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# *Xylella fastidiosa* subspecies and sequence types detected in *Philaenus spumarius* and in infected plants in France share the same locations

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## Abstract

In Europe, the meadow spittlebug *Philaenus spumarius* is the main known vector of the quarantine bacterium *Xylella fastidiosa*. So far detection and identification of *X. fastidiosa* has more often been performed from plant matrices than insects, mainly using a real-time PCR and multilocus sequence typing (MLST) approach. Detection of *X. fastidiosa* in its insect vectors would enhance knowledge of the epidemiologic situation in France, specifically in the already infected Corsica and Provence-Alpes-Côte d'Azur (PACA) regions. The aim of this study was to validate a methodological approach to detect *X. fastidiosa* in *P. spumarius*, analysed individually or in groups of 10, using real-time PCR and MLST, and to apply the approach to more than 4,000 individuals collected between 2015 and 2019 from infected areas. The corresponding results expanded our knowledge of the epidemiology of *X. fastidiosa* in France: (a) *X. fastidiosa* subsp. *multiplex* including the sequence types ST6 and ST7 were identified in the insect vector; (b) the rate of positive insects per infected area was as high as 33.3% in Corsica or 50% in the PACA region; (c) positive adults were found during winter; and (d) the bacterial load in *P. spumarius* (droplet digital PCR) usually ranged from  $10^3$  to  $10^4$  cells per insect, but could be as high as  $10^5$  or  $10^6$  cells per insect for some individuals (13%). The subspecies and sequence types detected in *P. spumarius* corresponded to the situation officially reported for plants in the same areas.

## KEYWORDS

bacterial load, detection methods, droplet digital PCR, MLST, *Philaenus spumarius*, *Xylella fastidiosa*

## 1 | INTRODUCTION

*Xylella fastidiosa*, endemic to the Americas, is a gram-negative bacterium that colonizes plant xylem vessels, transmitted by xylem sap-feeding insects. Five subspecies have been described:

*fastidiosa*, *multiplex*, *pauca*, *sandyi*, and *morus* (EFSA, 2018), causing different symptoms in a wide range of host plants. In fact, 595 plant species have been reported as either naturally or artificially infected hosts, belonging to 85 botanic families (EFSA, 2020).

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Since 2012 in Europe, imported coffee plants that were infected with several subspecies of *X. fastidiosa* have been intercepted by the authorities (Bergsma-Vlami *et al.*, 2015; Jacques *et al.*, 2016; Loconsole *et al.*, 2016; EPPO, 2019a), and since 2013, the bacterium has been detected on cultivated host plants in open orchards and fields, as well as in wild host plants. In 2013, the subspecies *pauca* was identified in olive trees, ornamental plants, and wild flora in Apulia, Italy (Saponari *et al.*, 2013). In 2015, the subspecies *multiplex* was reported in ornamental plants and wild flora in Corsica and the Provence-Alpes-Côte d'Azur (PACA) regions of France (Denancé *et al.*, 2017). One year later in Germany, the subspecies *fastidiosa* was detected on oleander (*Nerium oleander*) before being eradicated (EFSA, 2018). The subspecies *pauca*, *multiplex*, and *fastidiosa* were then reported in cultivated plants and wild flora on three Balearic Islands, Spain (Mallorca, Ibiza, and Menorca), while the subspecies *multiplex* was found to infect almond trees (*Prunus dulcis*) in continental Spain (Landa, 2017). More recently in 2018, the subspecies *multiplex* was reported from ornamental plants in Tuscany, Italy, and in Portugal (EPPO, 2019a).

Long-distance dispersal of *X. fastidiosa* occurs mainly via human-mediated movement of infected plants and propagating material, whereas natural spread over short distances relies on xylem sap-feeding insects. In the Americas, the role of *X. fastidiosa*'s vectors, such as sharpshooters and spittlebugs, in the epidemiology and spread of the disease has been widely studied and reviewed (Redak *et al.*, 2004; Chatterjee *et al.*, 2008; Krugner *et al.*, 2019). Recently in Europe, transmission testing has revealed that the spittlebugs *Philaenus spumarius*, *Philaenus italosignus*, and *Neophilaenus campestris* are efficient vectors in the Apulia region, Italy (Cornara *et al.*, 2017; Cavalieri *et al.*, 2019). Cornara *et al.* (2018) provided a state of the art study concerning the meadow spittlebug *P. spumarius*, which belongs to the order Hemiptera, family Aphrophoridae, is widely distributed in Europe, and is considered to be the main vector of *X. fastidiosa* in Italy. Being highly polymorphic and varying in colour from yellowish white to black, more than 50 synonym names have been given to this species. *P. spumarius* is a highly polyphagous species with a host list exceeding 1,000 plants (mainly dicotyledonous plants). The species is univoltine, overwintering as eggs. Once hatched, nymphs crawl to the closest green succulent plant and begin forming a spittle. After five instars (with development taking 5–6 weeks), adults appear in April under temperate climatic conditions and live until autumn. However, some individuals can survive in mild winters and remain active until the first days of the following spring. Nymphs have only limited dispersal capacity and adults are the main mobile stage, both actively and passively, because they can fly and, more often, jump. Seasonal movement of adults from the herbaceous vegetation to the canopy of trees and shrubs has been observed in late spring to early summer in Italy (Bodino *et al.*, 2019). The transmission of *X. fastidiosa* represents the most serious threat posed by the meadow spittlebug to agriculture and is a key determinant of *X. fastidiosa* establishment and dissemination in Europe. The bacteria are acquired and replicated in the insect foregut, but do not cross the gut barrier. Similarly, they do not circulate within the

other organs of the insect (Hopkins and Purcell, 2002), defining this transmission process as foregut-borne.

In France, from 2015 to 2019, the *X. fastidiosa* subspecies *multiplex* was identified from more than 40 plant species, and 166 infected areas were reported in PACA (Regional Food Authority [SRAL] of the PACA region, personal communication). Because Corsica has been classified as under containment since December 2017, the entire island is considered to be infected, and the “infected areas” are no longer counted. An infected area corresponds to a radius of 100 m around the first infected plants detected. Despite the eradication strategy and the specific surveillance scheme applied around the site in Menton (PACA) where the genotype ST53 of the *pauca* subspecies was detected in the myrtle-leaf milkwort (*Polygala myrtifolia*) in 2015, an olive tree was reported to be infected by this sequence type (ST) in August 2019. At the same time, the subspecies *multiplex* (ST unidentified) was reported from an olive tree in another location in PACA in the city of Antibes. This was the first time that an olive tree was officially identified as a host plant of *X. fastidiosa* subspecies *multiplex* and subspecies *pauca* in France. The presence and prevalence of vectors or potential vectors of *X. fastidiosa* was studied through a national project coordinated by the National Plant Health Laboratory of Montpellier, France, which involved several other partners. This project revealed not only the strong prevalence of *P. spumarius* in France, but also the occurrence of *N. campestris* (P. Reynaud, National Plant Health Laboratory of Montpellier, personal communication). As the bacterium had already been detected in *P. spumarius* in Apulia, Italy (Saponari *et al.*, 2014) and Corsica, France (Cruaud *et al.*, 2018), we decided to focus our work on this particular insect vector. So far, no vector transmission assay has been conducted in France, and the role of *P. spumarius* in the epidemiology of the disease is not fully understood. Nevertheless, all the progress made in the understanding of the traits that contribute to vector acquisition and transmission in *X. fastidiosa*, reported in several reviews (Redak *et al.*, 2004; Chatterjee *et al.*, 2008; Krugner *et al.*, 2019), will be helpful to interpret and inform our findings in the context of *X. fastidiosa* detection and spread in Europe.

The detection and identification of *X. fastidiosa* is preferentially performed directly in plants or insects rather than in pure culture, as the isolation of this bacterium is fastidious. Several molecular methods are available to detect and determine the subspecies of *X. fastidiosa*, and their applications were recently reviewed (Baldi and La Porta, 2017). Those validated and performed by the French National Reference Laboratory are (a) the real-time PCR developed by Harper *et al.* (2010) to detect *X. fastidiosa*, and (b) the multilocus sequence typing (MLST) scheme based on Yuan *et al.* (2010) to determine the subspecies in plant macerates. In fact, this real-time so-called “Harper PCR” is the most reliable, sensitive, and highly specific test for detecting *X. fastidiosa* (Modesti *et al.*, 2017; EPPO, 2019a). The detection scheme used on plants has been adapted to develop an efficient detection protocol that can be used to test *P. spumarius* individually (EPPO, 2019b). Recently, Cruaud *et al.* (2018) proposed another detection scheme to detect *X. fastidiosa* in whole *P. spumarius*, different from the one described in the EPPO protocol (EPPO, 2019b).

However, this scheme is not suitable for routine and high-throughput analyses, as subspecies identification is performed by applying nested PCR, which is known to increase the risk of false-positive results due to cross-contamination when performed in two steps (Llop *et al.*, 2000). Detecting the bacteria inside the vector is therefore a critical step towards understanding disease dissemination and spread, and there is a great and urgent need for a method that is easily applicable for high-throughput analysis without cross-contamination risks.

The aims of our study were to: (a) improve the detection methods for *X. fastidiosa* hosted by *P. spumarius*, in both individuals and pools of individuals; (b) study the prevalence of *X. fastidiosa* in *P. spumarius* collected in infected localities in France, and to compare it to the situation described in plants; (c) evaluate the bacterial load in *P. spumarius* collected in France; and (d) more generally improve our knowledge of the disease epidemiology in France and our ability to design ad hoc control strategies.

## 2 | MATERIALS AND METHODS

### 2.1 | *P. spumarius* samples

To evaluate and validate the methods applied to artificially contaminated samples, 1,178 insects belonging to the species *P. spumarius* were collected with a sweep net by the Hauts-de-France Regional federation for pest control (FREDON) and the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) Montpellier National Plant Health Laboratory in 2015 and 2017 in *X. fastidiosa*-free areas of France (Table 1). Moreover, 4,062 *P. spumarius* individuals in Corsica (3,820) and PACA (242) (Table 1), collected from 2015 to 2019 by official inspectors (FREDON Corse and FREDON PACA) in localities with infected areas, were tested with the validated protocol for epidemiological surveys.

Collected insects were identified by a morphological approach involving the use of dichotomous keys available in the literature for

these groups of insects (Ribaut, 1936, 1952; Della Giustina, 1989), and relying on the observation of external morphological characteristics associated with the study of genitalia, when required. Insects were stored at  $-20^{\circ}\text{C}$  in 96% ethanol.

### 2.2 | Bacterial strain

The *X. fastidiosa* subsp. *fastidiosa* ST2 (ATCC 35879) type strain was used to artificially contaminate the ground insect product (Table S1). Colonies were incubated on modified PWG (PWGm) solid medium for 3 weeks (EPPO, 2019b) at  $28^{\circ}\text{C}$  and then suspended in sterile demineralized water. The concentration of the suspension in cells/ml, used for artificial contamination or as PCR positive control, was quantified using immunofluorescence (IF), as indicated in the standard PM7/24 *Xylella fastidiosa* (EPPO, 2019b), and by droplet digital PCR (ddPCR) as described by Dupas *et al.* (2019).

### 2.3 | Bacterial sample preparation and artificial contamination of insects

Whole insects or insect heads removed manually from the body under a binocular microscope were ground according to Cruaud *et al.* (2018) or according to the following method: head or whole insect placed in a 2 ml microtube with 200  $\mu\text{l}$  of sterile demineralized water and 10 stainless steel beads (diameter 3 mm), then ground for 2 min at 30 Hz using an MM400 mixer mill (Retsch). The ground product was transferred to a new 2 ml microtube. The same grinding conditions were applied when working on a pool of insect heads (pools of 5 or 10 heads). Artificial contamination was carried out before grinding, by adding calibrated bacterial suspension. When working on pools of heads, artificial contamination was performed in order to have only one positive head in the pool.

<i>X. fastidiosa</i> status	Year of collection	Origin	No. of individuals
Free areas	2015	Occitanie <sup>a</sup>	31
	2015	Hauts-de-France <sup>b</sup>	363
	2017	Hauts-de-France	784
Infected areas	2015	Corsica <sup>c</sup>	330
	2016	Corsica	3,346
	2017	Provence-Alpes-Côte d'Azur <sup>d</sup>	28
	2017	Corsica	144
	2018	Provence-Alpes-Côte d'Azur	195
	2019	Provence-Alpes-Côte d'Azur	19

**TABLE 1** *Philaenus spumarius* collected in *Xylella fastidiosa*-free or infected areas in France

<sup>a</sup>Collected by LSV Montpellier.

<sup>b</sup>Collected by FREDON Hauts-de-France.

<sup>c</sup>Collected by FREDON Corse.

<sup>d</sup>Collected by FREDON Provence-Alpes-Côte d'Azur.

## 2.4 | DNA extraction methods

Two DNA extraction methods were compared. The first, referred to here as the “QuickPick” method, was similar to the method applied on plants to extract *X. fastidiosa* DNA (EPPO, 2019b). DNA extraction was performed using the QuickPick SML Plant DNA Kit (Bio-Nobile). After centrifugation of 200  $\mu$ l of ground product for 20 min at 20,000  $\times$ g at room temperature, the supernatant was discarded and the pellet was resuspended in 37.5  $\mu$ l of lysis buffer and 2.5  $\mu$ l proteinase K solution. The tubes were vortexed and incubated in a dry bath at 65°C for 20 min with regular stirring (1,500 rpm). They were then centrifuged for 5 min at 18,000  $\times$ g at room temperature, and the supernatant (around 40  $\mu$ l) was retrieved. The last part of the extraction was automated using a KingFisher mL or KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific). First, 62.5  $\mu$ l of binding buffer and 2.5  $\mu$ l of magnetic beads were added to the 40  $\mu$ l of supernatant, followed by three steps of washing by adding 125  $\mu$ l of washing buffer. DNA was finally eluted in 50  $\mu$ l of elution buffer.

The second method was the DNA extraction method described in the work of Cruaud *et al.* (2018) and performed here either on (a) the whole insect as described by Cruaud, or (b) the insect head, as previously presented here. The 200  $\mu$ l of ground product were centrifuged for 20 min at 20,000  $\times$ g at room temperature; the supernatant was discarded. The rest of the extraction was performed as described previously (Cruaud *et al.*, 2018). DNA was eluted in 50  $\mu$ l of TE buffer (1 $\times$ ).

For each DNA extraction, a negative control containing *X. fastidiosa*-free insect head ground in sterile demineralized water was added. For both DNA extraction methods, and for each concentration, three independent artificially contaminated ground head products were extracted at three different times, to obtain nine independent DNA extractions. To evaluate the DNA extraction methods, the Harper-loos duplex real-time PCR was applied, as described below, in duplicate or triplicate on each DNA extract, when testing the whole insect or only the head, respectively.

## 2.5 | *X. fastidiosa* detection in *P. spumarius* using the Harper-loos duplex real-time PCR

The detection test was performed using the Harper-loos duplex real-time PCR (Harper-loos duplex) combining XF-F/XF-R primers and an XF-P probe targeting the *rimM* gene specific to *X. fastidiosa* (Harper *et al.*, 2010), and an internal control targeting the 18S ribosomal gene of eukaryotes using 18S uni-F/18S uni-R primers and an 18S uni-P probe (loos *et al.*, 2009). Harper-loos duplex was carried out in a final volume of 20  $\mu$ l containing 1  $\times$  TaqMan fast universal master mix without AmpErase UNG (Applied Biosystems), 0.3  $\mu$ M XF-F and XF-R primers, 0.1  $\mu$ M XF-P probe, 0.15  $\mu$ M 18S-uni-F and 18S-uni-R primers, 0.05  $\mu$ M 18S-uni-P probe, 0.3  $\mu$ g/ $\mu$ l bovine serum albumen (BSA), and 2  $\mu$ l DNA. Reactions and fluorescence detection were performed on a Bio-Rad C1000 Touch thermal cycler/Bloc CFX96 Real optics module using a thermal cycling programme of: preincubation for 2 min at 50°C

and 10 min at 95°C; followed by 40 cycles of 10 s at 94°C and 40 s at 62°C; the heating ramp speed was fixed at 5°C/s. For each PCR plate, two positive amplification controls, *X. fastidiosa* subsp. *fastidiosa* (ATCC 35879) at 5  $\times$  10<sup>7</sup> cells/ml for targeting Harper, and DNA extract of *P. spumarius* head not artificially contaminated for targeting loos, and a negative amplification control (ultrapure water) were added. A sample was considered positive when an exponential curve was obtained with a C<sub>t</sub> value lower than 38 for Harper, as recommended by Harper *et al.* (2010), and a C<sub>t</sub> value lower than 30 for loos.

The analytical specificity of the Harper primers in the Harper-loos duplex was evaluated according to two criteria: (a) inclusivity (i.e., ability to detect target strains) as tested on 14 target strains belonging to all *X. fastidiosa* subspecies, repeated twice; and (b) exclusivity (i.e., ability to not generate positive results from nontarget strains) as tested on 40 nontarget strains belonging to *Xanthomonadaceae*, or bacteria, saprophytes, and phytoplasmas detected on the same host plants as *X. fastidiosa* (Table S1).

The analytical sensitivity (i.e., the lowest amount of target that can be reliably detected), with a detection rate of 100% for all replicates, of the Harper-loos duplex was evaluated on artificially contaminated ground head product, with a 10-fold dilution range of *X. fastidiosa* subsp. *fastidiosa* suspension at 10 to 10<sup>5</sup> cells/head. DNA of each bacterial concentration was extracted in triplicate on three different days, and DNA extracts were amplified in duplicate to obtain 18 data points for each concentration.

## 2.6 | Identification of the *X. fastidiosa* subspecies and sequence type by MLST

The following protocol used to determine the subspecies on collected insects was first validated on artificially contaminated ground head product to verify its reliability. The identification of the subspecies and the allocation to a sequence type (ST) was achieved using an MLST scheme (Yuan *et al.*, 2010). Partial sequences of seven housekeeping genes (*cysG*, *gltT*, *holC*, *leuA*, *malF*, *nuoL*, and *petC*) were amplified as described in the EPPO standard PM7/24 (4) (EPPO, 2019b) to identify subspecies from plant extract. BSA (0.3  $\mu$ g/ $\mu$ l) was added to the PCR mix specific to each housekeeping gene to limit the impact of possible PCR inhibitors that could be present in the insect head. Once the housekeeping genes were amplified, PCR products were sent to Genewiz for both forward and reverse sequencing. Geneious v. 11.1.2 Biomatters was used to edit, assemble, translate, and align the forward and reverse sequences of each sample. ST allocation was carried out according to the PubMLST website (<https://pubmlst.org/xfastidiosa/>).

## 2.7 | Detection and identification of *X. fastidiosa* in *P. spumarius* pools

DNA of pools of insect heads was extracted with the DNA extraction method presenting the best performance criteria in this study, that is, the QuickPick method. The analytical sensitivity,

with a detection rate of 100%, of the Harper-loos duplex was first evaluated and compared on artificially contaminated samples of 1, 5, and 10 heads with a range of 10-fold suspensions of *X. fastidiosa* subsp. *fastidiosa* (ATCC 35879) of  $10^5$ ,  $10^4$ , and  $10^3$  cells/head, cells/5 heads, or cells/10 heads. Samples corresponding to a given concentration were extracted separately in triplicate and amplified using the Harper-loos duplex performed in triplicate, to obtain nine results per assay. The subspecies identification was conducted applying MLST on DNA extracts, as described above.

## 2.8 | Analysis of insects collected in infected areas

The detection and identification of *X. fastidiosa* in insects collected in infected areas were conducted according to the validated methods described in this study (QuickPick method, Harper-loos duplex, and MLST). To compare individuals versus pools of 10 insect heads, for four areas, a set of the insects collected in Corsica in 2016 was analysed individually, and the remaining insects by groups of 10.

When working on pools, the positive amplification control used for targeting loos primers consisted of DNA extracted from 10 heads of *P. spumarius* collected from the infected areas. Two amplifications were performed by Harper-loos duplex per sample. A sample was considered positive regarding *X. fastidiosa* infection when the  $C_t$  value of at least one of the two replicates obtained for Harper's primers was lower than 38, and negative otherwise.

## 2.9 | Estimation of the rate of infected *P. spumarius* collected in infected areas

For the pools, the rate of *X. fastidiosa* positive insects and the  $1 - \alpha$  confidence interval of  $P$  were calculated according to calculation of the positive rate for seed lots (Maury *et al.*, 1986); with  $P$  the positive rate as a percentage,  $N$  the total number of tested groups,  $Y$  the number of negative groups, and  $n$  the size of the group (here  $n = 10$ ). The R `binom.test` function (R statistical software, v. 3.6.1; package `stats`, R Development Core Team) was used to assess statistical differences between positive rates obtained for individuals and the group.

## 2.10 | Assessment of bacterial load in *P. spumarius* collected in infected areas

The bacterial load was assessed by applying ddPCR on naturally infected *P. spumarius* tested individually, according to Dupas *et al.* (2019). The DNA volume added was 2  $\mu$ l, with addition of BSA to the reaction mix to a final concentration of 0.3  $\mu$ g/ $\mu$ l.

The reaction was validated when at least 10,000 droplets were generated. A sample was considered positive when at least two positive droplets with fluorescence amplitude equal to the positive

control were found. The concentration of the target in the insect was estimated by Quantasoft Analysis Pro software (Bio-Rad), as described by Dupas *et al.* (2019).

## 2.11 | Data and maps related to the *X. fastidiosa* epidemiologic picture

The French National Plant Protection Organization defined an official surveillance strategy for *X. fastidiosa* on plants (DGAL/SDQSPV/2018-482 and DGAL/SDQSPV/2017-653) in 2015, based on Commission Implementing Decision (EU) 2015/789 of 18 May 2015 ([https://eur-lex.europa.eu/eli/dec\\_impl/2015/789/2018-10-11](https://eur-lex.europa.eu/eli/dec_impl/2015/789/2018-10-11)). Since 2015, the National Plant Health Laboratory of Angers has been in charge of official analyses to detect *X. fastidiosa* in plant samples. The detection protocol applied for plants was thus described in MA039 ([https://www.anses.fr/fr/system/files/ANSES\\_LSV\\_MA039\\_V04.pdf](https://www.anses.fr/fr/system/files/ANSES_LSV_MA039_V04.pdf)), a method validated by intralaboratory comparisons by the laboratory, and that is included in the standard PM7/24 *Xylella fastidiosa* (EPPO, 2019b). The results of *X. fastidiosa* detection and identification in plant samples analysed from July 2015 to December 2019 in Corsica and PACA regions (generated independently of this study) and the results for *X. fastidiosa* detection and identification in insects (generated in this study) were mapped using R (R Core Team, 2013). The location points were the GPS coordinates where plants were collected, and the centre of the municipality where insects were collected.

## 3 | RESULTS

### 3.1 | Validation of detection and identification methods for *X. fastidiosa* in individuals and pools of *P. spumarius*

For *X. fastidiosa* detection, the two DNA extraction methods described above were compared on artificially contaminated ground head product and whole insects, with  $10^4$ ,  $10^3$  and  $10^2$  cells/head or cells/insect. The QuickPick method had lower  $C_t$  values than the Cruaud method, when applied on the head or on the whole insect, regardless of the grinding conditions and bacterial concentrations used (Table 2). The best analytical sensitivity was found for the QuickPick method: the detection threshold, with a detection rate of 100% on the 27 replicates, was set at  $10^3$  cells/head ( $C_t$  values ranging from 33.44 to 36.08) with the QuickPick method, compared to  $10^4$  cells/head or cells/insect ( $C_t$  values ranging from 33.48 to 36.82) with the Cruaud method. Similar results were obtained with the Cruaud method when using the whole insect or only the head. At a concentration of  $10^3$  cells/head, the repeatability of DNA extraction methods was 100% for the QuickPick method, 100% for the Cruaud method on whole insects, and 85% for the Cruaud method adapted to the insect head. Because  $C_t$  values obtained with DNA extracted by the QuickPick method were



**TABLE 2** (a) Analytical sensitivity comparison of Harper-loos duplex real-time PCR of DNA extracts when applying the QuickPick method or the Cruaud method. (b) Validation data of analytical sensitivity estimation of the Harper-loos duplex real-time PCR performed on DNA extracted with the QuickPick method

Method	Data	Concentration (cells/head or cells/insect)			Repeatability (%) <sup>a</sup>	
		10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>		
(a)	QuickPick method	Mean C <sub>t</sub> values	31.09	34.66	37.52	100
		SD	0.38	0.60	1.01	
		No. of assays with C <sub>t</sub> < 38	27/27	27/27	12/27	
	Cruaud method <sup>b</sup> (insect head)	Mean C <sub>t</sub> values	34.81	37.99	37.80	85
		SD	0.81	0.84	0.47	
		No. of assays with C <sub>t</sub> < 38	27/27	7/27	2/27	
	Cruaud method (whole insect)	Mean C <sub>t</sub> values	34.71	37.59	38.22	100
		SD	0.78	0.90	0.07	
		No. of assays with C <sub>t</sub> < 38	18/18	10/18	0/18	
(b)	Harper-loos duplex real-time PCR	Mean C <sub>t</sub> values	30.91	34.05	37.70	100
		SD	0.49	0.62	1.08	
		No. of assays with C <sub>t</sub> < 38	18/18	18/18	15/18	
		Detection rate (%)	100	100	83	

<sup>a</sup>Repeatability was evaluated at the limit of detection, at 10<sup>3</sup> cells/head or insect.

<sup>b</sup>Adapted from the Cruaud method: test on the head and not on the whole insect.

<sup>c</sup>Analytical sensitivity.

systematically the lowest, this automated DNA extraction method was selected for the final study.

Concerning the identification of *X. fastidiosa*, the analytical specificity of the Harper-loos duplex was confirmed with a result of 100% on pure bacterial strains for Harper primers. Indeed, as expected, all the 14 target strains were amplified, corresponding to 100% inclusivity, and none of the 40 nontarget strains were amplified, confirming 100% exclusivity (data not shown). Duplex did not affect the Harper real-time PCR performance, and no cross-reaction was obtained with the loos primers for bacterial strains. The detection threshold of the Harper-loos duplex applied to *P. spumarius* was 10<sup>3</sup> cells/head, as 100% of the 18 replicates presented a C<sub>t</sub> value lower than 38 (Table 2).

In order to use the detection methods described above on more than one head, the ground products of 1, 5, and 10 heads were compared. The macroscopic and microscopic observations revealed that the size of debris was similar and homogeneous in these three conditions. When applying the Harper-loos duplex, the mean C<sub>t</sub> values obtained were similar for one head and both pools of heads, regardless of the bacterial concentrations used for artificial contamination (Figure S1). Under these conditions, pooling insect heads did not influence the C<sub>t</sub> values for *X. fastidiosa* detection. The detection threshold, with a detection rate of 100% for all the replicates, of the Harper-loos duplex applied to the pools of 10 heads was found to be 10<sup>3</sup> cells/heads (Figure S1).

In addition, MLST was applied for 1, 5, and 10 heads at 10<sup>5</sup> cells/heads and only for 1 and 10 heads at 10<sup>4</sup> cells/heads. When applied on one head, the seven housekeeping genes were amplified for both bacterial concentrations. When applied on pools of 5 and 10 heads, the amplifications were also successful at 10<sup>5</sup> cells/5 heads and cells/10 heads. On the seven genes, five (*cysG*, *holC*, *leuA*, *nuoL*, and *petC*) were successfully amplified at 10<sup>4</sup> cells/5 heads and cells/10 heads. For the pools, the analytical sensitivity of the MLST is 10<sup>5</sup> cells/5 or 10 heads. Nevertheless, the identification of the subspecies was possible with a combination of two gene sequences, such as *cysG* and *malF*, as reported in the EPPO standard PM7/24 (4) (EPPO, 2019b) or with *cysG* and *holC*, *holC* and *nuoL*, *malF* and *glT*, or *malF* and *nuoL* (data not shown), which was controlled for the 87 STs included in the PubMLST website (<https://pubmlst.org/xfastidiosa/>).

### 3.2 | Analysis of insects collected in infected areas

The validated protocol described above, that is, the QuickPick-based DNA extraction method combined with the Harper-loos duplex, was applied on *P. spumarius* insects collected from infected areas in France.

From 2015 to 2017, 3,820 *P. spumarius* insects were collected in Corsica and analysed individually (1,550), or in pools of 10 individuals (2,270). As expected, amplifications were obtained for the loos internal control for all the tested *P. spumarius* samples. Among the 1,550 insects analysed individually, 193 (12.5%) were Harper-loos-positive (i.e., at least one of the two replicates had a  $C_t$  value lower than 38 for the Harper primers; Table 3). Among the 1,357 negative ones, 86 individuals (6.3%) presented  $C_t$  values ranging from 38 to 40 for the Harper primers. Among the 193 positive samples, 91% of the tested individuals had two real-time PCR replicates positive. The rate of infected *P. spumarius* identified in the prospected areas in Corsica ranged from 2.5% to 33.3%, depending on the locality (Table 3). At least one insect was detected as *X. fastidiosa*-positive within, or close to, the 15 examined infected areas in Corsica from 2015 to 2017. The MLST scheme was performed on 119 Harper-loos-positive individuals. The subspecies *multiplex* was allocated to 106 samples (89%) (at least two genes identified among the combinations presented in the Materials and Methods), including 86 (81.1%) for which the ST was fully identified with the seven genes: 37 as ST6 and 49 as ST7. Interestingly, MLST was also successful for 31 samples (29.2%) with  $C_t$  values ranging from 30 to 34.6 for the Harper primers (Figure 1).

Among the insects collected in 2016 from four different infected areas, 2,270 insects were analysed in pools of 10 and compared to 639 insects analysed individually (Table 4). Of the 227 tested pools, 143 (63%) were positive. For the individuals,  $C_t$  values ranged from 22.81 to 37.99, whereas they ranged from 20.46 to 37.90 for the pools. The MLST scheme was performed on 82 Harper-loos-positive pools. The *multiplex* subspecies was allocated to 73 samples (89%), including 63 (86.3%) for which the ST was fully defined: 22 as ST6 and 41 as ST7 (Table 4). As for the insects analysed individually, the proportion of ST7 identified was higher than those analysed in pools of 10. The trend was the same for the STs identified from the pools. MLST was again successful for samples with  $C_t$  values ranging from 30 to 32.02 (for the Harper primers). As shown in Table 4, the calculated rates of positive insects, associated with confidence intervals, were not significantly different ( $p > .05$ ) between insects analysed individually and pools of 10, for three out of the four considered infected areas. For areas 2015/145 (municipality of Ajaccio), 2016/032 (municipality of Serra-di-Ferro) and 650 m from 2017/015 (municipality of Sartène), the rates of positive individuals were 4%, 13.47%, and 4.39%, respectively, and for pools, the rates of positives were 5.1%, 12.0%, and 9.9%, respectively. The rates of positive samples were significantly different ( $p = .001$ ) only for the 2015/083 area (municipality of Ajaccio), for which these rates were 14.1% for individuals and 8.1% for groups, and the confidence interval was narrower for the pools (5.8 and 10.8) than for the individuals (10.1 and 19.0). This could be explained by the higher number of insects tested in pools than individually for this area.

In the PACA region, from 2016 to 2019, 242 *P. spumarius* individuals were collected and analysed individually or by pools of 10. Among the 130 insects individually analysed, eight were positive and allocated to the *multiplex* subspecies (one ST6 and seven ST7), with  $C_t$  values ranging from 27.12 to 35.28. Positive samples were identified

in five out of the 11 prospected areas, with the rate of *X. fastidiosa* positive insects ranging from 20% to 50% (Table 3). However, no positive results were reported among the 11 tested pools.

### 3.3 | Assessment of the bacterial load

In order to assess the *X. fastidiosa* load per *P. spumarius* individuals collected in France and found to be Harper-loos duplex-positive, the concentration of bacteria per head was calculated by performing ddPCR assays on insects tested individually. As the *rimM* gene targeted by the Harper real-time PCR (Harper *et al.*, 2010) is a single copy gene, the detection by ddPCR of one gene corresponds to one detected *X. fastidiosa* cell. Ten *P. spumarius* heads detected as positive (mean  $C_t$  values ranging from 25.83 to 34.04 for Harper primers) and three detected as negative (no  $C_t$  value obtained for Harper primers) were analysed by two independent ddPCR assays (Table S2). As expected, no positive droplets were detected for the three negative samples. The presence of *X. fastidiosa* was found in the DNA of all 10 known infected *P. spumarius*, as at least two positive droplets were detected for them. A curve was implemented to highlight the correlation between the  $C_t$  values obtained by Harper-loos duplex and the  $\log_{10}$  of the bacterial load estimated by ddPCR by combining the results of the two ddPCR assays, resulting in a correlation coefficient of 0.963 (Figure 2). According to the line equation ( $y = -3.0441x + 42.489$ ), ranges of  $C_t$  values were allocated to a range of bacterial load per head (Table S3). In our conditions, the 38 cut-off value corresponds to 29 cells/head.

This correspondence was applied to the 201 infected *P. spumarius* collected in Corsica and PACA and tested individually (Figure 3). The *X. fastidiosa* load detected in collected *P. spumarius* corresponded mainly to orders of magnitude from  $10^3$  (27%) to  $10^4$  (27%) cells per head, as the mean  $C_t$  values ranged from 27.27 to 33.36. However, the bacterial load reached lower values, of the order of magnitude of  $10^2$  for 17% and between 29 and 99 cells for 15% of the tested insect heads. Interestingly, higher values were also obtained, with an order of magnitude of  $10^5$  cells per head for 12% and  $10^6$  cells per head for 1% of the positive tested insect heads.

In Corsica, insects were collected between October and March, from 2015 to 2018. The distribution of the number of positive insects and their bacterial load (Figure 4) revealed that the highest bacterial load (more than  $10^5$  cells/head) in *P. spumarius* was reached in October, November, and December. Infected *P. spumarius* collected during winter, in January and March, showed a bacterial load of an order of magnitude of  $10^3$  cells or lower.

### 3.4 | Comparison with the geographic distribution of infected plants

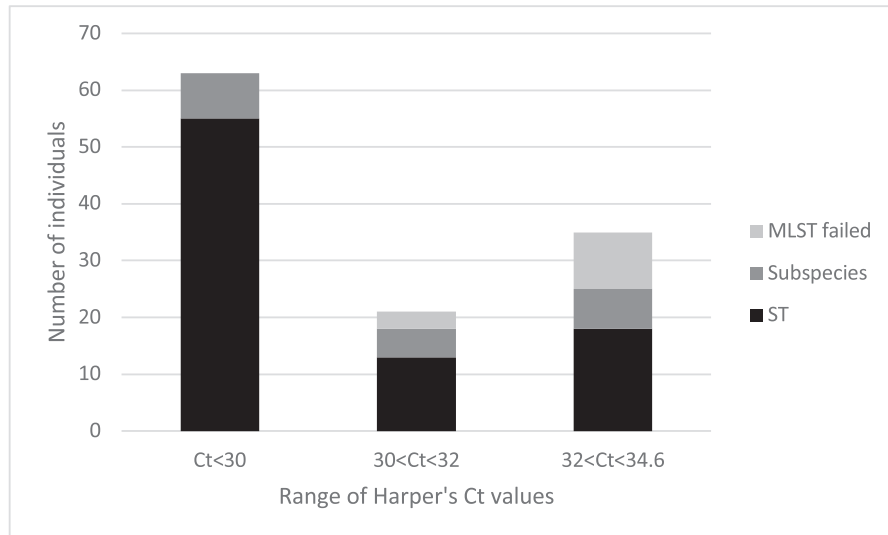
Maps presenting the distribution of infected plants and insects, collected independently at different times, revealed a strong



**TABLE 3** Detection of *Xylella fastidiosa* using Harper-loos duplex real-time PCR and sequence type (ST) identification by multilocus sequence typing (MLST) in all *Philaenus spumarius* collected in the Corsica and PACA regions from 2015 to 2019 and analysed individually

Region	Municipality	Infected area reference	Date of collection	n	Positive			
					No.	%	ST	
Corsica	Ajaccio	2015/037	Nov 2015	74	4	5.4	NA	
		2015/083	Nov 2015	140	21	15.0	ST6	
			Nov 2016	255	36	14.1	ST6	
			Jan/Feb 2017	20	0	0.0	–	
			2015/108	Dec 2016	1	0	0.0	–
			2015/145	Nov 2015	31	4	12.9	NA
				Dec 2016	25	1	4.0	NA
				Jan/Feb/Mar 2017	79	2	2.5	ST6
		Corbara	2016/068	Dec 2016	74	7	9.5	ST7
				Jan 2017	7	0	0.0	–
	Grosseto-Prugna	2015/172	Oct/Nov 2016	142	35	24.6	ST6/ST7	
		3.5 km from 2015/029	Oct/Nov 2016	217	32	14.8	ST6/ST7	
	Olmetto	2015/174	Nov 2015	21	4	19.0	NA	
	Porto Vecchio	2016/091	Nov 2016	3	1	33.3	NA	
	Sarrola Carcopino	2015/074	Nov 2015	14	1	7.1	NA	
	Sartène	2015/236	Dec 2015	28	2	7.1	NA	
		650 m from 2017/015	Oct/Nov 2016	114	5	4.4	ST7	
			Jan 2017	30	1	3.3	ST7	
	Serra-Di-Ferro	2016/032	Nov 2016	245	33	13.5	ST7	
			Jan/Mar 2017	8	2	25.0	ST7	
Zonza	2015/044	Nov 2015	4	1	25.0	NA		
	2015/176	Nov 2015	18	1	5.6	NA		
PACA	Antibes	2015/233	May/June 2018	16	0	0.0	–	
			May 2019	2	0	0.0	–	
	La Seyne-sur-Mer	2015/217–2016/019	Jun 2017	3	0	0.0	–	
			Nov 2017	2	1	50.0	ST7	
			May 2018	2	0	0.0	–	
	Mandelieu-la-Napoule	2018/048	Nov 2019	2	1	50.0	ST7	
		2016/013	Jun 2017	3	0	0.0	–	
	Mougins		Jun 2018	1	0	0.0	–	
		2016/077	Jun 2017	4	0	0.0	–	
	Nice		May/June 2018	32	0	0.0	–	
		2016/015	Jun 2017	2	0	0.0	–	
			May/June 2018	61	0	0.0	–	
	Saint-Laurent-du-Var	2018/005	Oct 2019	2	1	50.0	ST7	
		2015/218	Jun 2017	2	0	0.0	–	
			May/June 2018	40	0	0.0	–	
	Saint-Raphaël		Sep/Oct 2019	13	3	23.1	ST6/ST7	
		2018/003	May 2018	3	0	0.0	–	
	Villeneuve-Loubet	2016/014 BC	Jun 2017	2	0	0.0	–	
			May/June 2018	16	0	0.0	–	
		2016/014 BD	Jun 2017	10	2	20.0	ST7	
		May/June 2018	24	0	0.0	–		

Abbreviation: NA, MLST not applied.



**FIGURE 1** Frequencies of multilocus sequence typing (MLST) results according to Harper's  $C_t$  values obtained applying Harper-loos duplex real-time PCR for *Philaenus spumarius* collected in Corsica. ST: the allele numbers of the seven housekeeping genes were identified, allowing us to define the sequence type (ST). Subspecies: the allele numbers of fewer than seven housekeeping genes were identified, allowing us to define the subspecies. MLST Failed: the allele numbers identified did not allow us to define the subspecies, or no housekeeping gene was amplified

colocation of the subspecies and the STs identified in both plant and insect sampling (Figure 5). In fact, the *multiplex* subspecies was identified in *P. spumarius* collected both in Corsica and PACA, and the same STs (ST6 or ST7) were found in the corresponding prospected infected areas (Table S4). When there was a predominance of ST6 in plant samples, it was confirmed in *P. spumarius*, as in the municipality of Ajaccio, where ST6 was predominant in both plants and insects. Coinfection with ST6 and ST7 was reported for plants analysed previously (five in Corsica and six in PACA) by culturing or identification of a double peak for the gene *cysG* at the nucleic position that distinguishes both STs, but not for the analysed insects in this present study (Figure 5).

#### 4 | DISCUSSION

Since its first report in the Americas, several methods have been developed and used to detect *X. fastidiosa* in plants and insects, such as plating methods, microscopy, immunological methods, and more recently molecular methods (Baldi and La Porta, 2017; EPPO, 2019b). In this study, detection and identification methods for *X. fastidiosa* were evaluated and validated on artificially contaminated head ground product of *P. spumarius*. The combination of DNA extraction using the QuickPick SML Plant DNA Kit with Harper-loos duplex (loos *et al.*, 2009; Harper *et al.*, 2010) and an optimized MLST scheme (Yuan *et al.*, 2010; EPPO, 2019b), mainly used for plant surveillance, was found to be specific, reproducible, sensitive, and relevant for the detection of

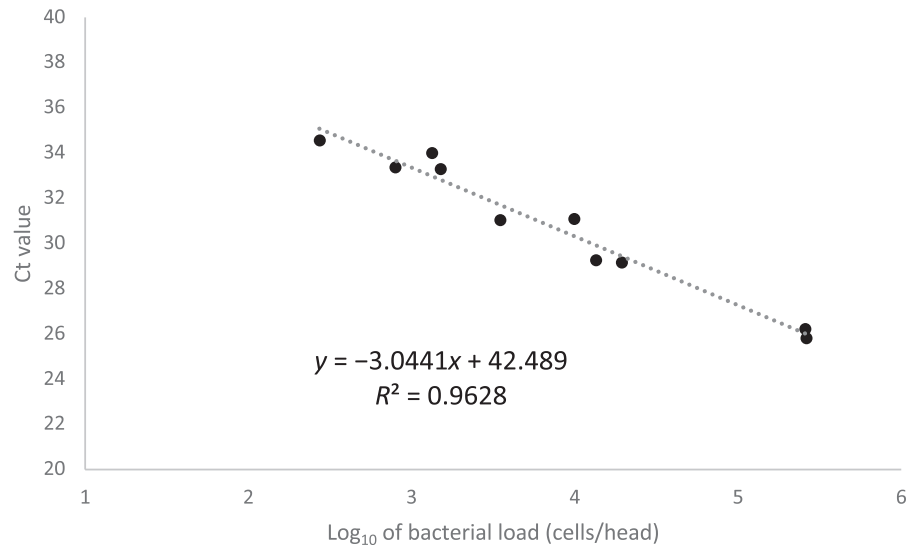
**TABLE 4** Results of the *Xylella fastidiosa* detection by Harper-loos duplex real-time PCR and *X. fastidiosa* identification by MLST in *Philaenus spumarius*

Infected area reference	Individuals						Groups						$p^a$
	n	Positive		ST	CI (%)		n	Positive		ST	CI (%)		
		No.	%		LL	UL		No.	%		LL	UL	
2015/083	255	36	14.12	ST6	10.09	19.00	65	37	8.08	ST6/ ST7	5.77	10.84	.001***
2015/145	25	1	4.00	ST6	0.10	20.35	22	9	5.12	ST6	2.61	9.05	.051 NS
2016/032	245	33	13.47	ST7	9.46	18.39	83	60	12.04	ST7	9.18	15.20	.491 NS
650 m from 2017/015	114	5	4.39	ST7	1.44	9.94	57	37	9.94	ST7	7.06	13.30	.057 NS

Note: *P. spumarius* was collected in four different infected areas in Corsica in 2016 and analysed individually and in groups of 10. The confidence intervals (CI) were estimated for individuals using a binomial test and for groups according to Maury *et al.* (1986). ST, sequence type.

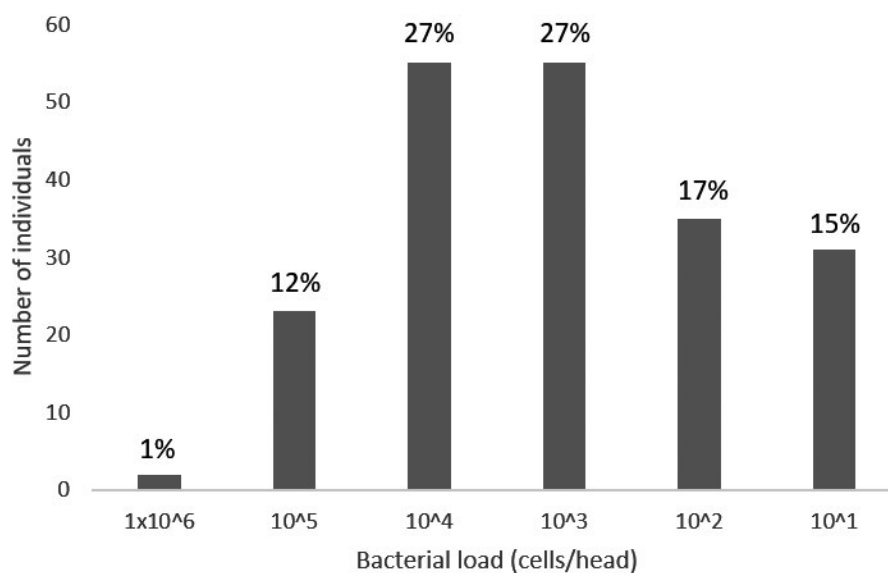
<sup>a</sup> $p$  value of the significant difference calculated between infection rates obtained for individuals and groups using a binomial test; \*\*\*significantly different at  $p < .01$ ; NS, not significantly different ( $p > .05$ ).

**FIGURE 2** Correlation between Harper's  $C_t$  values obtained applying Harper-loos duplex real-time PCR and  $\log_{10}$  of bacterial load (cells/head) obtained by applying droplet digital PCR assay on 10 *Philaenus spumarius* hosting *Xylella fastidiosa*. Dotted line: regression curve

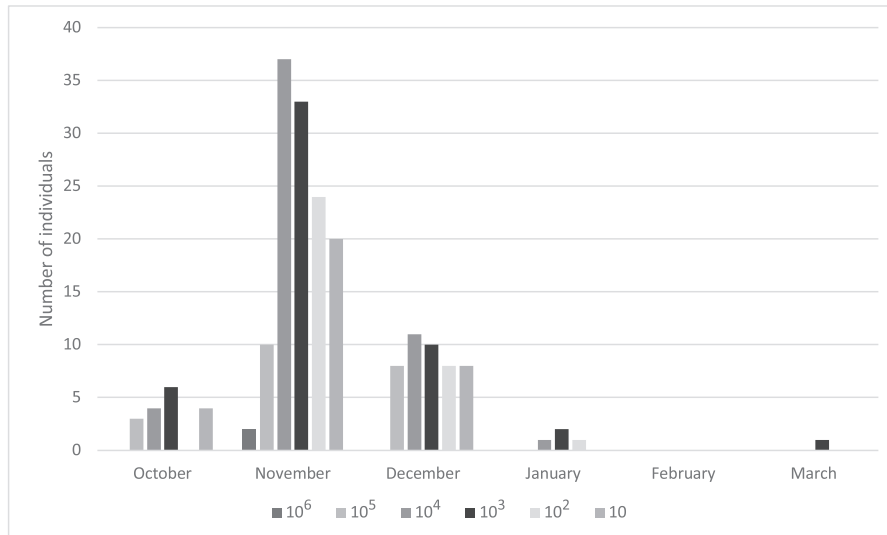


*X. fastidiosa* in *P. spumarius* collected in infected areas. So far, available protocols have been used to analyse *P. spumarius* one by one or in five-head pools (Cornara *et al.*, 2017; Cruaud *et al.*, 2018; Eppo, 2019b). We succeeded here in the efficient detection of *X. fastidiosa* in one head or pools of 10 heads of *P. spumarius*, with the same detection threshold of  $10^3$  cells, with a rate of detection of 100% of replicates, for testing one head and pools of 10 heads. Nevertheless, it allowed for the detection of bacteria with a concentration lower than the detection threshold of 100% evaluated at  $10^3$  cells/head, such as  $10^2$  and 10 cells/head. In order to validate the PCR analytical process in each PCR, an internal control (loos *et al.*, 2009) was added to Harper real-time PCR (Harper *et al.*, 2010). Although Cruaud *et al.* (2018) pointed out a lack of sensitivity of Harper real-time PCR in *P. spumarius*, the

sensitivity, reproducibility, and robustness of Harper real-time PCR and Harper-loos duplex were supported at the interlaboratory level, in the context of a test performance study (Euphresco project 2015-F-146, final report available at <https://zenodo.org/record/2656679#.XiGvK3dFzj>). That project focused on the evaluation of molecular methods to detect *X. fastidiosa* in the vector *P. spumarius*. Three laboratories from three different countries tested a panel of artificially contaminated ground insect products (two samples at  $5 \times 10^5$  cells/head; two samples at  $5 \times 10^4$  cells/head; four samples at  $5 \times 10^3$  cells/head; four negative samples; one positive amplification control), using the QuickPick DNA extraction method and Harper-loos duplex real-time PCR. These results also confirmed the reproducibility and sensitivity of this protocol.



**FIGURE 3** Distribution of the frequencies (in percentage) of bacterial load (cells/head) of *Xylella fastidiosa* subsp. *multiplex* estimated by Harper-loos duplex real-time PCR and droplet digital PCR assays in the 201 positive *Philaenus spumarius* collected in Corsica (193) and PACA (8)



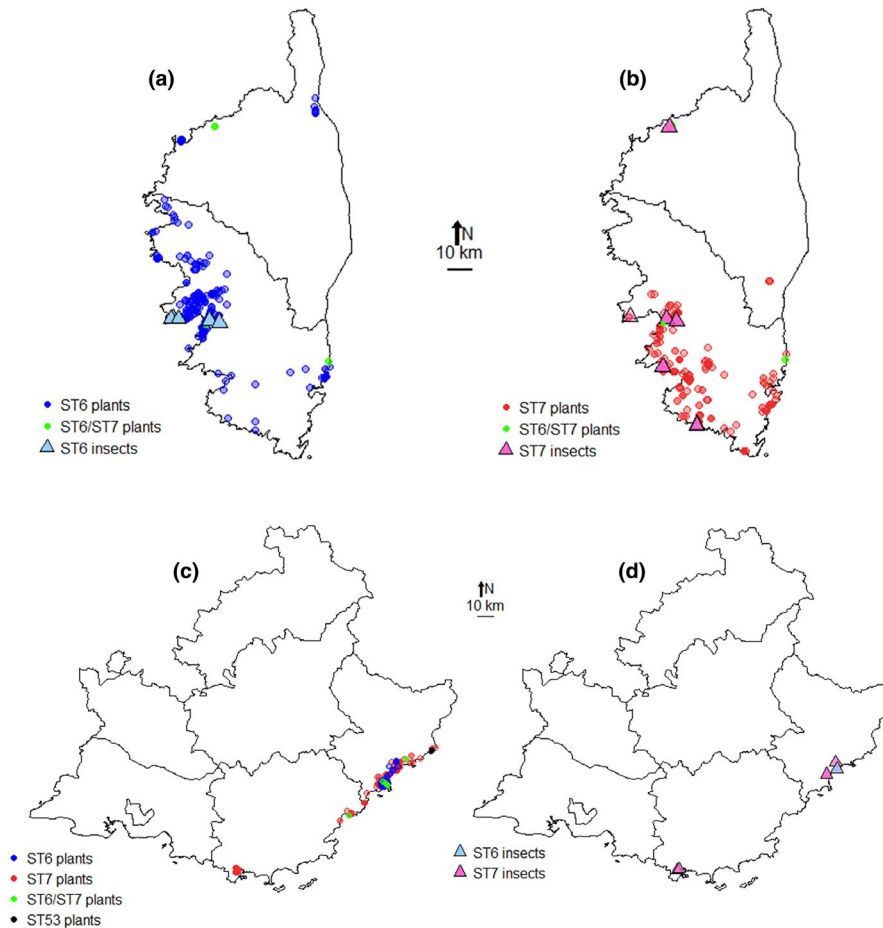
**FIGURE 4** Distribution of the *Xylella fastidiosa* bacterial load values (cells/head) estimated in the 193 positive heads from *Philaenus spumarius* insects collected in Corsica between October and March, from 2015 to 2018

These suitable and accurate methods to detect *X. fastidiosa* and to identify its subspecies in its vector *P. spumarius* could be applied for the surveillance of *X. fastidiosa*. The pool method is suitable for surveillance on a large scale, including large collection of insects in pest-free areas or in buffer zones. These methods were applied to 4,062 individuals collected from infected areas in France from 2015 to 2019. The high repeatability and reproducibility of the Harper-loos duplex was confirmed in our conditions, because for 91% of the positive samples both PCR replicates were positive, 9% of positive samples were close to the detection threshold limit ( $C_t$  close to 38), for which a replicate of both was positive ( $C_t$  lower than 38). The ddPCR assays allowed us to determine that a  $C_t$  value of 38 (Harper's cut-off) corresponds to a bacterial load of 29 cells/head, which is the technical limit of this real-time PCR protocol. All these results confirmed that the Harper-loos duplex is effective to detect *X. fastidiosa* in naturally infected *P. spumarius*. The MLST scheme of Yuan *et al.* (2010) is sensitive enough to identify the *X. fastidiosa* subspecies, as in 89% of the tested samples the *multiplex* subspecies was determined, and the ST was identified for 81.1% of them. MLST was also successful for Harper-positive samples with  $C_t$  values higher than 30, such as 34.6. This highlights the suitability of the MLST scheme of Yuan *et al.* (2010) to identify the subspecies and the ST of *X. fastidiosa* in *P. spumarius*. In this study, only the ST6 and ST7 sequence types of the *multiplex* subspecies were detected in *P. spumarius* collected in Corsica and PACA regions, in accordance with the official results reported in plants. In fact, the same STs were identified in insects and plants, as sequences were perfect matches to genotypes already present in the 12 sampled locations. Interestingly, in PACA the insect tests showed the presence of ST7 and ST6 in the infected area 2015/218, where only ST7 was identified in plants. This result highlights the relevance of combining insect and plant collections for *X. fastidiosa* surveillance, which will be included in future EU rules for *X. fastidiosa* surveillance. The presence of ST6 and ST7 in the same

plant samples has previously been described in France (Denancé *et al.*, 2017). Even if some *P. spumarius* were collected in areas where both STs were present, it is not surprising if ST6 and ST7 are not found in the same individual, as only 1.6% of typed plant samples (12 among 761 for which the ST was determined) were coinfecting by both STs. For this study, no collection of insects was performed in Menton where the subspecies *pauca* ST53 was found in 2015, and more recently in 2019.

The positive infection rate of the 3,820 *P. spumarius* collected in Corsica was estimated for each infected area to range from 2.5% to 33.3%, but the number of individuals collected and analysed individually was not identical between each area, and varied from 1 to 255. Cruaud *et al.* (2018) reported that the prevalence of *X. fastidiosa* in *P. spumarius* collected in October in Corsica ranged from 12.5% to 34.4%. Similarly, the positive infection rate of the 242 *P. spumarius* collected in PACA was estimated to range from 20% to 50%, but the number of individuals collected in each area was low and varied from 1 to 61. In Italy, bacterial prevalence in *P. spumarius* has also been investigated. Early studies reported 67% infected individuals among *P. spumarius* collected in November in ground vegetation from *X. fastidiosa* subsp. *pauca*-infected olive orchards in the Salento area (Saponari *et al.*, 2014). Cornara *et al.* (2017) showed that the percentage of infected *P. spumarius* was over 50% throughout the summer, with a maximum in August. In Alicante, Spain, where only *X. fastidiosa* subsp. *multiplex* ST6 was reported (EFSA, 2018), 27% of tested *P. spumarius* were positive (Cornara *et al.*, 2019).

The ddPCR technique, which was already developed to detect *X. fastidiosa* in plants (Dupas *et al.*, 2019), was applied to quantify the *X. fastidiosa* bacterial load hosted by *P. spumarius*. This method has previously been used to detect and quantify human pathogens in mosquitoes (Jongthawin *et al.*, 2016), but to our knowledge it was applied here for the first time to detect and quantify plant pathogenic bacteria in an insect. The ddPCR technique was a suitable



**FIGURE 5** Geographical distribution of the *Xylella fastidiosa* subspecies and sequence types (STs) identified in plants and *Philaenus spumarius* in Corsica and PACA regions. In Corsica: (a) distribution of ST6 (in blue) and coinfection ST6/ST7 (green) in plants (dots) and in *P. spumarius* (triangles); (b) distribution of ST7 (in red) and coinfection ST6/ST7 (green) in plants (dots) and in *P. spumarius* (triangles). In PACA: (c) distribution of ST6 (in blue), ST7 (in red), ST6/ST7 (green), and ST53 (black) in plants; (d) distribution of ST6 (in blue) and ST7 (in pink) in *P. spumarius*. For maps (a), (b), and (c), each dot corresponds to a plant sample, whereas for maps (a), (b), and (d), each triangle corresponds to the ST(s) identified in all *P. spumarius* tested in one or more areas for a given municipality

tool to estimate the population of *X. fastidiosa* in *P. spumarius*. The bacterial load assessed by applying ddPCR to *P. spumarius* collected in France was high when compared to values reported for other insects in the literature. Even though the bacterial load mainly ranged between  $10^3$  and  $10^4$  cells per head, it reached  $10^5$  cells and up to  $10^6$  cells in individuals collected in October, November, and December in Corsica, representing 13% of the infected individuals analysed individually in Corsica. Recently, Sicard *et al.* (2020) studied the instantaneous population growth dynamics of *X. fastidiosa* subsp. *fastidiosa* in plants and insects. Their study revealed that during the replication phase, the copy number of genes close to the origin of replication can be higher than those close to the terminus, but as *X. fastidiosa* grows very slowly, the ratio between these copy numbers (peak-to-trough, PTR) is not very high, less than two in plants and insects. In our study, we cannot exclude that some of the bacterial population in the collected *P. spumarius* were in the replication phase during the DNA extraction, and that the copy number of the gene *rimM* targeted by the real-time PCR Harper could be

higher than one copy. In the light of the study of Sicard *et al.* (2020), we suggest that the maximum estimated bacterial population in *P. spumarius* head could be modulated with a weak uncertainty of between  $5 \times 10^5$  cells/head and  $10^6$  cells/head. Ranieri *et al.* (2020) published a comparison of the foregut morphometry and fluid dynamics between two *X. fastidiosa* vectors, *P. spumarius* and the blue-green sharpshooter *Graphocephala atropunctata*. They showed that *P. spumarius* foregut morphology allowed the insect to host twice as many cells as *G. atropunctata*, which harbours around  $10^4$  cells per individual. These results are in accordance with the fact that the bacterial load of *X. fastidiosa* detected here in *P. spumarius* was found to be higher, compared to *G. atropunctata*. So far, in Europe, the population of *X. fastidiosa* subsp. *pauca* hosted by *P. spumarius* has been estimated in Italy by quantitative PCR (qPCR) at  $10^2$  to  $<10^4$  cfu per individual (Cornara *et al.*, 2016; Cavalieri *et al.*, 2019). When qPCR is based on a standard curve built with bacterial suspension calibrated by counting colonies on media, there is a risk of underestimating the population, as the bacterium easily forms aggregates (Cornara

*et al.*, 2016). The type of method used to estimate the bacterial load, such as qPCR or ddPCR (based on DNA copies detection), could explain the discrepancies of population estimation. Esteves *et al.* (2019) revealed that different STs, all belonging to the subspecies *pauca*, influenced the vector competence, such as acquisition and transmission rates. We can hypothesize that it could be the same for *P. spumarius*, which could explain the discrepancies of bacterial population size estimated in Italian individuals hosting ST53 and in French individuals hosting ST6 or ST7. However, the ST might not be the only factor explaining these differences of estimated bacterial load, as several factors (host plant, vector species, bacterial concentration in host plant, chitin degradation, temperature, crop management practices) represent traits influencing bacterial acquisition by vectors, multiplication in the vector, and transmission to plants, as discussed in a review by Sicard *et al.* (2018). It will be interesting to compare our results with bacterial load estimation in *P. spumarius* hosting subspecies *multiplex* in Italy, Spain, or Portugal (Landa, 2017; EPPO, 2019a).

We report here for the first time in Europe the presence of *X. fastidiosa*-infected *P. spumarius* adults during winter. Among 144 *P. spumarius* adults collected in Corsica during winter, 3.5% hosted *X. fastidiosa* with a bacterial load of an order of magnitude of 10 to 10<sup>3</sup> cells/head. Positive individuals were collected in 2017, in January (four positives from three different infected areas) and one in March. Unfortunately, we are not able to draw conclusions for the PACA region, as no *P. spumarius* was collected during winter. It was reported that in olive groves from southern Italy, a few adults may be present during winter, but it was not mentioned whether they were tested for *X. fastidiosa* (Bodino *et al.*, 2019). Cornara *et al.* (2016) showed that there is a correlation between the size of the bacterial population in the vector's foregut and the success in pathogen transmission. As the positive adults collected during winter in Corsica hosted low bacterial populations, the transmission of the bacteria could be less efficient than in autumn, when the bacterial load in insects is at its maximum. In the current context of global warming, in the future *P. spumarius* may be reported in winter not only in France, but also in other parts of Europe with mild winters, and where the extension of distribution of *P. spumarius* has already been predicted (Cruaud *et al.*, 2018). However, further studies with larger numbers of collected *P. spumarius* individuals will provide additional evidence about their impact on the spread and the epidemiology of the disease.

Our study provides efficient methods for the detection of *X. fastidiosa* and its identification at the subspecies level for epidemiologic study and surveillance in its host plants and its main known insect vector in Europe, *P. spumarius*. The analysis of around 4,000 *P. spumarius* individuals collected in France, either in Corsica or in PACA regions, allowed us to provide new insights into the high competence of *P. spumarius* to host the bacterium, and about *X. fastidiosa*-infected *P. spumarius* adults found in Corsica during winter, and therefore potentially infectious from the early period of the year. *X. fastidiosa* could rest over the winter in the insect and retain the same geographic distribution on different host plants, in the absence of

control measures, which might increase in the context of ongoing global climatic change. This new epidemiologic information should be considered when examining the establishment of insect vectors, and disease surveillance.

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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