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Specific detection and quantification of the phytopathogenic agent '*Candidatus* Phytoplasma prunorum'

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ABSTRACT

'*Candidatus* Phytoplasma prunorum' is a wall-less bacterium associated with European stone fruit yellows (ESFY), a severe disease of *Prunus* spp. (mainly apricot and Japanese plum trees). It can be spread by one insect vector, *Cacopsylla pruni*, and by the trade of infected material. The availability of PCR-based methods allowing a sensitive and specific detection of '*Ca.* P. prunorum' is crucial for this phytoplasma because, at present, it is uncultured and cannot be detected serologically. We developed a PCR test which, in contrast to the existing detection tools, provides a fast, specific and sensitive detection of '*Ca.* P. prunorum' in plants and insects. For studies requiring an absolute quantification of the phytoplasma titer, the same primers were used to develop a real-time PCR assay, including a standard for *C. pruni*. The sensitivity of these molecular tools was compared by serial dilutions and their specificity was assessed both *in silico* and experimentally for reference strains and field samples of the closely related phytoplasma '*Ca.* P. prunorum', '*Ca.* P. pyri' (pear decline agent) and '*Ca.* P. mali' (apple proliferation agent), as well as for representative strains of the '*Ca.* Phytoplasma' genus.

1. Introduction

Phytoplasmas are uncultured wall-less bacteria (class Mollicutes) that live in the phloem of their host plants and are transmitted by insect vectors belonging to the order Homoptera [1]. The Phytoplasmas are presently subdivided into 15 groups based on the similarity of their 16S ribosomal DNA (rDNA) sequences [2]. '*Candidatus* Phytoplasma prunorum' ('*Ca.* P. prunorum') is a member of the 16SrX group. It is associated with European stone fruit yellows (ESFY) [3,4], a disease affecting most of wild and cultivated *Prunus* species [5,6]. This disease is naturally vectored by only one insect species, *Cacopsylla pruni* [7]. The trade of infected planting material can also contribute to disease spread, and ESFY causes substantial economic loss due to the decline and death of the infected trees (mainly apricot and Japanese plum trees). Widespread in Europe, it is recorded as a quarantine disease by the main organizations in charge of plant protection. On cultivated stone fruit trees, ESFY generally induces yellows, tree decline, and vegetative disorders with typical symptoms such as an early bud break and leaf rolling [3]. However, the visual detection sometimes suffers from a lack of sensitivity and specificity. In fact, the nature and intensity of these symptoms can depend on the season, host plant, and strain of '*Ca.* P. prunorum' [8,9]; some infected plants can even be asymptomatic [10,11]. Moreover, several other phytoplasmas have been detected in *Prunus*, some of which have a significant prevalence. Peach trees (*Prunus persica*) clearly exemplify this situation because, in the orchard, some symptoms of ESFY, western X-disease, and peach yellow leaf roll (PYLR) are similar [12]. Peach trees naturally infected by '*Ca.* P. phoenicium' [13], '*Ca.* P. asteris' [14] or '*Ca.* P. australiense' [15] also displayed yellows symptoms. Such yellows may be confounded with ESFY symptoms and may result in an erroneous diagnosis. Thus, a more sensitive and specific diagnostic test is often necessary to complement the visual detection of ESFY either in epidemiological studies or for plant protection services in charge of disease management and imported plants quarantine.

As no serological test is available for ESFY and the phytoplasmas are yet uncultured, the current alternatives to symptom-based diagnosis rely on PCR amplification of a fragment of '*Ca.* P. prunorum' genome. PCR primers generally target the rDNA region. The generic primer pair fU5/rU3 [16] is often used for the sensitive detection of a wide range of phytoplasmas; the primer pair P1/P7 is also frequently used but it also amplifies other Mollicutes [17]. Plant samples sometimes contain epiphytic or endophytic bacteria,

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including other phytoplasmas or members of the genus *Acholeplasma* [18], the closest relative of the phytoplasmas. These bacteria, as well as chloroplasts, have been reported to generate false positives because some generic primers match shared portions of DNA [19,20]. The specific detection of '*Ca.* P. prunorum' in insects is challenging too, because they show no symptoms and they contain a wealth of gut and cuticle bacteria, plant pathogens (potentially including other phytoplasmas), as well as one obligatory endosymbiotic species and, frequently, secondary endosymbionts [21,22]. When the aim is to specifically detect the ESFY phytoplasma, the number of false positives can be reduced by designing primers on slightly less conserved zones of the rDNA region. More specificity has been achieved through the detection of subclades that includes the ESFY agent [16,23–25]. However, the corresponding primers were designed to amplify several phytoplasmas and they would not differentiate PYLR from ESFY on peach, for example. As a result, complementary tests are required when the specific identification of '*Ca.* P. prunorum' is at stake. A primer pair targeting a non-ribosomal DNA sequence has also been designed to detect '*Ca.* P. prunorum' but the specific detection was obtained at the expense of sensitivity [5], and thus the classical approach still relies on the amplification of an rDNA fragment, followed either by oligonucleotide hybridization [26] or more frequently by several enzymatic digestions [4,16], which are both time- and resource-consuming. In this article, our aim is to provide new sensitive and cost-effective protocols dedicated to the specific detection and/or real-time quantification of '*Ca.* P. prunorum' from plants and insects sampled either in experimental, commercial, or natural conditions.

2. Materials and methods

2.1. Sources of phytoplasmas and psyllids

X. Foissac (INRA, Bordeaux, France) kindly provided reference strains of '*Ca.* P. asteris' (European aster yellows), peach Western X, '*Ca.* P. mali' (strains AP15^R and AT), Stolbur C (strain StolC), '*Ca.* P. pyri' (strain PD1^R) and '*Ca.* P. phoenicium' (http://www.bordeaux.inra.fr/umr1090/coll_isola.htm). These phytoplasmas had been maintained in *Catharanthus roseus* (periwinkle), except '*Ca.* P. phoenicium' which was extracted from infected almond leaves. Our reference strains of '*Ca.* P. prunorum' (FO00, PO00 and TG01) are maintained in *Prunus marianna* GF 8-1 rootstocks. In order to further examine the robustness of this diagnostic test to the potential variability associated with field samples, additional samples were taken from a range of wild and domesticated *Prunus* species with ESFY symptoms, and our own collection was complemented by more reference strains and field samples of the 16SrX phytoplasma group, kindly provided by L. Carraro (University of Udine, Italy), W. Jarausch (AlPlanta, Neustadt/W., Germany) and A. Laviña (IRTA, Barcelona, Spain). Adult *C. pruni* were collected from shelter conifers at the end of their overwintering period. The psyllids were conserved at −80 °C in 1.5 ml Eppendorf tubes until DNA extraction.

2.2. DNA extraction

Total DNA was extracted from petioles of *C. roseus* or from 0.5 g of phloem from woody shoots of the fruit trees as described previously [27]. Plant material was collected under sterile conditions to prevent cross-contamination (especially from experimental hosts with a high phytoplasma titer). Total insect DNA was extracted following Marzachi et al. [28], and 1 μl of Glycoblue (15 mg/ml) (Ambion) was added to improve precipitation and to dye DNA pellets. Finally, plant and insect DNA was stored at −20 °C after resuspension, respectively in 100 μl and 30 μl of DEPC-treated water.

Table 1
Sequence of the primers designed to detect or quantify '*Ca.* P. prunorum' and *C. pruni* DNA.

Target/Name	Sequence (5′ → 3′)
'*Ca.* P. prunorum' 16S-ITS rDNA	
ESFYf (forward)	CCATCATTTAGTTGGGCACT
ESFYr (reverse)	ATAGGCCCAAGCCATTATTG
C. pruni 18S rDNA	
CPf (forward)	CAAGTACGTCCCCGTTGATCA
CPr (reverse)	GCTGGCTGACATCGTTTATGG

2.3. ESFY-specific PCR

Previous work on the 16S-ITS-23S rDNA sequences identified several primers differentiating some closely related '*Candidatus Phytoplasma*' species [24]. Based on the alignment of phytoplasma rDNA sequences obtained with Clustal W v1.83 [29], one of these primer pairs (fAT/rPRUS) has been modified to design new ESFY-specific primers (ESFYf/r, in Table 1) that allow more stringent PCR conditions. This primer pair amplified a 504-bp fragment and specifically matched the sequence of the reference strain of '*Ca.* P. prunorum' ESFY-G1^R (GenBank accession no. AJ542544). Primer ESFYr had at least three mismatches with the sequences of the other phytoplasmas, and the sequence corresponding to primer ESFYf was poorly conserved outside the 16SrX group (Fig. 1). Extensive BLAST searches against the NCBI GenBank database confirmed that these primers were highly unlikely to amplify DNA from other known organisms.

Each amplification reaction was performed in 20 μl containing 1 μl of template DNA, 1X PCR buffer and 0.5 unit of Taq DNA polymerase (Invitrogen), 1.5 mM $MgCl_2$, 125 μM of each dNTP, and 0.35 μM of each primer. The best balance between sensitivity and specificity was obtained with the following two-step PCR: denaturation at 94 °C for 1 min, followed by 20 cycles at 94 °C for 30 s, 65 °C for 20 s, and 72 °C for 45 s, and then 20 cycles at 94 °C for 30 s, 62 °C for 20 s, and 72 °C for 45 s. Then, 8 μl of the amplification product was analyzed by electrophoresis on 1.5% agarose gels in 0.5X TBE buffer, and visualized using a UV transilluminator after ethidium bromide staining. To check the robustness of the method, the amplification was carried out in two thermocyclers with different ramping rates: T1 (Biometra) and PT100 (MJ Research).

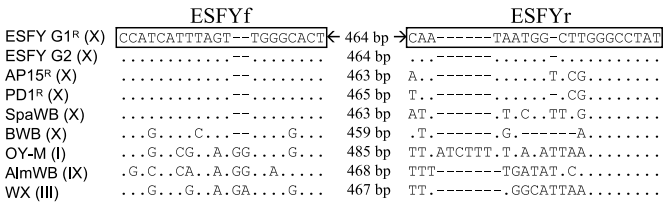

Fig. 1. Sequence alignment of the 16S-ITS rDNA region showing the specificity of the ESFYf/r primers in relation to other phytoplasmas belonging to the 16SrX group or occurring in stone fruit trees. The 16S rDNA groups [2] are indicated in parentheses. The GenBank accession no. for '*Ca.* P. prunorum' (ESFY-G1^R and ESFY-G2), '*Ca.* P. mali' (apple proliferation, strain AP15^R), '*Ca.* P. pyri' (pear decline, strain PD1^R), spartium witches' broom (SpaWB), buckthorn witches' broom (BWB), '*Ca.* P. asteris' (OY-M), almond witches' broom (AlmWB), and western X (WX) phytoplasmas are AJ542544, AJ542545, AJ542541, AJ542543, X92869, X76431/AJ583009, NC_005303, AF515637, and AF533231, respectively. In the fragment showed, PYLR (Y16394) has the same sequence as PD1^R.

Table 2
Origin of the *C. pruni* individuals from which the 18S rDNA sequences were obtained.

Identifier	Place collected in France[a]	Host plant	Date collected	GenBank identifier
AigPinus1	L'Espérou, G	*Pinus* sp.	Jan-03	DQ778629
MNspino1	Mas de Londres, H	*Prunus spinosa*	Feb-02	DQ778630
MNspino2	Mas de Londres, H	*P. spinosa*	Feb-02	DQ778631
TorMyro1	Torreilles, PO	*P. cerasifera*	May-02	DQ778632
TorMyro2	Torreilles, PO	*P. cerasifera*	May-02	DQ778633
TorSpino1	Torreilles, PO	*P. spinosa*	May-02	DQ778634
TorSpino2	Torreilles, PO	*P. spinosa*	May-02	DQ778635

[a] Nearest village; the department is abbreviated (G: Gard; H: Hérault; PO: Pyrénées-Orientales).

2.4. Quantitative real-time PCR

The specific primer pair ESFYf/r was also used for the real-time quantification of '*Ca.* P. prunorum'. An internal control suitable for a wide range of plants has already been described [30]; we used a reference gene from *C. pruni* to take account of any variability in the yield of insect DNA extractions.

2.4.1. Primers for C. pruni control

As no sequence was published for *C. pruni*, a fragment of the 18S rDNA was amplified by PCR and sequenced for 7 *C. pruni* caught on 3 different hosts (Table 2). DNA was extracted as described above and we used a protocol previously described by Miquelis et al. [31] to amplify a 944-bp DNA fragment. Then, the PCR products were directly sequenced with a MegaBACE 1000 automated sequencer (Amersham), and the sequences were deposited in GenBank (Table 2). Finally, after excluding the highly conserved regions of the 18S rDNA and one fragment showing intraspecific diversity, the primer pair CPf/r (Table 1) was chosen using the software Primer Express v1.0 (Applied Biosystems). The 92-bp portion of DNA amplified with CPf/r was completely identical for the 7 *C. pruni* sequences and also for *Trioza eugeniae* (GenBank accession no. U06482), the only homologous sequences available in the Psylloidea clade apart from one undetermined Psyllidae from Australia (GenBank accession no. DQ532498) with only one mismatch (on primer CPr). Thus, CPf/r primers are expected to amplify DNA from any *C. pruni*, and probably from any other *Cacopsylla*.

2.4.2. Calibration curves

After amplification of '*Ca.* P. prunorum' and *C. pruni* DNA with the primers ESFYf/r and CPf/r (respectively), the PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega) and cloned using the pGEM-T Easy Vector System II (Promega). A plasmid solution was obtained and purified with Wizard Plus SV Minipreps DNA Purification System (Promega), and an Ultraspec 3000 spectrophotometer (Amersham) was used to determine its concentration. We used 10-fold serial dilutions of these DNA references to generate calibration curves for the absolute quantification of rDNA targets in '*Ca.* P. prunorum' and *C. pruni*. The PCR efficiency (E) and the target copy number (N_T) in samples with an unknown number of templates were derived from the equation of the regression line fitted to the calibration curve [32] as $E = 10^{-S} - 1$ and, for any sample with its threshold cycle (C_T), measured by real-time PCR, $N_T = 10^{S \times C_T + I}$ (S and I being the slope and intercept of the regression line, respectively). Then, the estimated number of phytoplasmas (N_P) inside an individual insect was given by $N_P = a \times N_T/2 = 3.75 \times N_T$, a being the overall sampling factor and $N_T/2$ being the estimated number of phytoplasma genomes in the sample, as there are 2 rDNA operons per '*Ca.* P. prunorum' genome [33].

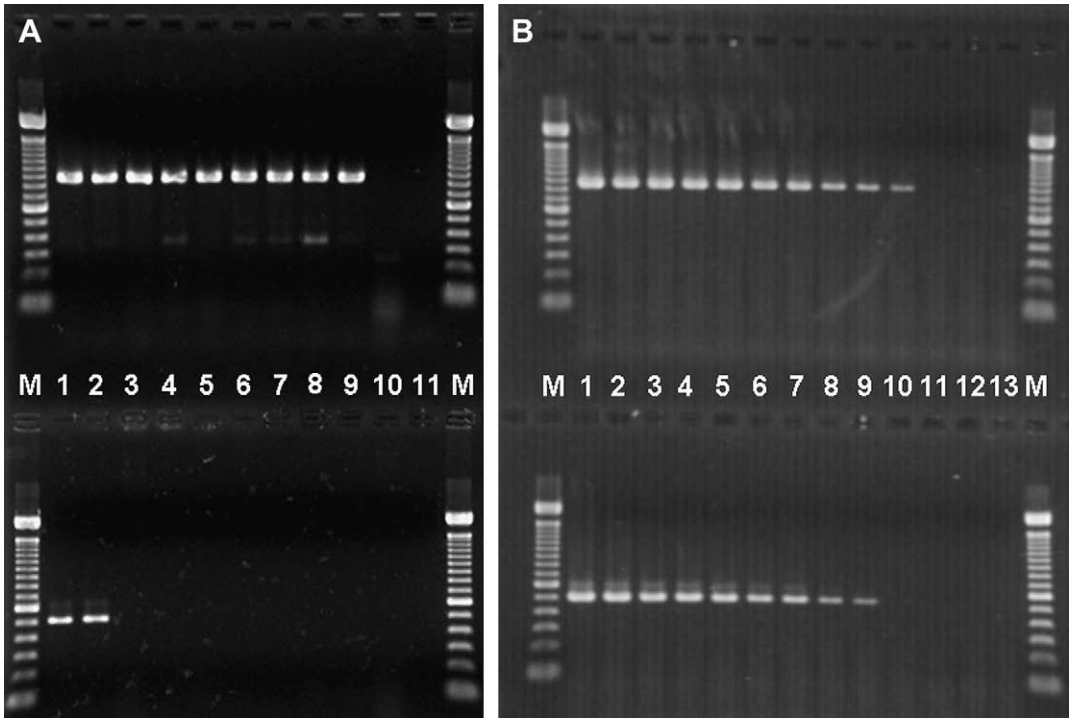

Fig. 2. Agarose gels (1.5%) of PCR products generated by the primers ESFYf/r (bottom) in comparison to the generic primers fU5/rU3 (top). (A) Specificity of the PCR diagnostic test with ESFYf/r. Lanes: (1–2) '*Ca.* P. prunorum' (strain FO00 from a *P. marianna* and a *Cacopsylla pruni*, respectively), (3) '*Ca.* P. asteris' (European aster yellows), (4) peach Western X, (5–6) '*Ca.* P. mali' (strains AP15^R and AT, respectively), (7) '*Ca.* P. phoenicium', (8) '*Ca.* P. pyri' (strain PD1^R), (9) Stolbur C, (10) healthy periwinkle, (11) healthy *C. pruni*, (M) 100 bp DNA marker. (B) Sensitivity of the PCR diagnostic test with ESFYf/r, estimated from 10-fold serial dilutions of '*Ca.* P. prunorum' (strain FO00) from 10^{-1} to 10^{-6} (odd lanes from 1 to 11) and from 5×10^{-2} to 5×10^{-6} (even lanes from 2 to 10), (12) healthy *P. marianna*, (13) PCR mix, (M) 100 bp DNA marker.

2.4.3. Real-time PCR amplification conditions

The real-time PCR reactions were performed with the qPCR Core kit for SYBR green I No ROX (Eurogentec), using the following reagents: 5 μl of plant or insect total DNA extract, 2.5 μl of 10X buffer and 0.625 unit of Hot GoldStar DNA polymerase, 0.3 μM (ESFYf/r) or 0.2 μM (CPf/r) of each primer, 0.2 mM of each dNTP, 4 mM of $MgCl_2$ for ESFYf/r (2 mM for CPf/r), 0.75 μl of SYBR green I, and sterile water to adjust the final volume to 25 μl. After a denaturation step at 95 °C for 10 min, 40 cycles alternating between 95 °C for 30 s, 63 °C for 30 s and 72 °C for 45 s were followed by a final cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. The analyses were performed using the Mx3000P real-time PCR system and software (Stratagene).

2.5. Comparison of the sensitivity of the new methods

The detection test with the generic primers fU5/rU3 [16] was used as a benchmark to check the specificity and sensitivity of our diagnostic test. Each amplification reaction was performed in 20 μl containing 1 μl of template DNA, 1X PCR buffer and 0.5 unit of Taq DNA polymerase (Invitrogen), 1.5 mM $MgCl_2$, 125 μM of each dNTP, and 0.5 μM of each primer, with the following PCR conditions: 92 °C for 1 min; then 35 cycles at 92 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and a final elongation step at 72 °C for 4 min.

A nested PCR protocol was also included in the comparison between the different methods because this procedure is often used wherever maximum sensitivity is expected. After a pre-amplification with the primer pair PA2F/R [34], 1 μl of a 30-fold dilution of the PCR product was re-amplified with ESFYf/r primers as described in Section 2.3. The dilution series required to assess the absolute sensitivity of the different ESFYf/r-based methods were prepared as follows: total DNA extracted from a *P. marianna* infected by '*Ca.* P. prunorum' (strain FO00) was diluted 10-fold; the phytoplasma concentration in this extract was determined by real-time PCR. It was then mixed with increasing volumes of DNA extract from a healthy *P. marianna*, providing each method with the same number of targets per sample. Note that real-time PCR uses 5 μl of DNA extract per reaction instead of 1 μl for the other methods, so the corresponding dilution series was 5 times less concentrated in order to reach the same amount of targets per reaction.

3. Results

3.1. Validation of the specific PCR

When experimentally tested on 9 strains corresponding to 7 different phytoplasmas (among which the closely related '*Ca.* P. pyri' and '*Ca.* P. mali'), the ESFYf/r primers consistently detected only '*Ca.* P. prunorum' while the other phytoplasmas were also amplified by fU5/rU3 (Fig. 2A). The same results were obtained when comparing 19 '*Ca.* P. prunorum' strains (originating from 6 *Prunus* species and 4 countries) to 7 '*Ca.* P. pyri' and 9 '*Ca.* P. mali' strains (Table 3). In addition, when using the primers ESFYf/r in routine diagnosis (on 290 adult *C. pruni* and 240 *Prunus spinosa*) we did not detect any unspecific amplification of other prokaryotic organisms present in total DNA extracts (data not shown). The primer pair ESFYf/r was slightly less sensitive than fU5/rU3 (limit dilutions: 10^{-5} and 5×10^{-6}, respectively) (Fig. 2B).

Blast searches in the NCBI GenBank database against all the available sequences from fruit tree phytoplasmas showed that the primer pair ESFYf/r matched all of the 9 '*Ca.* P. prunorum' sequences (originating from 3 *Prunus* species and 4 countries), while the primer ESFYr differed from the 5 '*Ca.* P. pyri' sequences by 3 point mutations and from the 8 '*Ca.* P. mali' sequences by 1 or 2 more 1-nt insertions; the primer pair fU5/rU3 perfectly matched these 22 sequences (Table 4).

Combining the evidence from experimental and *in silico* tests, no polymorphism could be detected in the sequences matched by the primer pair ESFYf/r within '*Ca.* P. prunorum' strains originating from 6 *Prunus* species and 6 countries. Finally, 11 additional publicly available sequences (EU168782–EU168783, EF560638–EF560645, U54988) are 5′-truncated and thus provide information only on the sequence matched by primer ESFYr. These sequences originating from various laboratory strains and from *C. pruni* collected on 3 different host plants are all perfectly matched by primer ESFYr, which further supports the observed absence of nucleotide polymorphism within '*Ca.* P. prunorum' on this DNA fragment (data not shown).

3.2. Validation of the real-time PCR

3.2.1. Quantification of 'Ca. P. prunorum' DNA

From the slope of the standard curve (Fig. 3B), a PCR efficiency of 84% was calculated during the real-time amplification of '*Ca.* P.

Table 3
Experimental tests showing the specificity of the primer pair ESFYf/r on samples originating from a range of *Prunus* species in Europe, in comparison with the closely related '*Ca.* P. pyri' and '*Ca.* P. mali'.

Phytoplasma strain[a]	Provided by[b]	Origin		PCR test with	
		Host plant	Country	fU5/rU3	ESFYf/r
'*Ca.* P. prunorum'					
FO00*		Prunus armeniaca	France	+	+
PO00*		*P. armeniaca*	France	+	+
TG01*		*P. salicina*	France	+	+
LNp*	LC	*P. persica*	Italy	+	+
LN-S1*	LC	*P. salicina*	Italy	+	+
LN-S2*	LC	*P. salicina*	Italy	+	+
ESFY-87*	LC	*P. salicina*	Italy	+	+
G32*	WJ	*P. armeniaca*	France	+	+
VilArmen45		*P. armeniaca*	France	+	+
ValArmen1		*P. armeniaca*	France	+	+
MonDom1		*P. domestica*	France	+	+
MNspino26		*P. spinosa*	France	+	+
SMCsali19		*P. salicina*	France	+	+
3889-32	WJ	*P. armeniaca*	Germany	+	+
3889-33	WJ	*P. armeniaca*	Germany	+	+
4062-30	WJ	*P. marianna*	Germany	+	+
AL ESFY1	AL	*P. armeniaca*	Spain	+	+
AL ESFY2	AL	*P. armeniaca*	Spain	+	+
AL ESFY3	AL	*P. armeniaca*	Spain	+	+
'*Ca.* P. pyri'					
PD1[R*]	XF	*Pyrus communis*	Germany	+	–
3703-9	WJ	*P. communis*	Germany	+	–
3703-20	WJ	*P. communis*	Germany	+	–
3703-21	WJ	*P. communis*	Germany	+	–
3703-26	WJ	*P. communis*	Germany	+	–
AL PD2	AL	*P. communis*	Spain	+	–
AL PD3	AL	*P. communis*	Spain	+	–
'*Ca.* P. mali'					
AP15[R*]	XF	*Malus domestica*	Italy	+	–
AT*	XF	*M. domestica*	Germany	+	–
LC AP1	LC	*M. domestica*	Italy	+	–
LC AP2	LC	*M. domestica*	Italy	+	–
LC AP3	LC	*M. domestica*	Italy	+	–
3934-34	WJ	*M. domestica*	Germany	+	–
4062-22	WJ	*M. domestica*	Italy	+	–
4062-23	WJ	*M. domestica*	Germany	+	–
4062-27	WJ	*M. domestica*	Germany	+	–

[a] Reference strains conserved *in planta* by the provider (denoted by *) or field samples.
[b] The phytoplasmas have been collected by the authors, unless otherwise stated : LC: L. Carraro (University of Udine, Italy); WJ: W. Jarausch (AlPlanta, Neustadt/W., Germany); AL: A. Laviña (IRTA, Barcelona, Spain); XF: X. Foissac (INRA, Bordeaux, France).

Table 4
In silico tests showing the specificity of the primer pair ESFYf/r on DNA sequences originating from a range of *Prunus* species in Europe, in comparison with the closely related '*Ca.* P. pyri' and '*Ca.* P. mali'.

Phytoplasma strain	GenBank identifier	Origin		Mismatches with	
		Host	Country	fU5/rU3	ESFYf/r[a]
'*Ca.* P. prunorum'					
ESFY-G1^R	AJ542544	*Prunus persica*	Germany	0	0
ESFY-G2	AJ542545	*P. armeniaca*	Germany	0	0
ESFY4	Y11933	*P. armeniaca*	Czech Republic	0	0
ESFY5	AY029540	*P. armeniaca*	Austria	0	0
ESFY-63	AJ575107	*P. armeniaca*	Spain	0	0
ESFY-142	AJ575108	*P. armeniaca*	Spain	0	0
ESFY-173	AJ575106	*P. armeniaca*	Spain	0	0
ESFY-215	AJ575105	*P. armeniaca*	Spain	0	0
ESFY-2102	AM933142	*P. salicina*	Spain	0	0
'*Ca.* P. pyri'					
PD1^R	AJ542543	*Pyrus communis*	Germany	0	3 m.
PD3	Y16392	*P. communis*	Italy	0	3 m.
973PD	AJ964959	*P. communis*	Spain	0	3 m.
Unnamed	AY949984	*P. communis*	Serbia	0[b]	3 m.
PYLR1	Y16394	*Prunus persica*	USA	0	3 m.
'*Ca.* P. mali'					
AP15^R	AJ542541	*Malus domestica*	Italy	0	3 m. + 1 ins.
AT	X68375 CU469464	*M. domestica*	Germany	0	3 m. + 1 ins.
AP1/93	AJ542542	*M. domestica*	France	0	3 m. + 1 ins.
APU	AF248958	*Unknown*	Italy	0	3 m. + 1 ins.
T-3	EF392654	*M. domestica*	Italy	0	3 m. + 2 ins.
T-16	EF392655	*Cacopsylla melanoneura*	Italy	0	3 m. + 1 ins.
147	EF392656	*M. domestica*	Italy	0	3 m. + 2 ins.
Unnamed	AY598319	*M. domestica*	Czech Republic	0	3 m. + 1 ins.

[a] All the point mutations (m.) and 1-nt insertions (ins.) correspond to primer ESFYr.
[b] For primer rU3 only (the sequence is 5′-truncated).

prunorum' DNA. The dissociation (melting) curves for '*Ca.* P. prunorum' samples (Fig. 3A) had a single peak at 85 °C (indicating that only the targeted fragment was indeed amplified); the amplification curves (Fig. 3C) were smooth and parallel over a wide range of concentrations. In addition, Fig. 3C shows that no amplification was obtained from a healthy *P. marianna* (curve 12), from a healthy periwinkle (curve 13), and from a healthy (i.e., ESFY-negative when tested with the primers fU5/rU3) *C. pruni* (curve 14), which demonstrates that the other prokaryotes usually carried by these hosts of the phytoplasma were not amplified. The late amplification obtained for '*Ca.* P. pyri' and '*Ca.* P. mali' (very closely related to '*Ca.* P. prunorum') indicated that the specificity of this method could be challenged by highly concentrated lab samples (Fig. 3C, curves 10 and 11, respectively). The two highly infected overwintering *C. pruni* that we tested contained around 15 million copies of the '*Ca.* P. prunorum' genome (1.46×10^7 and 1.63×10^7).

3.2.2. Quantification of C. pruni DNA

The PCR efficiency estimated from the slope of the standard curve (Fig. 4B) was 88% for *C. pruni* DNA. The shapes of the dissociation curves (single peak at 83 °C for *C. pruni* samples; Fig. 4A) and of the amplification curves (Fig. 4C) indicated that the real-time

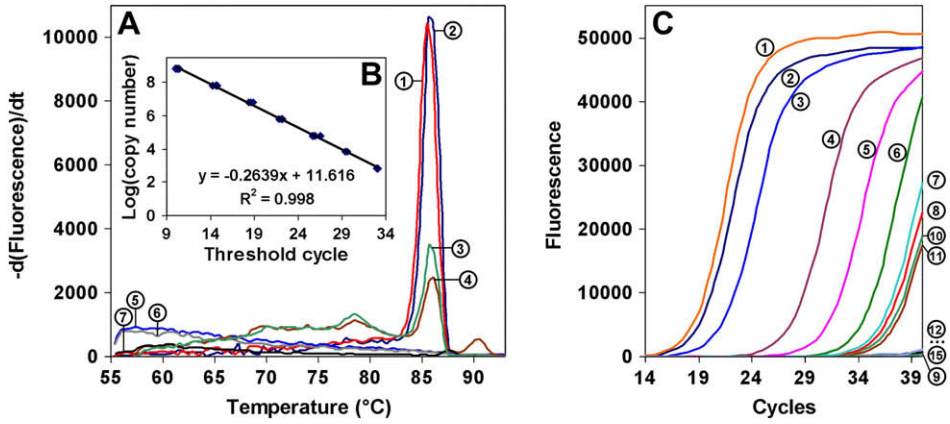

Fig. 3. Sensitivity and specificity of the real-time PCR amplification of '*Ca.* P. prunorum' DNA (16S rDNA region). (A) Dissociation curves for (1) a mature *C. pruni* infected by '*Ca.* P. prunorum', (2) a *P. marianna* infected by '*Ca.* P. prunorum' (strain FO00), (3) '*Ca.* P. pyri' (strain PD1^R), (4) '*Ca.* P. mali' (stain AP15^R), (5) a healthy *C. pruni*, (6) healthy periwinkle, (7) PCR mix. (B) Standard curve from serial dilutions of the plasmid solution. (C) Amplification curves for (1) a mature *C. pruni* infected by '*Ca.* P. prunorum', (2–9) serial dilutions of DNA extracted from a *P. marianna* infected by '*Ca.* P. prunorum' (strain FO00) with 2.76×10^6, 5.52×10^5, 10^4, 10^3, 10^2, 50, 20 and 10 targets, respectively, (10) '*Ca.* P. pyri' (strain PD1^R), (11) '*Ca.* P. mali' (stain AP15^R), (12) healthy *P. marianna*, (13) healthy periwinkle, (14) a healthy *C. pruni*, (15) PCR mix.

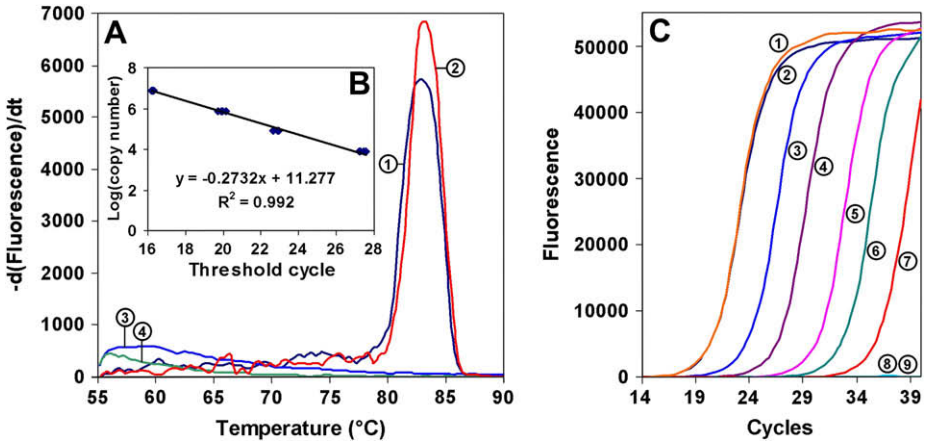

Fig. 4. Sensitivity and specificity of the real-time PCR amplification of *C. pruni* DNA (18S rDNA region). (A) Dissociation curves for (1) a mature *C. pruni* infected by '*Ca.* P. prunorum', (2) a healthy *C. pruni*, (3) a *P. marianna* infected by '*Ca.* P. prunorum' (strain FO00), (4) PCR mix. (B) Standard curve from serial dilutions of the plasmid solution. (C) Amplification curves for (1) a healthy *C. pruni*, (2–7) 10-fold serial dilutions of DNA extracted from a *C. pruni* infected by '*Ca.* P. prunorum' (from 10 to 10^{-5}), (8) a *P. marianna* infected by '*Ca.* P. prunorum' (strain FO00), (9) PCR mix.

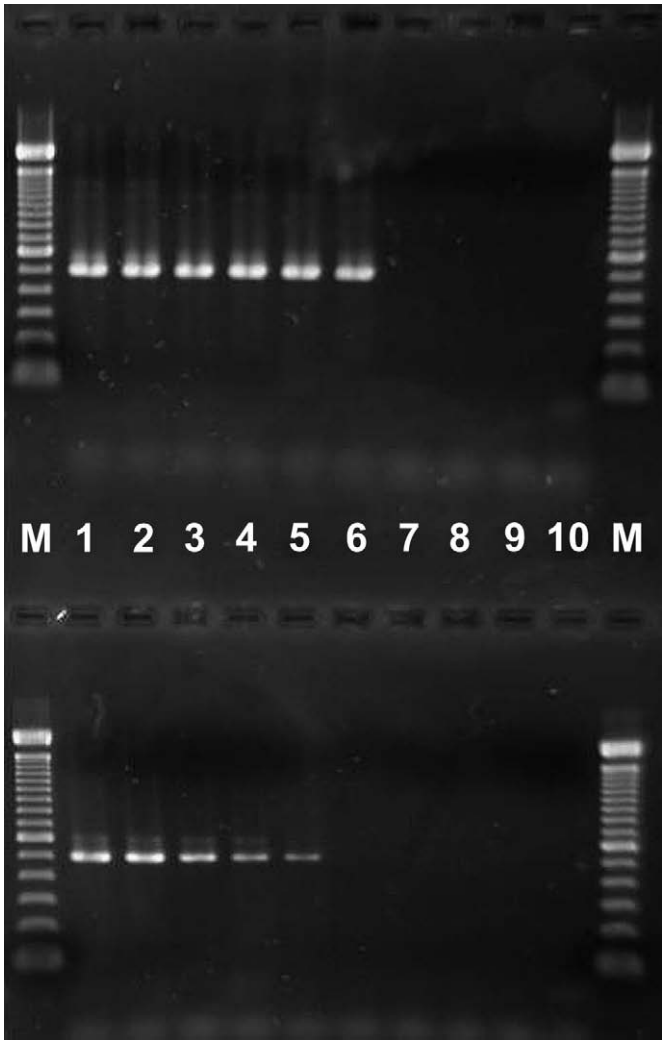

Fig. 5. Comparison of the PCR products generated by the primers ESFYf/r in simple PCR (bottom) or in nested PCR (top), visualized in 1.5% agarose gel. Lanes (1–8) serial dilutions of '*Ca.* P. prunorum' (strain FO00) with 5.52×10^5,10^4, 10^3, 10^2, 50, 20, 10, and 5 targets, respectively, (9) healthy *P. marianna*, (10) PCR mix, (M) 100 bp DNA marker.

amplification performed well. The primers CPf/r specifically amplified psyllid DNA, as shown in Fig. 4C by the absence of amplification from an ESFY-infected *Prunus* (curve 8) and by a similar amplification for healthy and infected psyllids (curves 1 and 2). A series of BLAST searches against the NCBI GenBank database further indicated that the primer pair CPf/r had no significant homology with known prokaryote sequences, and more generally with the rest of living organisms (except some phylogenetically related insects). The number of 18S rDNA targets measured in the samples from three adult *C. pruni* was so similar (mean: 3.48×10^5; coefficient of variation: 1.4%) that no correction was applied to the previously estimated numbers of phytoplasma per psyllid.

3.3. Comparison of the sensitivity of the new methods

The simple PCR can detect down to 50 targets (Fig. 5, bottom). This test is thus less sensitive than both the nested PCR (Fig. 5, top) and the real-time PCR (Fig. 3C, curve 8), which appeared to be equally sensitive (down to 20 targets).

4. Discussion and conclusions

Through this work, our aim was to provide specific and sensitive molecular methods in order to improve the diagnosis and study of European stone fruit yellows, a plant disease of significant economic concern. All the primers were based on the rDNA regions of the causal agent '*Ca.* P. prunorum' and of its vector *C. pruni*. The rDNA sequences are duplicated in '*Ca.* P. prunorum' [33], which doubles the sensitivity of the detection in comparison with other genes. In addition, the rDNA is the most frequently sequenced portion of the genomes throughout the tree of life. The nucleotide diversity in this zone within and between species is thus better known than for any other locus, which provides more confidence on the specificity of the corresponding PCR primers. However, the 16S rDNA is highly conserved within the 16SrX phytoplasma group: '*Ca.* P. prunorum' and '*Ca.* P. pyri' have 98.8% nucleotide identity in this zone [4]. Hence the interest of designing a primer on the subsequent internal transcribed spacer (ITS1), which is slightly less conserved. Even in this more variable portion, there are not many degrees of freedom to design good PCR primers, as '*Ca.* P. prunorum' and '*Ca.* P. pyri' have 98.5% nucleotide similarity in this zone [4]; thus there is a small residual trade-off between specificity and sensitivity.

The choice between the available molecular tools depends on the required level of specificity and sensitivity. If the issue is to detect any phytoplasma in a given sample, primers for the genus '*Ca.* Phytoplasma' should be used; further identification (included for new strains or species) can draw on RFLP analysis, ITS1 sequencing, or probe-based methods (e.g., [26] or TaqMan real-time PCR). However, if one just aims to detect the presence of '*Ca.* P. prunorum' in plants or insects, specific primers such as ESFYf/r are more cost-effective. These primers and the associated protocol described in this article constitute the first specific and sensitive PCR test for the detection of '*Ca.* P. prunorum' in plants or insects. This fast and cost-effective method is suitable for everyday diagnosis by researchers or plant protection services. In contrast to other primers (including fAT/rPRUS), ESFYf/r do not amplify '*Ca.* P. pyri' whose subtype PYLR (affecting peach trees) [4] has exactly the same sequence as '*Ca.* P. pyri' in the zone targeted by the primers (Table 4). The sensitivity is slightly lower than with the universal primers fU5/rU3; nevertheless, it can detect the pathogen down to 50 targets (Fig. 5), corresponding to 25 phytoplasmas per µl of DNA extract. If a higher level of sensitivity is required (e.g., for detecting the phytoplasma in a partially resistant plant species), ESFYf/r can be used in a nested PCR after a first round of amplification with an outer primer pair such as PA2F/PA2R [34]. As with the primers fU5/rU3, this procedure can detect around 20 targets (Fig. 5), corresponding to 10 phytoplasmas per µl.

In addition, when used with the SYBR green chemistry, the primers ESFYf/r provide quantitative information. The simplest approach consists in assessing the relative concentrations in different samples (e.g., comparison to a dilution series from an initial sample of unknown concentration). The approach described in this article is more informative: the absolute number of phytoplasmas in a given sample is estimated through comparing the real-time amplification of the tested DNA to serial dilutions of standard DNA of known concentration (i.e., plasmids containing the target 16S rDNA fragment). Such quantification relies on the assumption of a constant yield of DNA extraction. Using internal standards enables checking this assumption and/or evaluating the ratio of '*Ca.* P. prunorum' to host targets. Plant-specific standards have been defined by Christensen et al. [30]; in this study, we obtained the first sequences from *C. pruni* (each around 820 bp of the 18S rDNA) and we designed a primer pair (CPf/r) to quantify this target. The primers CPf/r are specific enough to avoid any cross-amplification from plant or phytoplasma material (Fig. 4A, curve 3; Fig. 4C, curve 8), but they are generic enough to be used in experiments involving other psyllid species. The estimated number of ESFY phytoplasma borne by infected *C. pruni* (around 1.5×10^7) seems to be in the lower range of previous measures for other phytoplasma species in their vector [35], but above the 2.47×10^5 '*Ca.* P. mali' genomes measured in *Cacopsylla picta* [36].

In addition to providing quantitative information, the real-time PCR is highly sensitive, and 20 targets can be detected (Fig. 3C, curve 8), corresponding to 2 phytoplasma genomes per µl. Because the annealing happens at a lower temperature than for the specific PCR test, the real-time PCR assay is not as specific: high titers of the closely related phytoplasmas ('*Ca.* P. pyri' and '*Ca.* P. mali') can be misinterpreted as low titers of '*Ca.* P. prunorum'. If such a high phytoplasma titer is expected in field samples, it would thus be preferable to dilute the samples or to use the proposed specific PCR detection test (if the quantitative information is not essential). Some of the real-time PCR protocols published for phytoplasmas have been successfully tested on the economically important 16SrX group (i.e., AP group). A few assays specifically target the eponym member of the AP group, responsible for apple proliferation [20,36,37], whereas more generic methods can amplify several phytoplasmas in this group, included '*Ca.* P. prunorum' [25,30,37]. Thus, our method complements the existing tests by providing a more specific detection and quantification for '*Ca.* P. prunorum'; in addition, an important internal control (of the consistency of DNA extraction from psyllids) is now available. This real-time PCR assay is mainly meant for quantifying the phytoplasma in plants or insects, but it can also be considered as a valuable alternative to the classical PCR procedure (in particular for high-throughput applications such as routine diagnosis, where real-time PCR is less expensive than conventional PCR) because it provides more information without the time- and resource-consuming post-PCR steps (e.g., electrophoresis, RFLP, nested PCR) that can also increase the risk of sample cross-contamination.

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References

[1] Weintraub PG, Beanland L. Insect vectors of phytoplasmas. Annu Rev Entomol 2006;51:91–111.
[2] The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. Int J Syst Evol Microbiol 2004;54:1243–55.
[3] Lorenz K-H, Dosba F, Poggi Pollini C, Llácer G, Seemüller E. Phytoplasma diseases of *Prunus* species in Europe are caused by genetically similar organisms. Z Pflanzenk Pflanzen 1994;101:567–75.
[4] Seemüller E, Schneider B. '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. Int J Syst Evol Microbiol 2004;54:1217–26.
[5] Jarausch W, Lansac M, Saillard C, Broquaire J-M, Dosba F. PCR assay for specific detection of European stone fruit yellows phytoplasmas and its use for epidemiological studies in France. Eur J Plant Pathol 1998;104:17–27.
[6] Carraro L, Loi N, Ermacora P. Transmission characteristics of the European stone fruit yellows phytoplasma and its vector *Cacopsylla pruni*. Eur J Plant Pathol 2001;107:695–700.
[7] Carraro L, Osler R, Loi N, Ermacora P, Refatti E. Transmission of European stone fruit yellows phytoplasma by *Cacopsylla pruni*. J Plant Pathol 1998;80:233–9.
[8] Jarausch W, Eyquard JP, Lansac M, Mohns M, Dosba F. Susceptibility and tolerance of new French *Prunus domestica* cultivars to European stone fruit yellows phytoplasmas. J Phytopathol 2000;148:489–93.
[9] Kison H, Seemüller E. Differences in strain virulence of the European stone fruit yellows phytoplasma and susceptibility of stone fruit trees on various rootstocks to this pathogen. J Phytopathol 2001;149:533–41.
[10] Carraro L, Ferrini F, Ermacora P, Loi N. Role of wild *Prunus* species in the epidemiology of European stone fruit yellows. Plant Pathol 2002;51:513–7.
[11] Torres E, Martín MP, Paltrinieri S, Vila A, Masalles R, Bertaccini A. Spreading of ESFY phytoplasmas in stone fruit in Catalonia (Spain). J Phytopathol 2004;152:432–7.
[12] Kison H, Kirkpatrick BC, Seemüller E. Genetic comparison of the peach yellow leaf roll agent with European fruit tree phytoplasmas of the apple proliferation group. Plant Pathol 1997;46:538–44.
[13] Abou-Jawdah Y, Karakashian A, Sobh H, Martini M, Lee I-M. An epidemic of almond witches' broom in Lebanon: classification and phylogenetic relationships of the associated phytoplasma. Plant Dis 2002;86:477–84.
[14] Anfoka GH, Fattash I. Detection and identification of aster yellows (16SrI) phytoplasma in peach trees in Jordan by RFLP analysis of PCR-amplified products (16S rDNAs). J Phytopathol 2004;152:210–4.
[15] Jones P, Arocha Y, Antesana O, Montilliano E, Franco P. First report of an isolate of '*Candidatus* Phytoplasma australiense' associated with a yellow leaf roll disease of peach (*Prunus persicae*) in Bolivia. Plant Pathol 2005;54:558.
[16] Lorenz K-H, Schneider B, Ahrens U, Seemüller E. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology 1995;85:771–6.
[17] Deng SJ, Hiruki C. Amplification of 16S rRNA genes from culturable and non-culturable Mollicutes. J Microbiol Methods 1991;14:53–61.

[18] Tully JG, Whitcomb RF, Rose DL, Bové J-M, Carle P, Somerson NL, et al. *Acholeplasma brassicae* sp. nov. and *Acholeplasma palmae* sp. nov., two non-sterol-requiring mollicutes from plant surfaces. Int J Syst Bacteriol 1994;44:680–4.
[19] Skrzeczkowski LJ, Howell WE, Eastwell KC, Cavileer TD. Bacterial sequences interfering in detection of phytoplasma by PCR using primers derived from the ribosomal RNA operon. Acta Hortic 2001;550:417–24.
[20] Baric S, Dalla-Via J. A new approach to apple proliferation detection: a highly sensitive real-time PCR assay. J Microbiol Methods 2004;57:135–45.
[21] Subandiyah S, Nikoh N, Tsuyumu S, Somowiyarjo S, Fukatsu T. Complex endosymbiotic microbiota of the citrus psyllid *Diaphorina citri* (Homoptera: Psylloidea). Zoolog Sci 2000;17:983–9.
[22] Spaulding AW, von Dohlen CD. Psyllid endosymbionts exhibit patterns of co-speciation with hosts and destabilizing substitutions in ribosomal RNA. Insect Mol Biol 2001;10:57–67.
[23] Lee I-M, Bertaccini A, Vibio M, Gundersen DE. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology 1995;85:728–35.
[24] Smart CD, Schneider B, Blomquist CL, Guerra LJ, Harrison NA, Ahrens U, et al. Phytoplasma-specific PCR primers based on sequences of the 16S–23S rRNA spacer region. Appl Environ Microbiol 1996;62:2988–93.
[25] Torres E, Bertolini E, Cambra M, Montón C, Martín MP. Real-time PCR for simultaneous and quantitative detection of quarantine phytoplasmas from apple proliferation (16SrX) group. Mol Cell Probes 2005;19:334–40.
[26] Poggi Pollini C, Bissani R, Giunchedi L. Occurrence of European stone fruit yellows phytoplasma (ESFYP) infection in peach orchards in Northern-Central Italy. J Phytopathol 2001;149:725–30.
[27] Maixner M, Ahrens U, Seemüller E. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. Eur J Plant Pathol 1995;101:241–50.
[28] Marzachi C, Veratti F, Bosco D. Direct PCR detection of phytoplasmas in experimentally infected insects. Ann Appl Biol 1998;133:45–54.
[29] Thompson JD, Higgins DG, Gibson TJ. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.
[30] Christensen NM, Nicolaisen M, Hansen M, Schulz A. Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. Mol Plant – Microbe Interact 2004;17:1175–84.
[31] Miquelis A, Martin J-F, Carson EW, Brun G, Gilles A. Performance of 18S rDNA helix E23 for phylogenetic relationships within and between the Rotifera–Acanthocephala clades. C R Acad Sci Ser III 2000;323:925–41.
[32] Rutledge RG, Côté C. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res 2003;31:e93.
[33] Marcone C, Seemüller E. A chromosome map of the European stone fruit yellows phytoplasma. Microbiology 2001;147:1213–21.
[34] Heinrich M, Botti S, Caprara L, Arthofer W, Strommer S, Hanzer V, et al. Improved detection methods for fruit tree phytoplasmas. Plant Mol Biol Rep 2001;19:169–79.
[35] Blomquist CL, Kirkpatrick BC. Frequency and seasonal distribution of pear psylla infected with the pear decline phytoplasma in California pear orchards. Phytopathology 2002;92:1218–26.
[36] Jarausch W, Peccerella T, Schwind N, Jarausch B, Krczal G. Establishment of a quantitative real-time PCR assay for the quantification of apple proliferation phytoplasmas in plants and insects. Acta Hortic 2004;657:415–20.
[37] Galetto L, Bosco D, Marzachi C. Universal and group-specific real-time PCR diagnosis of flavescence dorée (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insect vectors. Ann Appl Biol 2005;147:191–201.