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**Host plant identification in the generalist xylem feeder *Philaenus spumarius* through gut content analysis**

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(Article begins on next page)

1 **Host plant identification in the generalist xylem feeder *Philaenus spumarius***  
2 **through gut content analysis**

3

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11

12 **Running Title**

13 Gut content analysis of *Philaenus spumarius*

14

15 **Keywords**

16 Meadow spittlebug, vector, polyphagy, adults, chloroplast DNA, *Xylella fastidiosa*

17

## 18 **Abstract**

19 The meadow spittlebug *Philaenus spumarius* (Hemiptera: Aphrophoridae) is the main vector of the  
20 phytopathogenic bacterium *Xylella fastidiosa* in Europe, where the ST53 strain induces the olive  
21 quick decline syndrome causing the most severe economic damage in southern Italy. The wide  
22 range of plant species infected by *X. fastidiosa*, and the wide **host range** of *P. spumarius* suggest  
23 that a huge number of wild and cultivated plants may become infected by the pathogen following  
24 unintentional introduction events. Therefore, it is necessary to detail the host plant preference of the  
25 vector, in order to include preferred in-field plants in pathogen-targeted diagnostic efforts. This will  
26 allow the identification of main sources for *X. fastidiosa* acquisition by *P. spumarius*; such plant  
27 species represent an important target for rational disease management. Here we investigated the  
28 host plants of *P. spumarius* in north-western Italy, a region where *X. fastidiosa* is still not present  
29 but is regarded as a primary threat. We designed a new molecular diagnostic tool targeting  
30 chloroplast DNA, to characterize the gut content of single *P. spumarius* adults. The newly set up  
31 nested PCR/sequencing-based identification protocol was proven to be useful for retrieving  
32 sequences from the two last different host-plant used by *P. spumarius*, even if a limited persistence  
33 of intact chloroplast DNA was reported in the spittlebug gut. We propose this protocol as a new tool  
34 for supporting research on xylem feeders biology that could be particularly useful for highly  
35 polyphagous species like *P. spumarius*. **Furthermore, the method could** assist monitoring of *X.*  
36 *fastidiosa* invasion, by contributing to the study of vector ecology and **pathogen epidemics**.

37

## 38 **Introduction**

39 The plant pathogen *Xylella fastidiosa* is a Gram negative bacterium belonging to  
40 Gammaproteobacteria (Chatterjee et al., 2008). It resides in xylem vessels of infected plants,  
41 inducing serious symptoms related to the occlusion of xylem vessels due to bacterial colonization  
42 and plant response (Sicard et al., 2018). Three distinct subspecies have been recognized based on  
43 genomic identity, namely *fastidiosa*, *multiplex* and *pauca* (Marcelletti & Scortichini, 2016). The  
44 subspecies *fastidiosa* causes one of the most troubling diseases, Pierce's Disease (PD) of grapevine,  
45 as well as leaf scorch diseases to coffee and oleander, whereas the subspecies *multiplex* is related to  
46 almond leaf scorch (Baldi & La Porta, 2017). *X. fastidiosa* ssp. *pauca* strains are the agents of citrus  
47 variegated chlorosis, a severe disease widespread in South American citrus producing areas  
48 (Cordeiro et al., 2014), and of olive quick decline syndrome, which rapidly devastated olive  
49 production in Apulia region of Italy, demonstrating the huge damaging potential of this pathogen in  
50 Europe (Strona et al., 2017). However, *X. fastidiosa* is widely generalist: its host range encompasses  
51 more than 560 plant species, with a rapid increase of reports on new infected plants following its  
52 spread in Europe from 2013 (EFSA, 2018). Its capability to infect a large number of plants is  
53 furtherly exacerbated by its transmission. Indeed, *X. fastidiosa* is **transmitted** by xylem feeding  
54 hemipterans, within the infraorder Cicadomorpha, belonging to the families Cicadellidae (subfamily  
55 Cicadellinae), Cercopidae, Cicadidae and Aphrophoridae (Redak et al., 2004). Low specificity has  
56 been reported for the interaction between *X. fastidiosa* strains and their vector species, and  
57 potentially all xylem feeders can be vectors (Almeida et al., 2005). Moreover, many vectors are  
58 highly polyphagous and widely distributed, being capable to easily move among habitats (Redak et  
59 al. 2004; Cornara et al., 2018a; Krugner et al., 2019), further increasing the dissemination potential  
60 of *X. fastidiosa*.

61 Since the first detection of *X. fastidiosa* in olive trees in Italy in 2013, relevant resources have been  
62 employed to study the spread of this pathogen and the consequent disease outbreaks in Europe and

63 in the Mediterranean basin, where *X. fastidiosa* is considered one of the most dangerous agricultural  
64 threats. Hence, the intensification of studies regarding *X. fastidiosa*-related epidemics is required for  
65 designing effective control strategies (Sicard et al., 2018). The main vector of *X. fastidiosa* in Italy  
66 is *Philaenus spumarius* L. (Hemiptera: Aphrophoridae), which is a cosmopolitan and highly  
67 polyphagous species, having the potential to widely expand the pathogen distribution in Europe  
68 (Cornara et al., 2018a). Since *P. spumarius* can feed on a broad range of monocotyledons and  
69 dicotyledons (Dongiovanni et al., 2019), and the plant composition at the landscape scale is  
70 predicted to affect its spatial distribution (Santoemma et al., 2019), understanding the feeding  
71 preference of vectors is a crucial issue for the management of *X. fastidiosa*.

72 The molecular analysis of gut content has been widely applied to delineate cryptic trophic  
73 behaviour of insects (Pompanon et al., 2012); PCR-based techniques have been commonly used to  
74 assess predator-prey and parasitoid-host relations (Sheppard & Harwood, 2005; Garipey et al.,  
75 2007). In herbivorous insects, the molecular analysis of gut content has been proposed as a method  
76 for elucidating multiple plant use by single individuals (Hereward & Walter, 2012). Most of the  
77 work has been focused on chewing species (Matheson et al., 2008; Jurado-Rivera et al., 2009;  
78 Pumariño et al., 2011; Avanesyan, 2014; De la Cadena et al., 2017), as a large amount of  
79 chloroplast DNA – the target for molecular analyses – can be retrieved from their gut. Conversely,  
80 analysing the gut content of sap-feeding insects may be considerably challenging, considering the  
81 low DNA load of plant sap. However, recently Rodney Cooper et al. (2016) successfully detected  
82 plant DNA from the phloem feeder *Bactericera cockerelli* (Sulc) (Hemiptera: Triozidae). It has  
83 been suggested that plant DNA may be ingested by sap feeders following stylet contamination  
84 during penetration into the parenchyma cells before reaching vascular tissues (Pearson et al., 2014;  
85 Rodney Cooper et al., 2016). Hence, not only phloem feeders but also xylem feeders may be  
86 exposed to plant DNA during probing of plant tissue, since during this phase the stylets cross  
87 periderm and parenchyma cells (Miranda et al., 2009; Cornara et al., 2018b). However, at present

88 the gut content of xylem feeding insects has never been molecularly investigated to search for plant  
89 DNA.

90 In this study, we tracked the presence of plant-related DNA in the gut of *P. spumarius*, by  
91 developing a specific protocol for xylem feeders, to identify host plants used by adult individuals of  
92 *P. spumarius*. We evaluated the persistence of target chloroplast DNA in the digestive tract of the  
93 spittlebug over a period of three days in the absence of plant substrate or after a plant change. The  
94 aim of this work was to assess the time range allowing molecular detection of plant-related DNA in  
95 the gut content of a polyphagous xylem feeder. **Such a molecular tool** could provide a support to  
96 classical bioassays in improving the knowledge on its plant host range, to investigate its seasonal  
97 movement based on food sources availability. Moreover, tracking the diet of single spittlebug  
98 individuals could be applied to all the other vector species recognized in Europe – which are close  
99 relatives of *P. spumarius* (Cavaliere et al., 2019) – assisting *X. fastidiosa* monitoring efforts in  
100 vectors. Indeed, the identification of the last host plants used by infected specimens will allow  
101 recognizing possible infection reservoirs, since no latency is needed for *X. fastidiosa* transmission by  
102 *P. spumarius* (Cornara et al., 2016).

103

## 104 **Materials and methods**

### 105 **Insects**

106 Adult *P. spumarius* individuals were collected by means of an entomological sweep net in July  
107 2018 in wild areas in north-western Italy (Piedmont and Lombardy regions) (Table 1), where both  
108 annual and perennial herbs were present, as well as tree species, all potentially hosting the  
109 spittlebug. Parts from all plants where insects had been captured were collected for identification.  
110 Insects were maintained at the DISAFA laboratories for at least 7-10 days in two distinct lab  
111 rearings in mesh cages (580 × 580 × 600 mm) containing potted *Digitaria ciliaris* (Retz.) Koeler or

112 *Medicago sativa* L. plants, under outdoor conditions in a sheltered place. Weeds other than *D.*  
113 *ciliaris* or *M. sativa* emerging from the pot soil were manually removed daily. Furthermore, other  
114 adult specimens (6 males and 4 females) were picked immediately after field collection and  
115 preserved at -20°C for molecular analyses (named group 5 in Figure 1).

### 116 **Rearing trials on different feeding substrates**

117 *P. spumarius* from the lab rearings were divided in four subgroups differing for the food source  
118 (Figure 1). Insects in groups 1-3 were taken from the rearing on *D. ciliaris*, while those in group 4  
119 were obtained from the rearing on *M. sativa*. Spittlebugs dedicated to group 1 were kept on *D.*  
120 *ciliaris* plants and then collected for molecular analyses, whereas adults in groups 2 and 3 were  
121 moved to a new feeding substrate, consisting of an artificial diet or a *M. sativa* seedling,  
122 respectively, for three days. The artificial diet was prepared with 0.7 mM L-glutamine, 0.1 mM L-  
123 asparagine, and 1 mM sodium citrate, pH 6.4, according to Killiny & Almeida (2009), and was  
124 supplied by using an artificial feeding system as described by Gonella et al. (2015). Insect mortality,  
125 integrity of the membrane containing the diet, and moisture in the feeding systems were checked  
126 daily for insects maintained on the artificial diet; only live specimens were kept for molecular  
127 analysis. *P. spumarius* individuals dedicated to group 4 were moved from the rearing on *M. sativa*  
128 to a *D. ciliaris* seedling for 3 days. At the end of the experiments, 10 live insects were collected  
129 from each group (namely, 5 males and 5 females for group 1; 6 males and 4 females for group 2; 7  
130 males and 3 females from group 3; 4 males and 6 females for group 4); spittlebug whole bodies  
131 were preserved at -20°C for molecular analyses.

### 132 **DNA extraction, PCR, and sequencing**

133 A molecular diagnostic protocol was set up to assess whether the visual host plant identification  
134 could be confirmed, and if the genetic material of possible previous host plants could be retained in  
135 and detectable from the gut of *P. spumarius*. Total DNA was extracted from the whole body of *P.*  
136 *spumarius* samples by using the QIAamp® PowerFecal® DNA Kit (Qiagen, Italy), according to the

137 manufacturer's instruction, with the following modification: insect tissues were lysed and  
138 homogenized with a sterile pestle in an Eppendorf tube with 750 µl of PowerBead Solution, then  
139 the homogenate was transferred in the Bead tube to proceed with the protocol indicated by the  
140 supplier. Subsequently, the DNA was submitted to nested PCR targeting the chloroplast region  
141 between the *trnL* and *trnF* genes, by using primer pairs partially modified from Taberlet et al.  
142 (1991; 2007), optimized to cover a wider range of plants from mixed DNA sources. Specifically,  
143 direct PCR was performed with the modified forward primer Fc1 (5'-  
144 CGRAATYGGTAGACGCTACG-3') coupled with the reverse primer Rf (Taberlet et al., 1991),  
145 targeting the *trnL-trnF* region. Products of direct PCR reactions were submitted to 1:40 dilution  
146 with sterile water and amplified in nested PCR with the modified forward primer Fg1 (5'-  
147 GGGYRHTCCTGRKCCAA-3') and the reverse primer Rd (Taberlet et al. 1991), targeting the *trnL*  
148 intron. All reactions were performed 25 µl reaction mixture containing 1 × PCR buffer (Solis  
149 Biodyne, Estonia), 0.12 mM of each dNTP, 0.3 mM of each primer, 1 U of HOT FIREPol® Taq  
150 polymerase (Solis Biodyne) and 2 µl of DNA template. The cycling conditions were as follows (for  
151 both direct and nested PCRs): 95°C for 15 min; 35 cycles of 95°C for 30 s, 48°C for 45 s and 72°C  
152 for 1 min; and 7 min at 72°C. Amplicons from the nested PCRs were sequenced at Eurofin  
153 Genomics S.r.l [Via Bruno Buozzi, 2, 20090 Vimodrone (Milano), Italy]; sequences were subjected  
154 to Nucleotide BLAST analysis against nr database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). For  
155 some, **When** direct sequencing did not allow to obtain a single clean sequence (**in samples**  
156 **belonging to groups 2-4**), amplicons were cloned using a pGEM® T-easy Vector Cloning Kit  
157 (Promega, Italy), and five clones from each amplicon were sequenced. Moreover, PCR products  
158 obtained from field-collected insects (group 5 samples) were immediately cloned as described  
159 above, and 10 clones from each amplicon were sequenced.

160

## 161 **Results**

162 A total of 144 *P. spumarius* adults were captured in the field both on herbs, shrubs and trees. The  
163 plants belonged to 18 species in 11 different families: Aceraceae (one species), Asteraceae (four  
164 species), Betulaceae (two species), Convolvulaceae (one species), Equisetaceae (one species),  
165 Fabaceae (three species), Fagaceae (one species), Poaceae (one species), Rosaceae: (two species),  
166 Pinaceae (one species), and Vitaceae: (one species) (Table 2).

167 After insect collection and rearing on different sources, we tested DNA samples by chloroplast-  
168 targeted nested PCR. After amplification of all of the 10 samples from spittlebugs fed for at least  
169 one week on *D. ciliaris* (group 1), a 450 bp-long amplicon was obtained (Figure 2); all sequences  
170 obtained from these PCR products were clean and referable to *D. ciliaris* (Table 3). These results  
171 indicated that a sufficient amount of amplifiable and clean DNA from plant chloroplast was  
172 achievable from the digestive tract of a xylem feeder such as *P. spumarius*. Furthermore, a  
173 minimum continuous exposure to a single plant of seven days was demonstrated to be sufficient to  
174 avoid the presence of “contaminant” chloroplast DNA from plants possibly consumed by the  
175 specimens before collection, allowing the experiment continuation.

176 As a second step we evaluated how long viable chloroplast DNA could persist in the gut of *P.*  
177 *spumarius*, by using an artificial feeding system containing a chloroplast-free liquid diet. High  
178 mortality was recorded in adults fed with the artificial diet: a total number of 97 specimens were  
179 individually used to obtain 10 surviving for three days, with a 89.7% mortality rate. After DNA  
180 extraction from the 10 *P. spumarius* specimens found alive after being maintained for three days on  
181 artificial diet, PCR reactions on these samples produced amplicons ranging from 200 to 300 bp.  
182 However, none of the resulting sequences were directly readable, and even after cloning only 100-  
183 150 bp-long sequences were obtained, unrelated to the target chloroplast *trnL* gene and hence  
184 referable to artefacts.

185 Additional trials were carried out to establish the influence of a host plant shift on the results of  
186 molecular plant identification. After PCR analysis on DNA samples from both group 3 and group 4

187 *P. spumarius* individuals (spittlebugs transferred from *D. ciliaris* to *M. sativa*, or from *M. sativa* to  
188 *D. ciliaris*, respectively, Figure 1), amplicons ranging from 350 to 450 bp were always obtained  
189 (Figure 2). Considering group 3 samples, 6 out of 10 sequenced amplicons were still clearly  
190 referable to *D. ciliaris*, whereas the remaining four PCR products (40%) were cloned. All of the  
191 Operational Taxonomic Units (OTUs) from five sequenced clones for each amplicon were related  
192 to *M. sativa* (Table 3). Conversely, 8 out of 10 sequences from group 4 samples were directly  
193 readable; four of them were OTUs related to *D. ciliaris* and four to *M. sativa*, while the remaining  
194 two amplicons (20%) were cloned. In both cases all of the five sequenced clones were referable to a  
195 single OTU; one of these sequences had as the closest relative *D. ciliaris* and the other *M. sativa*.  
196 As a final result, half of the 10 samples from group 4 were related to *D. ciliaris* and half to *M.*  
197 *sativa* (Table 3).

198 To assess the suitability of this protocol for the collected *P. spumarius* populations, we finally  
199 tested 10 randomly selected adults directly preserved after field collection (referred to as group 5).  
200 The target chloroplast gene was successfully amplified from all of the 10 samples coming from this  
201 group, producing amplicons ranging from 200 to 500 bp. These products were cloned and 10 clones  
202 for each sample sequenced; the results of sequencing are indicated in Table 4. Overall, a total of 11  
203 single plant species were identified through this method; all of them belonged to the host range  
204 recorded during the field survey (Table 2). Six of the identified plant species were woody plants and  
205 five were herbs. In eight samples, all of obtained OTUs were related to a single plant species, even  
206 though in one case two distinct OTUs affiliated to the same species were found. In the remaining  
207 two samples, OTUs related to two different species were retrieved; either belonging to tree or  
208 herbaceous plants. In one sample a total of three OTUs were obtained; however, two of them were  
209 referable to a single plant species. No amplicon containing OTUs affiliated to more than two plant  
210 species was found.

211

## 212 Discussion

213 The meadow spittlebug *P. spumarius* is known to be a highly polyphagous species being able to  
214 feed on monocotyledonous and dicotyledonous plants either as nymph or adult (Ossiannilsson,  
215 1981, Cornara et al., 2017, Cornara et al., 2018a; Di Serio et al., 2019). The identification of the  
216 proper host plants is simplified by the stationary behaviour of the nymph, as they produce and  
217 reside in a protective froth. On the contrary, the simple collection of adults on a plant species does  
218 not necessarily indicate that they had actively used that plant, due to the high mobility of the stage;  
219 for this reason, alternative methods to identify the actual host plants of single adults are required.  
220 The molecular analysis of plant-related DNA in the digestive tract of *P. spumarius* to identify its  
221 gut content confirmed previous results supporting the use of this technique on insects that feed on  
222 saps (Rodney Cooper et al., 2016). Specifically, we provided the first nested PCR-based  
223 identification of the gut content in xylem feeders. Insects with this feeding behaviour are thought to  
224 retain a very low plant-related DNA load. Hence, a major challenge is to successfully amplify  
225 potential mixed chloroplast DNA deriving from multiple host plants used by insects with such a  
226 little plant DNA concentration in their gut. Indeed, even though our newly designed primers were  
227 conceived to be universal, they may display different affinity levels with specific taxa (Bista et al.,  
228 2018; Piñol et al., 2019) resulting in biased amplification of single plant OTUs from an insect **that**  
229 had actually fed on many hosts. Up to three distinct OTUs were found in a single field-collected  
230 specimen, corresponding to two plant species always belonging to the observed host range,  
231 confirming that multiple hosts can be detected with this method. The relatively low number of  
232 distinct host plants detected by this method may be related to i) limited number of ingested/probed  
233 plants, or ii) differential DNA degradation based on inversely proportional time length of the  
234 feeding period on different hosts. The real number of different plant species used by an adult  
235 individual of *P. spumarius* during its life cycle is not known; however, studies on its feeding  
236 preference showed that adults may switch from an host to another in a few hours, and they may

237 perform several feeding events in a short time (Markheiser et al., 2020). These reports, along with  
238 the high mobility of *P. spumarius* adults, suggest that more than two plants have been used by  
239 collected adults before being sampled. On the other hand, the DNA of plants used for a limited time  
240 may have been degraded during the digestion process. Molecular analysis of chloroplast DNA from  
241 the gut of herbivorous insects feeding on seeds or roots allowed detection of the provided food  
242 source after three days of digestion (Wallinger et al., 2013; 2015); however, no indication on the  
243 real persistence of plant DNA from multiple hosts in the gut of xylem feeders is presently available.  
244 Moreover, plant identity was reported to affect post-feeding DNA detection success (Wallinger et  
245 al., 2013), suggesting that the different host plants may undergo a different fate once inside the  
246 insect gut.

247 We investigated the effect of a host plant switch on the newly designed molecular method. The  
248 retrieval of OTUs related to both plants sequentially provided to *P. spumarius* in experiments 3 and  
249 4 (nearly in a 1:1 ratio) supports to the absence – or the limited presence – of PCR-related bias  
250 produced by using the proposed primer pairs, at least considering the two plant species in the  
251 families Poaceae and Fabaceae. However, we must take into account that the possible differential  
252 detection of the two hosts may be related to different amounts of ingested chloroplast DNA and/or  
253 different responses to the insect digestion, consistently with the results obtained from field-collected  
254 adults. Accordingly, in some samples the detected plant did not correspond to the last plant species  
255 being supplied, suggesting that the range of data being potentially achieved by using this protocol is  
256 not restricted to the last plant consumed by the spittlebug, but it may depend on the amount of DNA  
257 ingested by each single tested adult from the two host plants, or to the DNA quality inside the insect  
258 gut at the time of collection. The retrieval of chloroplast DNA from an earlier provided plant after  
259 host switch is consistent with previous results reported for psyllids, where the DNA of the first host  
260 plant was found up to one week after insects were moved to a different species (Rodney Cooper et  
261 al., 2016). However, in these experiments we never found OTUs related to both host plants in the

262 same individual sample, even when cloning was required, despite the insects had actually  
263 consecutively fed on the two species. Even though we cannot rule out the possibility that  
264 sequencing a higher number of clones from each sample would allow obtaining sequences from  
265 both the plant species, this result indicates that single PCR reactions may support the preferential  
266 amplification of an individual target sequence, and this must be taken into account for data  
267 interpretation.

268 Another unknown aspect, when analysing field-collected specimens, is the time lapse from the last  
269 feeding event to collection; experiment 2 was set up exposing insects to an artificial diet to establish  
270 the persistence of plant DNA in the gut of *P. spumarius*. However, very low survival rates were  
271 observed for insects used in this experiment. Previous evidences have been provided on the marked  
272 recalcitrance of *P. spumarius* to accept artificial diets as food sources (Cornara et al., 2019). It is  
273 likely that the low percentage of adults capable to survive for three days had accidentally ingested  
274 the diet after piercing the membrane by chance. Molecular analyses performed on these insects did  
275 not result in successful amplification of the chloroplast DNA of the plant used by *P. spumarius*,  
276 most probably because of partial degradation of the little amount of plant DNA, which was still  
277 present after three days in which insects were maintained on the artificial feeding systems. It must  
278 be pointed out that such a degradation was only moderately visible in the experiments involving a  
279 switch of host plant (groups 3-4); therefore our results may be either suggestive of i) a partial  
280 interference of the diet components with chloroplast DNA integrity in the insect gut, or ii) marked  
281 tissue alteration in the digestive tract of almost starving individuals. In the light of these results, we  
282 can conclude that stable chloroplast DNA can be retrieved in the gut of *P. spumarius* only after very  
283 short time from the end of feeding.

284 Determining the host plants of *P. spumarius* is a key step parallel to *X. fastidiosa* monitoring  
285 actions, necessary in areas where the pathogen has not been recorded yet for facing its rapid spread  
286 in Europe. The molecular tool described here can support field observations, especially in

287 uninfected areas, where the knowledge of the main food sources of single adults could be very  
288 useful to immediately drive the control measures in case of pathogen detection in a plant (either  
289 wild or cultivated) in that area, even before finding infected vectors. In case of detection of  
290 individuals infected by the pathogen, it will be still possible to achieve information on the plants  
291 that have been used by these infected specimens, allowing their rapid eradication. Extensive  
292 monitoring of *X. fastidiosa* infection sources is very important in north-western Italy even though  
293 the pathogen has not been recorded in this region yet, since it includes several areas classed as  
294 climatically suitable for the pathogen (EFSA, 2019). Notably, *P. spumarius* was reported as  
295 abundant (almost reaching the density of 2 adults per plant) on olive trees in north-western Italy all  
296 throughout the olive growing season, suggesting a high risk of pathogen outbreaks in case of *X.*  
297 *fastidiosa* invasion (Bodino et al., 2019; 2020). Moreover, in our field collections, we commonly  
298 found *P. spumarius* adults on grapevine in grapevine growing sites during summer, which might be  
299 a serious issue in case of presence of Pierce's disease, which **has** been recently recorded in Europe,  
300 since the spittlebug **has** been demonstrated to transmit the strain being the causal agent to grapevine  
301 (Moralejo et al., 2019). However, we must point out that it is impossible to cover the full host range  
302 of an individual during its entire life, because the only chloroplast DNA that can be retrieved  
303 belongs to a limited number of host plants used by *P. spumarius* in the previous few days. Hence,  
304 considering that *X. fastidiosa* is persistent in in the foregut of its vectors (Chatterjee et al., 2008), if  
305 a specimen had been infected long before collection, some plant species representing a source for  
306 pathogen acquisition may remain unidentified. For this reason, this method could be more  
307 effectively applied in early phases of adult appearance and soon after the first detection of *X.*  
308 *fastidiosa* infection in a specific area, when the pathogen can be presumed to have been recently  
309 acquired by putatively infected insects.

310 Additional application of the proposed technique may be found in investigating the ecology of *P.*  
311 *spumarius*. The molecular identification of the host plant(s) used by single adults can be used to

312 establish the seasonal behaviour of this polyphagous insect, which has been earlier observed to  
313 move from herbaceous hosts to bushes and trees over the summer (Cornara et al., 2017). Our results  
314 showed that about half of field-collected specimens contained DNA from herbaceous species, while  
315 in the other half DNA from tree species was found, suggesting a transition among different host  
316 types, in agreement with our field samplings and with the collection period. Indeed, adults are most  
317 often found on arboreous species in mid-late summer, probably because of a typical grassland  
318 reduction during the warmer season. **In contrast**, herbaceous host plants are most frequently  
319 reported in early summer and autumn, **as females lay eggs** mainly on shrivelled grass near the soil,  
320 **since** the end of August **and until late season** (Halkka et al., 1967; Bodino et al., 2019). However, it  
321 is worth of remark that our survey was conducted in north-western Italy, which falls in the Cfa  
322 climate type according to the Köppen-Geiger classification (Peel et al., 2007), **displaying a**  
323 **tempered and humid climate with hot summer. Differently**, southern **and** coastal northern Italy,  
324 where the behaviour of *P. spumarius* was **previously** recorded (Bodino et al., 2019; 2020), **both** fall  
325 in the Csa type, with hot and dry summer (Peel et al., 2007). The climatic conditions of north-  
326 western Italy prevent the complete desiccation of the turf, allowing the meadow spittlebug adults to  
327 exploit this source also during summer, being less forced to migrate towards the woodland. A deep  
328 screening of *P. spumarius* populations occurring in distinct areas may further clarify this  
329 behavioural trait, to verify to what extent it is affected by differential environmental conditions  
330 determining plant species composition, and consequently food and shelter availability.

331 In conclusion, in this work we provided new insights on the identification of host plants of *P.*  
332 *spumarius* adults, contributing to elucidate its **feeding behaviour** in north-western Italy, where it  
333 may become a serious threat in case of introduction of *X. fastidiosa*, as this region includes many  
334 olive- and grapevine-growing areas. Furthermore, we demonstrated for the first time that the  
335 feeding behaviour of a xylem feeding insect such as *P. spumarius* sustains the amplification of plant

336 DNA. Our results provide a useful tool for better understanding the spread of *X. fastidiosa* in just  
337 invaded areas, contributing to the study of vector ecology and disease epidemiology.

338

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344

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464

465 **Table 1** Sampling sites of field collected adult *P. spumarius* used in this study.

<b>Municipality</b>	<b>Region, Province</b>	<b>Gps coordinates WGS84 (EPSG:4326)</b>
Caselle	Piedmont, Turin	45°05'50.3"N 7°28'14.6"E
Chieri	Piedmont, Turin	45°01'12.0"N 7°47'13.1"E
Grugliasco	Piedmont, Turin	45°04'01.4"N 7°35'29.4"E
Lonate Pozzolo	Lombardy, Milan	45°35'28.2"N 8°42'40.0"E
Morimondo	Lombardy, Milan	45°20'57.2"N 8°55'37.1"E

466

467

468 **Table 2** Number of adult *P. spumarius* collected in this study on different host plants.

Plant species	No. collected specimens
<b>Herbaceous host plant</b>	
<i>Achillea millefolium</i> L.	7
<i>Arrhenatherum elatius</i> (L.) P. Beauv. ex J. Presl & C.	4
<i>Cirsium arvense</i> (L.) Scopoli	11
<i>Convolvulus arvensis</i> L.	2
<i>Equisetum arvense</i> L.	1
<i>Medicago sativa</i> L.	15
<i>Rubus ulmifolius</i> Schott	1
<i>Solidago gigantea</i> Aiton	3
<i>Taraxacum officinale</i> Weber ex F.H. Wigg	10
<i>Trifolium pratense</i> L.	5
Total herbaceous hosts	<b>59</b>
<b>Woody host plant</b>	
<i>Acer campestre</i> L.	17
<i>Carpinus betulus</i> L.	5
<i>Corylus avellana</i> L.	9
<i>Picea pungens</i> Engelman	18
<i>Prunus avium</i> L.	16
<i>Quercus rubra</i> L.	3
<i>Robinia pseudoacacia</i> L.	1
<i>Vitis vinifera</i> L.	16
Total woody hosts	<b>85</b>
<b>Total number</b>	<b>144</b>

469

470

471 **Table 3** Results of sequencing of chloroplast *trnL* gene amplicons from *P. spumarius* adults reared  
 472 on different food substrates, according to insect groups described in Figure 1. The number of plant-  
 473 related OTUs is indicated for each insect sample, while for each OTU the ratio between the number  
 474 of sequences and the total number of tested insect for each group is reported.

475

Food source (Insect group)	No. retrieved plant OTUs	OTU proportion	Closest relative (NCBI Accession Number)
<i>D. ciliaris</i> (1)	1	10/10	<i>Digitaria ciliaris</i> (LC118761)
<i>D. ciliaris</i> to artificial diet (2)	0	0/12	-
<i>D. ciliaris</i> to <i>M. sativa</i> (3)	2	6/10	<i>Digitaria ciliaris</i> (LC118761)
		4/10	<i>Medicago sativa</i> (KP174818)
<i>M. sativa</i> to <i>D. ciliaris</i> (4)	2	5/10	<i>Digitaria ciliaris</i> (LC118761)
		5/10	<i>Medicago sativa</i> (KP174818)

476

477

478 **Table 4** Results of sequence analysis obtained after cloning the chloroplast *trnL* gene amplified  
 479 from field-collected *P. spumarius* adults (indicated as insect group 5 in Figure 1). The number of  
 480 plant-related OTUs is indicated for each insect sample, while for each OTU the ratio between the  
 481 number of sequences and the total sequenced clones for each insect sample (OTU proportion) is  
 482 reported as well as the obtained sequence length.

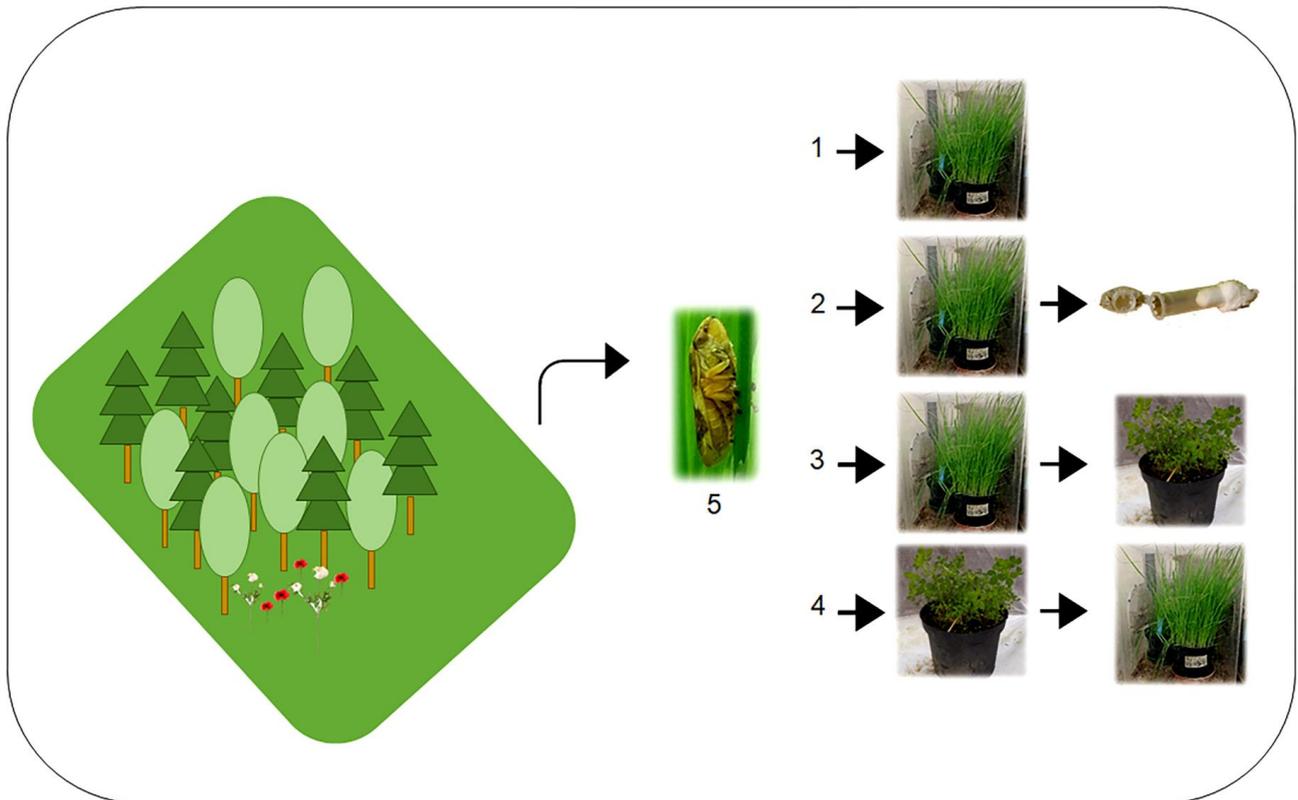
<b>Insect sample ID</b>	<b>No. plant OTUs</b>	<b>OTU proportion</b>	<b>Sequence length (bp)</b>	<b>Closest relative (NCBI Accession Number)</b>	<b>% sequence identity</b>
Ps18.1	1	10/10	310	<i>Corylus avellana</i> (KF718348)	100% (310/310 bp)
Ps18.2	1	10/10	246	<i>Convolvulus arvensis</i> (MF621879)	100% (246/246 bp)
Ps18.3	2	8/10	472	<i>Picea pungens</i> (EF440560)	99% (467/472 bp)
		2/10	471	<i>Plantago lanceolata</i> (AY101952)	100% (471/471 bp)
Ps18.4	2	7/10	396	<i>Achillea millefolium</i> (EU128988)	99% (395/396 bp)
		3/10	396	<i>Achillea millefolium</i> (EU128988)	99% (391/396 bp)
Ps18.5	1	10/10	423	<i>Acer campestre</i> (KU522504)	100% (423/423 bp)
Ps18.6	1	10/10	303	<i>Equisetum arvense</i> (GQ428069)	99% (301/303 bp)
Ps18.7	1	10/10	327	<i>Carpinus betulus</i> (AF327579)	99% (326/327 bp)
Ps18.8	3	4/10	312	<i>Arrhenatherum elatius</i> (MH569076)	100% (312/312 bp)
		2/10	312	<i>Arrhenatherum elatius</i> (MH569076)	99% (308/312 bp)
		4/10	312	<i>Convolvulus arvensis</i> (KC786130)	100% (312/312 bp)
Ps18.9	1	10/10	513	<i>Robinia pseudoacacia</i> (NC_026684)	99% (510/513 bp)
Ps18.10	1	10/10	223	<i>Quercus rubra</i> (KU186951)	99% (222/223 bp)

483

484

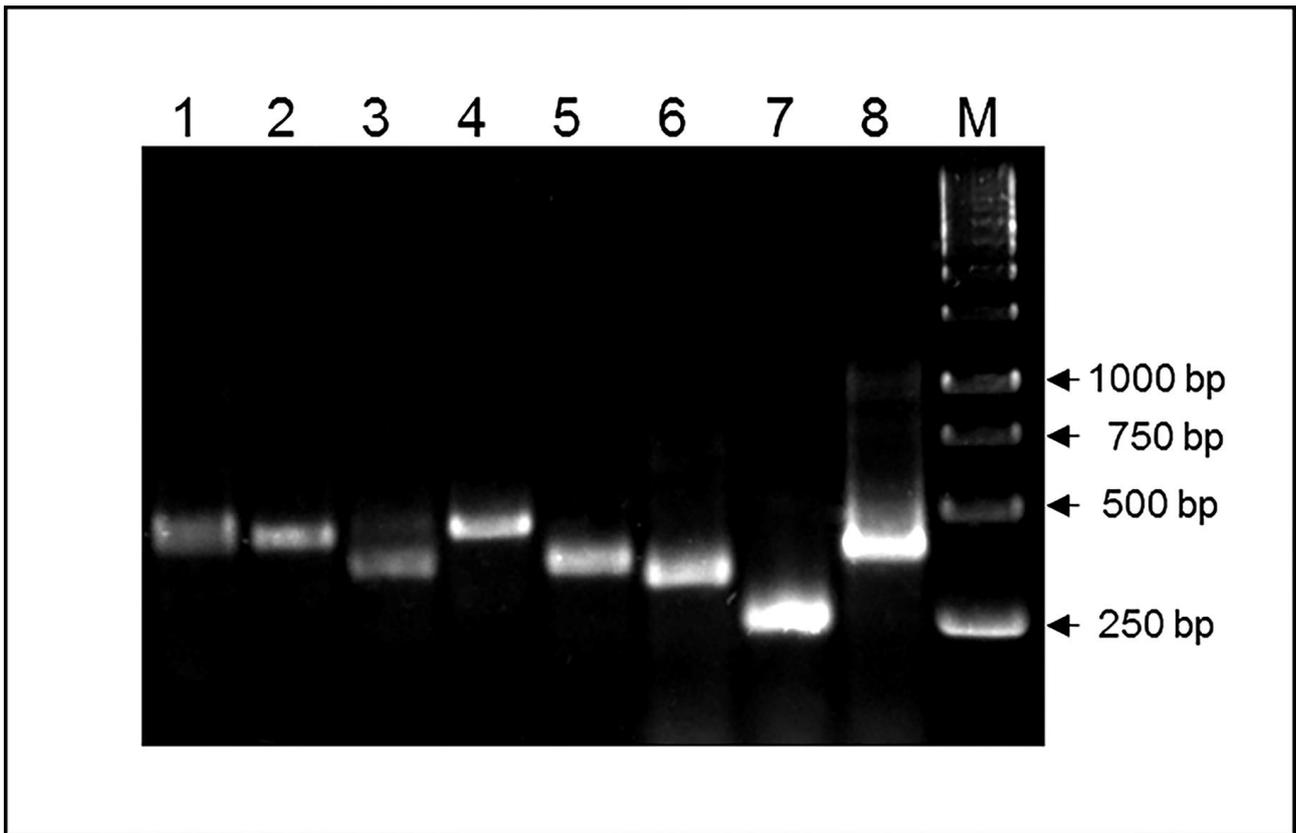
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487

488 **Figure 1** Experimental plan used for this work. Adult *P. spumarius* individuals were collected from  
 489 meadows close to forest areas in north-western Italy and then reared on potted plants of *D. ciliaris*  
 490 (1-3) or *M. sativa* (4) for at least 7 days. Afterwards, a first group of spittlebugs from the *D. ciliaris*  
 491 rearing was directly collected and submitted to molecular analysis (1); whereas a second group of  
 492 individuals was transferred on an artificial feeding system for 3 days (2). Additionally, a further  
 493 group of specimens from *D. ciliaris* was moved to potted *M. sativa* plants (3), while the spittlebugs  
 494 from the *M. sativa* rearing were transferred to *D. ciliaris* isolated plants (4) for other 3 days. A final  
 495 group of insects was preserved for molecular analysis directly after field collection for the  
 496 validation of the diagnostic method and for final persistence assessment of chloroplast DNA in the  
 497 gut of *P. spumarius* (5).



498

499 **Figure 2** Electrophoresis of chloroplast *trnL* gene amplicons obtained from one *P. spumarius* adult  
 500 reared for at least 7 days on *D. ciliaris* (1); two specimens maintained on *D. ciliaris* and then moved  
 501 to *M. sativa* (2-3); two specimens maintained on *M. sativa* and then moved to *D. ciliaris* (4-5); and  
 502 three field collected individuals (6-8), shown in Table 1 as Ps18.1 - Ps18.3. Sequences obtained  
 503 from amplicons 1, 2, and 4 were referable to *D. ciliaris* (450 bp); sequences from amplicons 3 and 5  
 504 were referable to *M. sativa* (350 bp); whereas sequences from field-collected specimens (amplicons  
 505 6-8) are indicated in Table 1.