          | 2016 49 | 43 | 16 | St5 (14); St11 (1); St19 (1) | 4 | 2 (St5) |
| No insect (control)        | 2015 — | — | — | — | 3 |
|                          | 2016 — | — | — | — | 5 |

Table 3. Identification of CaPsol insect vectors by transmission trials to grapevine and molecular analyses in 2015 and 2016. Sympt, potted grapevines with typical BN symptoms (yellowing and downwards rolling of leaves and lack of cane lignifications).
Interestingly, the main host plants of *A. makarovi* are important reservoir of CaPsol; thus, also this insect could be involved in the CaPsol spreading to grapevine. Considering the lack of information about host plants and feeding preferences of *L. striatella* and *P. alienus/confinis*, further study are needed to investigate their potential on BN epidemiology.

The novel findings acquired in this study evidenced that the BN epidemiology in vineyard agro-ecosystems is more complex than previously known. In fact, even if tested *H. obsoletus* specimens harboured principally the bindweed-related phytoplasma strains, found prevalent within the vineyard, they were captured by netting exclusively around the borders and in the vineyard neighbourhood. For that reason, it is reasonable to hypothesize that ‘Ca. *P. solani*’ spreading to vineyard borders could be due to *H. obsoletus* and its transmission within the vineyard could be due to the feeding activity of the alternative vectors. Other studies are needed to investigate if the alternative insect vectors could be able to acquire and transmit CaPsol using the infected grapevines as source of inoculum. Furthermore, the polyvoltinism of these alternative insect vectors increases the probability of acquiring and transmitting the phytoplasma during the growing season. Accurate investigation on the ability of CaPsol acquisition related to developmental stage, the latency period, and transmission efficiency of the alternative vectors will be necessary to improve the knowledge of BN epidemiology.

The BN management, based mainly on the weeding of herbaceous plants hosting both the phytoplasma and the vectors, should consider that the eight alternative insect vectors, identified in the present study, live mainly on grasses (Table 5) and not on broad-leaves as *H. obsoletus* and *R. panzeri*.

| Species                          | Number of insects Analyzed | rbcL PCR-positive | Plant in the insect gut | GenBank closest relative plant (Acc. No.) | % Identity |
|----------------------------------|----------------------------|-------------------|-------------------------|------------------------------------------|------------|
| *Hyalesthes obsoletus* Sign., 1865 | 23                        | 1                 | 1 *Vitis vinifera* (MG946878) | 99                                       |            |
| *Dicranotropis hamata* (Boh., 1847) | 8                         | 2                 | 1 *Urtica dioica* (MG946931) | 99                                       |            |
| *Laodelphax striatella* (Fall., 1826) | 10                        | —                 | —                       | —                                        | —          |
| *Dictyophara europaea* (L., 1767) | 10                        | 6                 | 3 *Vitis vinifera* (MG946878) | 99                                       |            |
|                                 |                            |                   | 1 *Crepis elongata* (IQ933285) | 98                                       |            |
|                                 |                            |                   | 1 *Urtica dioica* (MG946931) | 98                                       |            |
|                                 |                            |                   | 1 *Pisum sativum* (MG917089) | 99                                       |            |
| *Philaeus spumarius* (L., 1758)   | 16                        | 11                | 7 *Vitis vinifera* (MG946878) | 99                                       |            |
|                                 |                            |                   | 1 *Vicia cracca* (KP699058) | 98                                       |            |
|                                 |                            |                   | 1 *Potentilla heptancha* (MG742490) | 96                                  |            |
|                                 |                            |                   | 1 *Urtica dioica* (MG946931) | 94                                       |            |
|                                 |                            |                   | 1 *Daucus pumilus* (KX832312) | 98                                       |            |
| *Aphrodes makarovi* Zachv., 1948  | 1                         | 1                 | 1 *Peltophorum pterocarpum* (AM232423) | 95                                  |            |
| *Cicadella viridis* (L., 1758)   | 24                        | 8                 | 5 *Vitis vinifera* (MG946878) | 98                                       |            |
|                                 |                            |                   | 1 *Crepis capillaris* (KM360738) | 98                                       |            |
|                                 |                            |                   | 1 *Laportea interrupta* (KM586531) | 87                                  |            |
|                                 |                            |                   | 1 *Pisum sativum* (MG917089) | 95                                       |            |
| *Euscelis incisus* (Kbm., 1858)   | 22                        | 20                | 10 *Vitis vinifera* (MG946878) | 99                                       |            |
|                                 |                            |                   | 2 *Vicia sp.* (KP699053) | 98                                       |            |
|                                 |                            |                   | 1 *Potentilla sp.* (MG742490) | 96                                       |            |
|                                 |                            |                   | 1 *Crepis sp.* (KF602078) | 93                                       |            |
|                                 |                            |                   | 1 *Pisum sativum* (MG859922) | 95                                       |            |
|                                 |                            |                   | 1 *Corylus sp.* (MF996575) | 99                                       |            |
|                                 |                            |                   | 1 *Xanthoceras sorbifolium* (KP08923) | 81                                  |            |
|                                 |                            |                   | 1 *Ternstroemia gymnanthera* (MFI79490) | 96                                  |            |
|                                 |                            |                   | 1 *Musa coccinea* (MH603431) | 96                                       |            |
|                                 |                            |                   | 1 *Nicotiana sylvestris* (KM025249) | 98                                  |            |
| *Euscelidius variigatus* (Kbm., 1858) | 17                  | 10                | 6 *Vitis vinifera* (MG946878) | 98                                       |            |
|                                 |                            |                   | 2 *Nicotiana sp.* (KU199713) | 98                                       |            |
|                                 |                            |                   | 1 *Berberidopsis carallina* (EU002274) | 96                                  |            |
|                                 |                            |                   | 1 *Quina glaziouii* (JX664069) | 78                                       |            |
| *Psammotettix alienus/confinis* (Dhlb, 1850) | 23 | —                 | —                       | —                                        | —          |

Table 4. Identification of the plants present in the gut of the insect species tested in CaPsol transmission trials by rbcL gene amplification and sequence analysis.
Table 5. Host plants, hosting/vectoring CaPsol, occurrence, and biology of alternative CaPsol vectors.

| Species                     | Host plant               | Survival on grapevine | Host/vector of CaPsol | Abundance, development cycle, adult presence in Northern Italian vineyards |
|-----------------------------|--------------------------|-----------------------|-----------------------|---------------------------------------------------------------------------|
| Dicranotropis hamata        | Poaceae<sup>40,41,52</sup> | not available         | host<sup>51</sup>     | Uncommon – 2 generations/year. Overwintering as nymphal stage. Adult presence: mid-April - end of October<sup>40,41</sup> |
| Lasiodiplax straitella      | Poaceae, Juncacea, Cyperacea<sup>40,41,52</sup> | not available         | host<sup>56</sup>     | Common – 2 generations/year. Overwintering as egg stage. Adult presence: beginning of June - end of November<sup>40,41,52</sup> |
| Dicyophara europaea         | Polyphagous<sup>52</sup> | 2–6 days<sup>53</sup> | host<sup>52,54</sup>  | Common – 1 generation/year. Overwintering as egg stage. Adult presence: end of June - beginning of October<sup>52</sup> |
| Philaeus spumarius          | Polyphagous<sup>40,41,52</sup> | >10 days<sup>55</sup> | host<sup>54</sup>     | Common – 1 generation/year. Overwintering as egg stage. Adult presence: May - beginning of November<sup>40,41,53</sup> |
| Aphrodes mukarovi           | Urtica dioica, Taraxacum, Cirsium<sup>40,45,52</sup> | not available         | host<sup>51</sup>     | Uncommon – 1 generation/year. Overwintering as egg stage. Adult presence: End of May - beginning of November<sup>40,45,52</sup> |
| Euscelis incisus            | Poaceae, Fabaceae<sup>40,41,52</sup> | 3 days<sup>57</sup>   | vector<sup>35</sup>   | Common – 3 generations/year. Overwintering as nymphal stage. Adult presence: February - mid of November<sup>40,41,52</sup> |
| Euscelidius variigatus       | Poaceae<sup>40,41,52</sup> | 3 days<sup>57</sup>   | vector<sup>34</sup>   | Common – 3 generations/year. Overwintering as adult stage. Adult presence: Mid of April - October<sup>40,41,52</sup> |
| Psalmotettix alienus        | Poaceae<sup>40,41,52</sup> | not available         | host<sup>54</sup>     | Common – 2/3 generations/year. Overwintering as egg stage. Adult presence: Mid of June - beginning of October<sup>40,41,52</sup> |
| Psalmotettix confinis       | Poaceae<sup>40,41,52</sup> | not available         | —                     | Common – 2/3 generations/year. Overwintering as egg stage. Adult presence: Mid of June - beginning of October<sup>40,41,52</sup> |

Methods

The survey was conducted in Franciacorta grape-growing area, a gently-rolling hilly zone in Lombardy Region (Northern Italy) bordering Lake Iseo. The Franciacorta is the most important Italian area for the production of sparkling wines with bottle fermentation. The grapes (more than 1,200 ha) are Chardonnay, Pinot noir, Pinot blanc and Erbamat (autoctonous variety).

The BN incidence was investigated since 2012 in 30 vineyards by symptoms observations, molecular CaPsol investigation and spatial distribution analysis of vectors and symptomatic grapevines. In one of these, the population density of *H. obsoletus* was very low within the vineyard and the adults were localized only on nettle along the borders, while few individuals of *R. panzeri* were observed in the nearby forest. The BN incidence in this vineyard was extremely high (around 30% of symptomatic grapevines in 2012 and more than 50% in 2015) with scattered distribution of symptomatic grapevines no correlated with known vector spatial distributions.

To identify alternative insect vectors, molecular investigation on Auchenorrhyncha community and symptomatic grapevines (in 2013 and 2014) and transmission trials (in 2015–2017) were conducted.

Characteristics of target vineyard.

The investigation on insect vectors of CaPsol was conducted in a 10-year old Chardonnay organic vineyard divided into two parts bordering a forest composed of broadleaf species (mainly *Castanea sativa* Mill., *Fraxinus ornus* L., *Quercus pubescens* Willd.) (N 45°35′38.12″, E 10°09′34.32″). In the both parts of the vineyard, the rows were north-south oriented and grapevines, on Kober 5BB rootstock, were trained using the Guyot system (distance between rows 2 m, plant distance along the row 0.8 m, for a total of 3876 vines). Ground cover was typical of Northern Italy with spontaneous grasses and broadleaf species. Among the agricultural practices adopted, spring weeding on the row, mowing between rows and two insecticide treatments with pyrethrum (applied at the end of June) against *Scaphoideus titanus* Ball should be mentioned because they could interfere with vineyard colonization by insects.

Insect and grapevine samples collection.

During 2013 and 2014, insects were monitored and captured every week, from May to September, by yellow sticky traps (placed within, around the borders and in the neighbourhood of the vineyard in a regular grid), sweep entomological net and pooter. The traps (21 cm × 40 cm, SuperColor Giallo<sup>8</sup>, Serbios) were positioned in the canopy of the grapevines on the support wire and on grass other causes of similar symptoms (e.g. partial broken canes, *Stictocephala bisonia* symptoms) was made each year by the same two people. They inspected both sides of the plants in order to exclude vineyards (30 in 2013 and 2014) using the CTAB-based protocols described by Marzachi *et al*.<sup>30</sup>

Leaf petioles of symptomatic grapevine plants were also collected and stored at ~30 °C. Observation of BN symptoms was made each year by the same two people. They inspected both sides of the plants in order to exclude other causes of similar symptoms (e.g. partial broken canes, *Stictocephala bisonia* Kopp and Yonke activity).

Molecular identification and characterization of *Ca. P. solani* in insects and grapevines.

Total nucleic acids were extracted from 1150 insect pools (621 in 2013 and 529 in 2014) and leaf petioles of 60 grapevines (30 in 2013 and 2014) using the CTAB-based protocols described by Marzachi *et al.*,<sup>44</sup> and Angeli *et al.*,<sup>45</sup> respectively.

Specific detection of CaPsol was conducted by direct PCR using StampF/StampR0 primer pair followed by nested PCR with the StampF1/StampR1 primer pair, using mixtures and PCR conditions as described by Fabre *et al.*,<sup>29</sup> Total nucleic acids from periwinkle (*Catharanthus roseus* L. G. (Don)) plants infected by phytoplasma strains EY1 ("Ca. P. ulmi"), STOL (*Ca. P. solani"), and AY1 ("Ca. P. asteris") were used as reference controls. Total
nucleic acids from healthy periwinkle and PCR mixture devoid of nucleic acids were used as negative controls. The presence of the nested PCR products was verified through electrophoresis on 1% agarose gel and visualized under UV transilluminator.

Fifty-eight and 54 stamp PCR products (StampF1/StampR1), amplified from insect specimens and grapevine samples, respectively, were sequenced in both strands (Sanger method, 5X coverage per base position) by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences were assembled by the Contig Assembling Program and trimmed to the annealing sites of the nested PCR primer pair in the software BioEdit, version 7.2.6.46.

Nucleotide sequences of stamp gene, amplified from the CaPsol strains detected in the examined grapevine samples and insect specimens, were aligned using the ClustalW Multiple Alignment program in the software BioEdit and analysed by Sequence Identity Matrix to estimate their genetic diversity. Stamp sequence variants, identified in the study, were aligned and compared with representative sequences of previously defined sequence variants35; a nucleotide sequence identity of 100% was necessary for the attribution to such sequence variants.

‘Ca. P. solani’ phylogenetic analysis. Stamp gene nucleotide sequences of CaPsol representative strains of GuSt (stamp) sequence variants, identified in this and in previous studies35, were aligned and used for generating unrooted phylogenetic trees by Neighbor-Joining method performed using the Jukes-Cantor model and bootstrap replicated 1000 times in the MEGA6 software47. All positions with less than 95.0% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Transmission trials. Based on the results obtained from Auchenorrhyncha community description and molecular analysis of CaPsol strains, insects to be tested in transmission trials for their CaPsol vectoring activity to grapevine were selected using the following three criteria simultaneously satisfied in at least 2013 and/or 2014: (i) high abundance (>25 individuals collected in the vineyard over the vegetative season); (ii) high infection rate (>10% of CaPsol-infected insect pools); (iii) the harbouring of CaPsol strain characterized by stamp sequence variant found also in grapevine.

A total of 550 individuals of the 10 selected insect species were captured in the examined vineyard on three sampling days in 2015 (June 11 and 25; July 7) and five days in 2016 (June 28; July 6 and 21; August 3 and 28). The specimens of each insect species were captured, on each sampling date, using sweep net and pooter on the canopy of symptomatic grapevines and on the grassing near them. The captured insects were kept in jars for transport to the laboratory for their classification. Collected insects were kept alive and in the conditions required for survival and good fitness maintenance. After the classification, the insects were forced to feed on symptomatic grapevines and plants of grapevines on the grassing near them. The captured insects were kept in jars for transport to the laboratory for their classification. Collected insects were kept alive and in the conditions required for survival and good fitness maintenance. After the classification, the insects were forced to feed on asymptomatic and PCR-tested phytoplasma-free potted grapevines (cv. Chardonnay) previously treated with hot water. Forty-four transmission trials were conducted in a greenhouse under controlled conditions [25 ± 3°C, 70 ± 5 RH 16:8 (L:D) daily light cycle], located in Verona province (45°20′N, 11°13′03.28″E), and left till the end of adult survival. After this period, the plants were kept in an insect-free greenhouse in both years. On each date, one phytoplasma-free grapevine plant was maintained without insects as control. Dead insects were stored at −30°C. CaPsol was detected by nested PCR-based amplification of stamp gene35 and by SYBR Green real-time amplification assay35 using as templates the total nucleic acids extracted from both the individual insect specimens and the petioles of grapevine plants collected in October 2015 and July 2016 for the trials performed in 2015, and in October 2016 and July 2017 for the trials performed in 2016. In real-time amplification assay, only amplified PCR products showing a Tm of 81.5 ± 0.2°C and a Ct < 37 were associated with the presence of CaPsol, as previously described35. CaPsol strains detected in insects and plants were characterized by nucleotide sequence analyses of stamp amplicons as described above.

Plants eaten by the insects. In order to identify the plant species on which the insects have had their last meal, a molecular characterization of the gut contents has been performed for 154 insect specimens captured in the examined vineyard in 2016 simultaneously to those captured for transmission trials. A fragment (750 bp) of rbcL gene, coding for the plant plastid ribulose-bisphosphate carboxylase large subunit, was amplified by PCR using primers rbcL1F/rbcL724R as previously described30. Nucleotide sequences of rbcL gene, amplified from insect specimens, were sequenced in both strands (Sanger method, 5X coverage per base position) by a commercial service (Eurofins Genomics). Nucleotide sequences were assembled by the Contig Assembling Program, trimmed to the annealing sites of primer pair in the software BioEdit, compared to GeneBank through BLAST analysis (http://www.ncbi.nlm.nih.gov/blast).

Data availability
All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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F.Q. N.M. and P.A.B. conceived and designed the entire research. F.S., M.F. and N.M. conducted the open field survey; F.Q., A.M., A.P. and P.C. performed molecular biology analyses. F.Q., F.S., A.P. and N.M. analyzed data. F.Q. N.M. and P.A.B. conceived and designed the entire research. F.S., M.F. and N.M. conducted the open field survey; F.Q., A.M., A.P. and P.C. performed molecular biology analyses. F.Q., F.S., A.P. and N.M. analyzed data. F.Q. and N.M. wrote the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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