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Detection and identification of '*Candidatus* Phytoplasma prunorum' in *Prunus* germplasm

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Abstract

The molecular characterization of the causal agent of diseases associated with several symptoms such as decline, yellowing, leaf roll and off-season growth in stone fruits made it possible to determine a common etiology, and the name ´European stone fruit yellows´ phytoplasma was proposed. Recently, the new taxonomical species description within the genus is '*Candidatus* Phytoplasma prunorum'. A 2-yr survey was carried out in two different *Prunus* collections of the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) including European and Japanese plum genotypes of various species and several interspecific hybrids used as rootstocks. Both off-season growth in winter and decline were observed. In order to identify the phytoplasma suspected as a causal agent, two different PCR methods were applied to all inspected trees. The first method was a nested PCR with 16Sr X group-specific primers followed by RFLP analysis. The second method was a direct PCR with specific primers for '*Ca* P. prunorum' (Eca1/Eca2). In the most symptomatic trees the presence of this phytoplasma was confirmed by at least one of the methods; negative results were obtained in asymptomatic trees. The nested PCR-RFLPs analysis was confirmed as a reliable method for routinary diagnosis rather than direct PCR.

Additional key words: ESFY, nested-PCR, off-season growth, RFLPs.

Resumen

Detección e identificación de '*Candidatus* Phytoplasma prunorum' en germoplasma de *Prunus*

La caracterización molecular del agente causante de enfermedades asociadas a varios síntomas, como el enrollamiento clorótico, anticipación de la floración y la brotación, leptonecrosis o amarillamiento en los frutales de hueso permitió determinar una etiología común, el fitoplasma 'European stone fruit yellows', recientemente clasificado taxonómicamente como '*Candidatus* Phytoplasma prunorum'. Durante dos años se llevó a cabo una prospección en dos colecciones del Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón, que comprende diferentes especies e híbridos interespecíficos, de origen europeo y japonés, dentro del género *Prunus*. Se observaron síntomas de anticipo de floración y brotación en invierno y cierto decaimiento. Con objeto de identificar al posible fitoplasma causante de la enfermedad se aplicaron dos métodos en todos los árboles inspeccionados. El primero fue una PCR anidada con cebadores específicos del grupo 16Sr X seguida de un análisis de los fragmentos de restricción por RFLP. El segundo método fue una PCR con cebadores específicos de '*Ca* P. prunorum' (Eca1/Eca2). En los árboles más sintomáticos se confirmó la presencia de los fitoplasmas por uno de los dos métodos, obteniéndose resultados negativos en los árboles asintomáticos. El primer método resultó ser más fiable que la PCR directa como método de diagnóstico rutinario.

Palabras clave adicionales: anticipo de floración, ESFY, PCR anidada, RFLPs.

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Abbreviations used: AP (apple proliferation), ACLR (apricot chlorotic leaf roll), CITA (Centro de Investigación y Tecnología Agroalimentaria de Aragón), CTAB (cetyl trimethyl ammonium bromide) ESFY (European stone fruit yellows), PCR (polymerase chain reaction), PD (pear decline), PLN (plum leptonecrosis), rDNA (ribosomal DNA), RFLP (restriction fragment length polymorphism), TE (tris ethylendiaminetetraacetic acid).

Introduction

Several fruit species are affected by decline, yellowing, leaf curl, and floral virescence, diseases associated with phytoplasmas. These diseases include three economically important disorders of temperate fruit trees, 'pear decline' (PD), 'apple proliferation' (AP) and 'European stone fruit yellows' (ESFY). In fruit trees these diseases are transmitted by vectors and grafting. Vectors are sucking insects, mainly leafhoppers and psyllids, but until now only *Cacopsylla pruni* has been described as a vector of ESFY (Carraro *et al.,* 2001; Jarausch *et al.,* 2001b), having been identified in several regions of Spain (Laviña *et al*., 2004; Sabate *et al.*, 2007).

ESFY was firstly described as a decline of Japanese plum in Italy (Goidanich, 1933) and it has been reported in several countries in Europe, as a serious problem in some Mediterranean countries, such as Spain (Sánchez Capuchino and Forner, 1973; Torres *et al.*, 2004), France (Desvignes and Cornaggia, 1982; Jarausch *et al.*, 1998), and Italy (Carraro *et al.*, 1998), where apricot and Japanese plum, the most sensitive species, are widespread. Until Lorenz *et al.* (1994) determined the common etiology of these diseases and proposed the single name 'European stone fruit yellows' (ESFY), the disease had been designated according to the symptoms observed in stone fruit trees such as 'apricot chlorotic leaf roll' (ACLR) (Morvan, 1977), 'plum leptonecrosis' (PLN) (Giunchedi *et al.*, 1978), and 'peach yellowing' (Poggi Pollini *et al.*, 1993).

The symptoms may change depending on the cultivar and the time of observation. The most typical ones are yellowing and leaf roll in summer and off–season growth in winter. The latter causes an increase in the risk of frost damage during this unusual growing period in Spain because the sprouted buds are not able to stand the low temperatures registered during winter. Very often the disease has been confused with abiotic stresses, such as iron deficiency, etc. Climate is another factor influencing the expression of phytoplasma diseases. The practice of naming a phytoplasma after the associated plant syndrome has caused confusion because it has shown that phylogenetically different phytoplasmas can cause similar symptoms on the same host species (Firrao *et al.,* 2005). For years, the lack of an accurate diagnosis of the pathogen has been the main obstacle for their characterization.

The AP, PD and ESFY agents are among the most intensively studied phytoplasmas, using both molecular and biological methods. The 16S rDNA sequence data indicated that all of them are closely related and constitute, together with the 'peach yellow leaf roll' (PYLR) phytoplasma, a cluster designated the 'AP phytoplasma group' (Seemüller *et al.,* 1998) or 16Sr-X group (Lee *et al.,* 1998). According to phylogenetic relationships based on the 16S rDNA gene phylogeny and restriction fragment length polymorphism (RFLP) profiles, 14 to 20 phytoplasma groups have been described (Seemüller *et al.,* 1998; Lee *et al.,* 2000). More recent work based also in phylogenetic analysis has revealed that AP, PD, and ESFY agents are closely related differing only in 16-19 nt positions in their 16S rDNA, which corresponds to 98.6-99.1% sequence similarity (Seemüller and Schneider, 2004). The three phytoplasmas AP, PD and ESFY showed clear differences in vector transmission and host-range specificity. Supporting data for distinguishing agents at species level were obtained by examining other molecular markers. The names of '*Candidatus* Phytoplasma mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum' respectively were given for the three phytoplasma species. 'Peach yellow leaf roll' phytoplasma (PYLR) showed a 99.6% 16Sr DNA sequence similarity to PD sequence. Seemüller and Schneider (2004) proposed that this phytoplasma should be regarded as a subtype of '*Ca.* P. pyri'. These studies have shown the need for advancement in the detection methods for an early diagnosis of the phytoplasma diseases affecting stone fruits, avoiding thus the confused diagnosis often observed at very advanced status. Most *Prunus* species can support the multiplication of '*Ca.* P. prunorum' (Lorenz *et al.,* 1994; Jarausch *et al.,* 2000a). Besides Japanese plum and apricot, the disease has also been reported in almond (*P. amygdalus* Batsch.) and peach (*P. persica* L. Batsch) in southern Europe (Poggi Pollini *et al.,* 1993, 2001; Marcone *et al.,* 1996). Other species used as rootstocks have been described with different susceptibilities to '*Ca.* P. prunorum', those derived from *P. domestica* and *P. cerasifera,* being less affected when compared with those derived from *P. insititia*. Peach rootstocks and other hybrids are slightly more affected (Kison and Seemüller, 2001). Moreover the phytoplasma has also been detected in wild species, including *Prunus spinosa, Fraxinus excelsior, Rosa canina, Celtis australis, Corylus avellana* (Jarausch *et al.,* 2001a; Carraro *et al.,* 2001, 2002), which may play an important role in the epidemiology.

Two different PCR-based methods were tested to assess the presence of '*Ca.* P. prunorum' in two *Prunus* collections from the CITA. The first one included sever-

al Japanese and European plum varieties and the second included several intra- and inter-specific hybrids used as rootstock. A test was also conducted to ascertain the reliability of both methods, with the aim of selecting the most adequate for routine diagnosis in a new collection where new rootstocks and cultivars should be planted.

Material and methods

Phytoplasma reference strains

The DNAs of the phytoplasma reference strains used in this study were '*Ca.* P. mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum'. Phytoplasmas were maintained in periwinkle (*Catharanthus roseus) and* were kindly provided by Jean Luc Danet, Laboratoire de Biologie Cellulaire et Moléculaire, UMR GDPP IBVM, INRA-Bordeaux, France.

Plant material

The survey concerned 16 Japanese plum cultivars (*Prunus salicina*): 'Angeleno', 'Ambra', 'Casselman', 'El Dorado', 'Fortune', 'Friar', 'Frontier', 'Golden Japan', 'Laroda', 'Nubiana', 'Orotava', 'Queen Ann', 'Queen Rosa', 'Santa Rosa', 'Simka' and 'Songold'; eight European plum cultivars (*P. domestica*): 'Arandana', 'President', 'R.C. Bavay', 'R.C. d´Or', 'R.C. d´Oullins', 'R.C. Verte', 'Ruth Gerstetter', 'Stanley'. The rest of the genotypes were rootstocks including three genotypes of *P. cerasifera* Myrobalan: 'Myrobalan 605', 'Myrobalan VZ' and the selection 'Trihybrid'; five interespecífic hybrids of different origin and used as rootstocks: 'GF-8.1' (*P. cerasifera* × *P. munsoniana*); 'Miral-5' (*P. cerasifera* × *P. amygdalus??*), 'Deep Purple' (*P. besseyi* × *P. salicina*); 'Bruce', (*P. salicina* × *P. angustifolia*) and 'Citation' (*P. persica* × *P. salicina)*.

All the genotypes were grafted at planting time on either 'Monpol' or 'Montizo' rootstocks, two pollizo plums (*P. insititia*). Off-spring growth was observed in two years (2004 and 2005) in winter during rest. The following springs a mixed sample of leaves was collected from the two replicates of the collections for all genotypes. The trees were located at the Centro de Investigación y Tecnología Agroalimentaria de Aragon in Zaragoza, in two different plots, one for all fruit cultivars, except 'Orotava', and the other one for the rootstocks and 'Orotava'. The two plots, although 1 km away from the other, have the same edaphoclimatic conditions.

Phytoplasma detection

'*Ca.* P. prunorum' detection was carried out by using two molecular PCR-based techniques during two consecutives years. The sampling was done in the springs of 2004 and 2005. Total DNA was isolated from fresh or frozen (-20º) 0.05 g midrib tissues using the CTAB method (Doyle and Doyle, 1987), resuspended and stored in a TE buffer at –20ºC. The DNA concentration was measured and calculated with a spectrophotometer (Gene Quant II, RNA/DNA Pharmacia).

Method 1.- This method is a nested-PCR amplification of ribosomal DNA. The first amplification was carried out in a total volume of 20 µL of reaction mixture containing 40 ng of DNA, 100 µM each dNTP, 0.5 u *Taq* DNA polymerase (Invitrogen), 0.2 µM of each of the universal primers P1/P7 (Schneider *et al.,* 1995), 3 µM $MgCl_2$ and 1X *Taq* buffer (Invitrogen). One microlitre of the previous amplification was used as a template for the second step. The second amplification mixture contained the same components but with the specific primers of ´AP group´ f01/r01 (Lorenz *et al.,* 1995). After an initial denaturation at 94ºC for 2 min, the cycling parameters were 35 cycles of denaturation at 94ºC for 45 s, annealing at 53ºC for 1 min and extension at 72ºC for 2 min for primers P1/P7 and 35 cycles of 45 s at 94ºC, 1 min at 55ºC and 2 min at 72ºC for primers f01/r01. A final extension at 72ºC for 5 min was made in both cases. These primers amplify a fragment of approximately 1050 bp in the 16S rDNA gene of 'AP group'. Those samples testing positive in nested PCR assays were submitted to RFLP analysis. RFLP analysis was carried out by digestion with *Rsa*I enzyme incubating 20 µL of a reaction mixture containing 10 µL of the final amplification product of the nested-PCR, 0.25 u μL^{-1} of *Rsa*I (Invitrogen) and 1X *Rsa*I buffer (Invitrogen) for 1 hour at 37ºC.

Method 2.- The second method was a direct amplification in a total final volume of 20 µL containing 40 ng of DNA, 125 nM dNTPs, 0.5 u Taq DNA Polymerase (Invitrogen), 0.5 µM of each primer Eca1/Eca2 (Jarausch *et al.,* 1998), 3 µM $MgCl_2$ and 1X *Taq* buffer. The expected band was 237 bp long. PCR parameters were, an initial denaturation at 95ºC for 1 min, 40 cycles of 30 s at 94ºC, 30 s at 55ºC, 30 s at 72ºC, and a final extension at 72ºC for 4 min. In both cases PCR products

were subjected to electrophoresis in 1.7% agarose gels stained with ethidium bromide and visualized by UV light.

In order to verify the results, each of the PCR methods and digestions were performed three times for each sample.

Results

Off-season growth in winter

As the incidence of visual examinations varied greatly between cultivars and rootstocks, only the off-season growth in winter was taken into account for symptoms assertion.

The main symptoms observed were an early bud breaking and a growth decrease. Other symptoms of the disease were not taken into account because they varied greatly and occurred only occasionally. Early bud breaking symptoms (Table 1) were observed both years in winter in the annual visual examination, in seven Japanese plum cultivars and in the 'Trihybrid' Myrobalan. This last genotype presented a progressive decline. Since in the first year only some leaves remained at the top, these were the ones used for sampling. Four interspecific hybrids showed symptoms during the winter 2003-04, 'Citation', 'Deep Purple', 'GF-8.1' and 'Miral-5', but 'Bruce' only during the 2004-05 winter. No symptoms were observed during the two years in the eight European plum cultivars and in two Myrobalan genotypes, 'Myrobalan VZ' and 'Myrobalan 605'. The genotypes were considered with symptoms when either flower or leave buds were visible. Symptom severity was stronger in all the genotypes in the rootstock plot, where the interspecific hybrids and myrobalan genotypes were located, since the leaves were visible in all the branches at the end of January. However, in the plot where the Japanese and European cultivars were located, only flowers were visible in the branches.

Presence of '*Ca.* P. prunorum'

In 2004, method 1 allowed detecting '*Ca*. P. prunorum' in 10 of the 12 symptomatic trees (Table 1); those genotypes were six Japanese plum cultivars and four interspecific hybrids. The same results were obtained in 2005 with the exception of 'Angeleno', in which it was not possible to detect the presence of phytoplasma.

Using method 2 (direct PCR), from the seven symptomatic trees of Japanese plum observed in 2004, only four yielded the expected 237 bp-long PCR product. In 2005, for the four cultivars previously tested positive with direct PCR, only 'Queen Ann' remained positive (Table 1). Regarding the six interspecific hybrids studied the genotypes 'Citation', 'GF-8.1' and 'Miral-5' yielded the expected PCR product in 2004. However, in 2005 the phytoplasma was detected in 'Bruce' by 16S nested PCR but no amplification was obtained with 'Deep Purple'. Only two of the previously positive rootstocks, 'Citation' and 'GF-8.1', yielded a PCR product in 2005. Although symptoms were visible in the 'Trihybrid' Myrobalan and 'Orotava', it was not possible to detect the phytoplasma during the 2-yr survey.

Four Japanese plums were amplified with both PCR methods in 2004 and two accessions, namely 'Golden Japan' and 'Laroda', were tested positive with 16S nested PCR, 'Orotava' was the only symptomatic genotype for which no amplification was obtained either by nested-PCR or direct PCR. On the other side, 'Myrobolan 605' and 'Myrobolan VZ' were asymptomatic trees and no amplifications were observed. None of the two methods showed the presence of the '*Ca*. P. prunorum' in the *Prunus* European plum cultivars.

The RFLPs analysis of amplicons (Fig. 1) from phytoplasma detected in these 10 genotypes confirmed that '*Ca.* P. prunorum' was present in these samples since the restriction profile was indistinguishable from that of the reference isolate. From the seven symptomatic trees of Japanese plum observed in 2004, only four yielded the expected size of 237 bp after amplification with the specific primers Eca1/Eca2. All the asymptomatic genotypes were negative for the presence of '*Ca.* phytoplasma prunorum'.

Discussion

Most of the Japanese plums and genotypes including Japanese plum showed in their lineage a close relationship between the presence of symptoms and '*Ca*. P. prunorum' detection. However, the two PCR-based methods did not provide the same reliability in phytoplasma detection in symptomatic *Prunus* trees. The 16S nested PCR/RFLPs method was more accurate in relating the symptoms and the presence of phytoplasmas, since even in trees showing weak symptoms such as 'Laroda' and 'Golden Japan' it was possible to detect the phytoplasma. The RFLPs analysis of amplicons from

Table 1. Presence of early bud breaking in 2004 and 2005 on different genotypes of several *Prunus* species maintained in rootstock and cultivar collections, and presence (+) or absence (-) of '*Candidatus* phytoplasma prunurum' as detected by nested PCR-RFLPs analysis and direct PCR with specific primers Eca1-Eca2

Species	Genotype	2004			2005		
		Symptoms	Method 1	Method 2	Symptoms	Method 1	Method 2
Japanese cultivars							
P. salicina	Angeleno	+	+	+	+	-	-
P. salicina	Ambra	-	-	-	-	-	-
P. salicina	Casselman	-	-	-	-	-	-
P. salicina	El Dorado	-	-	-	-	-	-
P. salicina	Fortune	-	-	-	-	-	-
P. salicina	Friar	+	+	+	+	+	-
P. salicina	Frontier	-	-	-	-	-	-
P. salicina	Golden Japan	+	+	-	+	+	-
P. salicina	Laroda	+	+	-	+	+	-
P. salicina	Nubiana	+	+	+	+	+	-
P. salicina	Orotava	+	-	-	+	-	-
P. salicina	Queen Ann 05	+	+	+	+	+	+
P. salicina	Queen Rosa 06	-	-	-	-	-	-
P. salicina	Santa Rosa	-	-	-	-	-	-
P. salicina	Simka	-	-	-	-	-	-
P. salicina	Songold	-	-	-	-	-	-
European cultivars							
P. domestica	Arandana	-	-	-	-	-	-
P. domestica	President	-	-	-	-	-	-
P. domestica	R.C. Bavay	-	-	-	-	-	-
P. domestica	R.C. d´Or	-	-	-	-	-	-
P. domestica	R.C. d'Oullins	-	-	-	-	-	-
P. domestica	R.C. Verte	-	-	-	-	-	-
P. domestica	Ruth Gerstetter	-	-	-	-	-	-
P. domestica	Stanley	-	-	-	-	-	-
Rootstocks							
P. cerasifera	Myrobalan VZ	-	-	-	-	-	-
P. cerasifera	Myrobalan 605	-	-	-	-	-	-
P. cerasifera??	Trihybrid	+	-	-	+	-	-
P. salicina × *P. angustifolia*	Bruce	-	-	-	+	+	-
P. salicina × *P. persica*	Citation	+	+	+	+	+	+
P. besseyi × *P. salicina*	Deep Purple	+	+	-	+	-	-
P. cerasifera × *P. munsoniana*	GF-8.1	+	+	+	+	+	+
P. cerasifera × *P. amygdalus?*	Miral -5	+	+	+	+	+	-

phytoplasmas detected in symptomatic trees confirmed that phytoplasmas belonging to the 16SrX-B group were present in the genotypes studied, since the position of restriction sites with *Rsa*I was in agreement with the digestion of the 16SrDNA sequences of 'Ca. P. prunorum', distinguishable from those of both '*Ca.* P. mali' and '*Ca.* P. pyri' (Torres *et al.,* 2004; Firrao *et al.,* 2005). There were two exceptions, 'Orotava' and 'Trihybrid', where no analytical results were obtained even if they showed symptoms in both years. The explanation for this outcome may be attributed to the orchard maintenance. Both genotypes were in a rootstock collection plot, maintained at the original size since planting resulting in higher trees. We assume that in both years sampling was irregular and difficult due to the low number of leaves remaining, therefore a low phytoplasma titer, although clear death symptoms were observed in the lower part of the trees. We could also assume that in declining plants, PCR inhibitors, such as phenolic compounds may be present in high concentration and might therefore interfere with the efficiency of the diagnostic assay.

In 'Angeleno' and 'Deep Purple', the phytoplasma was not detected in the second year, probably because

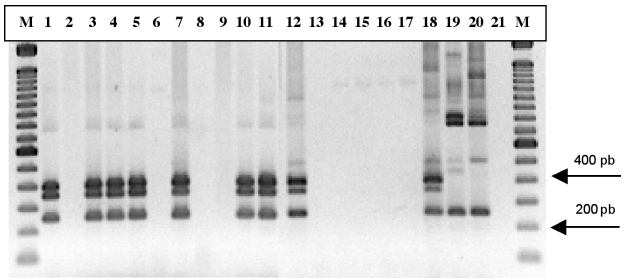

Figure 1. *Rsa*I digestion of nested-PCR products (method 1) from representative *Prunus* genotypes. Positive lanes: 1) Friar, 3) Golden Japan, 4) Laroda, 5) Nubiana, 7) Citation, 10) Miral-5, 11) GF-8.1, 12) Deep Purple; Negative lanes: 2) Caselman, 6) El Dorado, 8) Myrobalan 605, 9) Myrobalan VZ, 13) Arandana, 14) President, 15) R.C. Bavay, 16) R.C d'Or, 17) R.C. d'Oullins. Reference isolates were 18) '*Candidatus* phytoplasma prunurum', 19) '*Candidatus* phytoplasma pyri', 20) '*Candidatus* phytoplasma mali'; 21) phytoplasm-free host plant; M-100 pb ladder (Invitrogen).

of the disease progression. In the hybrid 'Bruce', the phytoplasma was detected in winter in an additional assay (data not shown) with the method PCR-RFLPs. That could be explained by a seasonal colonization pattern already reported for *Prunus* species, since phytoplasmas seem to survive in winter in the aerial parts of the tree (Jarausch *et al.,* 1999), suggesting that our sampling time could have been late. In the European plums, the absence of symptoms agrees with the negative results obtained with both detection techniques. This outcome is in agreement with previous studies (Carraro *et al.,* 1998), even though no resistance to '*Ca.* P. prunorum' has been reported for the genus *Prunus* (Jarausch *et al.,* 2000b). The primers Eca1/Eca2 were selected in a DNA region exhibiting a high degree of variability (Jarausch *et al.,* 1998) and this could be the reason why this primer pair did not fish out all phytoplasma isolates. It could be argued that this pair is not appropriate for the detection of '*Ca.* P. prunorum' in Spain, as it has already been reported for apricot *(P. armeniaca*) in Spain (Jarausch *et al.,* 2000a). This report confirms that off-season growth and plum leptonecrosis in Japanese plum (*P. salicina*) and interspecific hybrids including the myrobalan (*P. cerasifera*) genotypes are indeed due to '*Ca.* P. prunorum' (Jarausch *et al.,* 2001a; Torres *et al.,* 2004). The direct PCR method may be considered a less sensitive technique than the nested PCR, which requires a lower amount of DNA template. The genotypes studied gave the same RFLP profile, which suggests that the disease is due to one single phytoplasma.

Although a species could be reported as a phytoplasma host, but tolerant at the same time, our observations point out that the severity of the disease along the years can lead to tree death. Several examples support this hypothesis, as is the case of 'Trihybrid', belonging to the myrobalan group, considered highly tolerant to the disease (Carraro *et al.,* 2001, 2002), but also a phytoplasma host (Jarausch *et al.,* 2001b; Kison and Seemüller, 2001; Carraro *et al.,* 2002). 'Trihybrid', showed similar symptoms in two consecutive years to those trees where the phytoplasma was detected, and probably died as a consequence of an advanced status of ESFY. Another example is the hybrid 'Deep Purple' affected by ESFY and infected by the phytoplasma, during the first year but not during the second, when a more advanced decline was observed. Both of them are myrobalan (*P. cerasifera*) and/or Japanese plum hybrids (*P. salicina*), and are considered phytoplasma hosts (Carraro *et al.,* 2002). These results agree with several reports of 'GF-8.1' *(P. marianna)* and with other interespecific hybrid descriptions as hosts for '*Candidatus* phytoplasma prunorum' (Jarausch *et al.,* 2000a).

Since the rootstock in the grafted trees were not tested at planting time for phytoplasma infection and no susceptibility study for these two rootstocks has been reported yet, it cannot be concluded that the infection is not due to the rootstock, although rootstocks of the same species (*P. insititia)* have been found susceptible (Kison and Seemüller, 2001). Among the genotypes studied, those of American origin, 'Citation', 'Bruce' and 'Deep Purple', could have already been infected by the phytoplasma at planting. The genotypes in the rootstock collection would undergo an incubation period of several years, of variable length depending on the species (Carraro *et al.,* 2001; Torres *et al.,* 2004; Tedeschi *et al.,* 2006). An additional test with buds of the same origin has confirmed this hypothesis (data not shown).

The phytoplasma cells titer in the phloem of infected plants is often very low in woody plants and may vary with the season and the plant species (Marzachi, 2004). Since the non-ribosomal Eca1/Eca2 primers have shown good results for nested PCR (Jarausch *et al.,* 2001b) we assume that an enrichment procedure of the DNA extraction could improve the results (Ahrens and Seemüller, 1992).

For the time being we have not detected the phytoplasma in asymptomatic trees, even though there are reports of detection in apricot trees, the most sensitive species among all *Prunus* (Torres *et al.,* 2004), as well

as in other several *Prunus* species (Carraro *et al.,* 2004). In the future, this disease could become a reason for concern if control measures are not applied. A less time-consuming and reliable protocol, such as q-PCR (Galetto *et al.,* 2005) should be applied in order to detect this disease.

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