Transmission of '*Candidatus* Phytoplasma pyri' by naturally infected *Cacopsylla pyri* L. to peach, an approach to the epidemiology of Peach yellow leaf roll (PYLR) in Spain

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Abstract

Peach orchards in the North East of Spain were severely affected in 2012 by a previously unreported disease in this area. The symptoms included early reddening, leaf curling, decline, abnormal fruits, and in some cases death of the peach trees. All the infected peach samples were positive for '*Candidatus* Phytoplasma pyri', while none of them was infected by the '*Ca*. P. prunorum'. In this work, potential vectors able to transmit '*Ca*. P. pyri' from pear to peach and between peach trees were studied and their infective potential was analyzed at different times of the year. Transmission trials of the phytoplasma with potential vectors to an artificial feeding medium for insects and to healthy peach trees, were conducted. Additionally, isolated phytoplasmas were genetically characterized to determine which isolates were able to infect peach trees. Results showed that the only insect species captured inside peach plots that was carrier of the '*Ca*. P. pyri' phytoplasma was *Cacopsylla pyri* L. Other insect species captured and known to be phytoplasma transmitters were present in very low numbers, and were not infected with '*Ca*. P. pyri' phytoplasma. A total of 1928 individuals of *C. pyri* were captured in the peach orchards with a percentage of phytoplasma carriers around 49%. All the peach trees exposed to *C. pyri* in 2014 and 65% of 2015 were '*Ca*. P. pyri' infected one year after exposure, showing that this species is able to transmit the phytoplasma to peach trees. Molecular characterization showed that some genotypes are preferentially determined in peach.

Keywords: phytoplasma, insect vector, *Cacopsylla,* PCR-detection, MLST

Introduction

Phytoplasmas are parasitic cell wall-less bacteria inhabiting the phloem sieve cells of infected plants (Lee *et al*., 2000). They are transmitted from plant to plant by phloem-feeding insect vectors and have unique genome architectures having lost important metabolic genes as a result of their host-dependent lifecycles (Bai *et al.,* 2006; Christensen *et al*., 2005; Dickinson *et al*., 2013; Hogenhout *et al*., 2008).

The most economically important phytoplasma diseases of fruit trees in Europe are those caused by phytoplasmas belonging to the ribosomal group 16Sr-X, known as apple proliferation (AP) group. Diseases within this group are Apple proliferation (AP), European stone fruit yellows (ESFY), Pear decline (PD) and Peach yellow leaf roll (PYLR), caused by '*Candidatus* Phytoplasma mali', '*Ca.* P. prunorum' and *'Ca.* P. pyri', respectively (Seemüller & Schneider, 2004). All are present in Europe while in América only '*Ca*. P. pyri' is present. They induce some characteristic symptoms as yellowing, reddening, loss of vigor, early blooming and in some cases a general collapse and death of the trees.

These three phytoplasmas are closely related because they differ in less than 2-5% of the 16SrDNA nucleotide sequence, even though they are considered different *Candidatus* species because they present clear differences in vector transmission and host-range specificity (Seemüller & Schneider, 2004)**.** The 16Sr-X group phytoplasmas are naturally transmitted by psyllids of the genus *Cacopsylla* (Jarausch & Jarausch, 2010), and by vegetative multiplication. European diseases of the 16Sr-X phylogenetic group are sometimes interconnected by common host plant affiliations and the feeding preferences of their psyllid vectors (Jarausch & Jarausch, 2010). *'Ca.* P. pyri' causes PD and PYLR, nowadays, PD probably occurs wherever pear is grown in Europe and North America,

and constant losses and new outbreaks are continuously being observed. Apparently, the disease development and the symptoms expressed are highly influenced by the rootstock and by the stage of the disease (Seemüller *et al*., 2011). The reported vectors for *'Ca.* P. pyri' are *Cacopsylla pyri* (Linnaeus, 1758), *C. pyricola* (Foerster, 1848) and *C. pyrisuga* (Foerster, 1848), found in different ecological regions and causing direct damage on pear trees. *C. pyri* has been identified as the main vector of PD in Spain and in the Mediterranean area (Carraro *et al*., 1998a; Garcia-Chapa *et al*., 2005;). It is a pest in Catalonia, with resistances to the authorized insecticides and where up to five or six generations can be produced every year, these are the reasons why it seems to be difficult to control (Artigues *et al*., 1995).

Differences in virulence and genetic diversity of the three temperate fruit tree phytoplasmas in Europe, '*Ca.* P. mali', '*Ca.* P. pyri' and '*Ca.* P. prunorum', has been established by multilocus sequence analysis of the *aceF, imp, secY* and *pnp* genotypes by Danet *et al*., 2011, revealing a great diversity within this group. Some Spanish and Azerbaijan '*Ca.* P. pyri' isolates possess both '*Ca.* P. pyri' and '*Ca.* P. prunorum' genetic markers, indicating for the first time the existence of inter-species recombination between these two phytoplasmas. To allow these recombination events, these two phytoplasmas must share a common host or vector. Moreover, after an extended set of genes for homologous recombination was discovered in '*Ca.* P. mali,' and considering that members of the group 16Sr-X possess a linear chromosome, a single recombination event between their chromosomes can trigger the exchange of many gene markers at the same time (Kube *et al*., 2008).

In Catalonia (Northeastern Spain), '*Ca*. P. pyri' has been identified in the most important pear crops areas in Lleida, where it is endemic and affects all varieties analyzed so far. The highest number of infected and more affected orchards belonged to the 'Jules Guyot'

variety, but it has been also identified in Abate Fetel, Bartlett, Williams, Conference, Ercolini, and other varieties (Garcia-Chapa *et al*., 2003a).

In contrast, Peach yellow leaf roll disease (PYLR) was described for the first time in western North America producing important losses in peach orchards. The highest incidences were found nearby pear orchards in northern California and the disease seemed to be restricted to this area (Purcell *et al*., 1981). The infection induces a rapid decline of the peach tree, downward-rolled leaves in late summer and often swollen midribs. Surprisingly, similarity examination on 16S rDNA has determined that PYLR phytoplasma is most closely related to '*Ca.* P. pyri' than the other phytoplasma of the 16Sr-X group, and was thus considered as a subtype of '*Ca.* P. pyri' (Seemüller & Schneider, 2004). Molecular techniques and experimental transmission assays allowed to determine that the psyllid *C. pyricola*, the vector of '*Ca*. P. pyri' in North America is also the vector of this phytoplasma to peach, causing PYLR in this area (Blomquist & Kirkpatrick, 2002). Therefore, the spread of PYLR appeared to be dependent on adjacent pear orchards, where the vector supposedly reproduces and acquires the phytoplasma (Blomquist & Kirkpatrick, 2002; Purcell *et al*., 1981).

In 2012, a previously unreported syndrome of peach trees was reported in Lleida (Northeastern Spain), (Sabaté *et al*., 2014). The symptoms seemed to be produced by phytoplasmas and included early reddening, leaf curling, decline, abnormal fruits, and in some cases, chlorosis and death of the peach trees. The disease was found in a wide range of varieties and rootstocks suggesting vector transmission; in this area *C. pruni* was not reported before and *C. pyri* was abundantly found. Furthermore, the frequency of ESFY appearance on *Prunus persica* in Lleida is very restricted, *C. pruni* is not present and '*Ca.* P. pyri' is in almost every pear orchard (García-Chapa *et al*., 2005; Sabaté *et al*., 2016;). All the infected peach trees were positive for PD phytoplasma, while none of the samples

was infected by the ESFY agent. The work from Sabaté *et al.*, (2014) was the first report of PD phytoplasma infecting peach trees in Europe, and it is suggested that these important outbreaks can follow a pattern similar to North American PYLR. However, the isolates found (Acc. No. HG737345 and HG737344) were genetically closer to some European or Eastern PD strains (Acc. No. AJ542543, and FN 600725) than to North American '*Ca.* P. pyri' (Acc. No. U54990, U54989 and AF400588). Due to the potential severe impact of the disease, more information was needed concerning this novel disease reported. In this work, the potential vectors of PYLR, and their transmission capacities were studied during different times of the year. Transmission trials with *C. pyri* to healthy peach plants and artificial feeding medium were carried out. Additionally, isolated phytoplasmas were genetically characterized by studying five different genes using a multilocus sequence analysis to determine which isolates were able to infect peach trees.

Material and methods

Location and characteristics of the plots used in this study

The study was carried out in four plots with 400 trees each: two peach orchards where PYLR disease had already been previously identified and two pear orchards affected by '*Ca*. P. pyri' and located neighboring every peach orchard (Plot 1 pear/Plot 2 peach and Plot 3 pear/Plot 4 peach). All of them were located in a radium of 4 km in Lleida (Catalonia). The area is characterized by a Continental-Mediterranean climate, with irrigated intensive fruit production.

Insect vectors identification

With the aim to identify the insect species responsible for '*Ca*. P. pyri' transmission from pear to peach trees or between peach trees, insect capture and species identification studies were carried out in the four affected orchards. Insects were collected from June

2015 to February 2016 with four yellow traps per plot placed at 1.5 m high; two traps were placed inside the plots and two in the trees of the edges between the crops. The traps were replaced every two weeks, and insects belonging to families known to include phytoplasma vectors were classified following Hodkinson and White (1979) and stored at -20ºC before to be analyzed by PCR for phytoplasma presence.

Transmission trials of *'Candidatus Phytoplasma pyri'* to feeding medium

In October and November 2015, *C. pyri* adults were collected using the beating tray method in the peach orchards and in February 2016 in the pear orchards, because they were not present on peach. On each sampling data, 100 individuals of *C. pyri* were collected. The psyllids were placed on falcon tubes for 1 day to avoid false detection of PD phytoplasma due to the potential presence of residual phytoplasmas adhering to the stylet tissues. After the first day, each psyllid individual was transferred to 1.5 ml microcentrifuge tubes used as insect chambers. The caps were filled with 200 µl of 5% sucrose, 0.5% sorbitol and 9.4 mg/l of NCTC 135 medium (Sigma) and sealed with Parafilm (Garcia-Chapa *et al*., 2005). Each tube, was kept at 23-25 ºC until the insect died or maximum for 72 h. The time of death was recorded. In the laboratory the adults were separated by gender.

Phytoplasma detection in plants, insects and feeding medium

***DNA extraction from plants and insects*:** DNA from plant samples was isolated from approximately 1.0 g of fresh plant material: leaf midribs, buds or stems, using the phytoplasma-enrichment procedure of Ahrens & Seemüller (1992).

DNA extraction of insects was done according to Garcia-Chapa *et al*., (2005). Insects were ground in an extraction buffer (100 mM Tris-HCl at pH 8.0, 2% cetyltrimethylammonium bromide (CTAB), 1.4M NaCl, 20 mM EDTA and 0.2 % 2-

mercaptoethanol). The slurry was incubated for 10 min at 65ºC. After incubation, an equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and samples centrifuged for 10 min at 12,000 rpm. The supernatant was collected and the nucleic acids precipitated with an equal volume of 2-propanol, following 30 min incubation at –20ºC. DNA was pelleted at 12,000 rpm for 30 min. The pellet was washed with 70 % ethanol and resuspended in 50 µl sterile water.

***DNA extraction from feeding medium*:** Two hundred µl of the feeding medium were transferred to a 1.9 ml microcentrifuge tube with 900 µl of buffer TNE (SDS 1.1 %, 150mM NaCl, 2mM EDTA, 10 mM Tris-HCl at pH 8), 100µl of 5M Guanidine Hydrochloride and 50 µl of proteinase K (20 mg/ml). The mixture was incubated for 2 h at 58ºC and centrifuged for 10 min at 13000 rpm. An equal volume of chloroform was added to the supernatant and centrifuged for 10 min at 13000 rpm, 500µl of the supernatant were mixed with a DNA purification resin (Wizard minipreps, Promega) in a 1.5 ml microcentrifuge tube and mixed by inverting the tube several times. The mixture was transferred to a new spin column-vacuum adapter and a vacuum was applied to pull all the liquid through the column. One ml of 2-propanol was added and vacuum again applied. Finally, the spin column was transferred to another sterilized 1.5 ml microcentrifuge tube, and the DNA was eluted by adding 50µl of nuclease-free water and the sample was centrifuged at 13,000 rpm for 1 minute. Also, directly feeding medium diluted 1:10 in HPLC water without previous DNA extraction was assayed for PCR amplification.

The phytoplasma detection on extracted DNA was carried out using the Real time-PCR technique in a Step One^{TM} Instrument (Life Technologies). A first amplification with universal primers was carried out for the screening of positives using the primers and the TaqMan probe described in Christensen *et al*., (2004). A total volume of 10 µl containing

2 µl of DNA template and 8 µl of the following mixture was used: 0.3 µM forward primer (Ch-f), 0.9 µM reverse primer (Ch-r), 0.2 µM probe (Ch-p) and 1× TaqMan universal PCR Master Mix (Applied Biosystems). The qPCR cycling program was: 2 min at 50ºC for the UNG activation, 10 min at 95ºC, followed by 45 cycles at 95ºC for 15 s and 60ºC for 1 min. A Ct coefficient under 27 was considered strong positive sample and a Ct between 27 and 33 was considered a weak positive sample. Positive samples where amplified again by Real time-PCR with the specific primers for '*Ca*. P. pyri' detection to verify that this was the phytoplasma present in plants, insects and in the feeding medium (Nikolic *et al*., 2010).

Transmission of '*Candidatus* Phytoplasma pyri' to healthy peach trees

In September 2014 and in July, August, September and October 2015, *C. pyri* individuals were collected from the plots and transferred to 10 peach trees obtained from *in vitro* culture every month, totaling 50 tested plants. Groups of 30 *C. pyri* individuals were caged in every tree during a week. Another group of 10 healthy peach trees were used as negative controls each year. Insects were collected one week after inoculation and analysed for 'Ca. P. pyri'. The symptoms of the inoculated trees were followed and phloem and petioles analysed by Real-time PCR one year later.

Molecular characterization of '*Ca*. P. pyri' isolates from plants and insects

Amplification of sequences belonging to different genes were carried out by nested-PCR. Genes, *imp*, *pnp*, *aceF* and *secY*, were amplified with the primers described in Danet *et al*., (2011). A fragment of the ribosomal gene 16S was also amplified and sequenced. For this gene, the first amplification was done with the universal primers P1/P7 (Deng & Hiruki, 1991; Smart *et al*., 1996) located at the 16S rDNA and 23S rDNA gene, respectively. In the second amplification, general primer pair R16F2n/R16R2 (Gundersen

et al., 1996) and the specific one fO1/rO1 was used (Lorenz *et al*., 1995). This primer amplifies the 16S rDNA gene from DNA of all phytoplasma belonging to the Apple proliferation (AP) group, producing a fragment of about 1050 bp in length (Lorenz *et al*., 1995). The amplification conditions are those described by Danet *et al.*, 2011 and Garcia-Chapa *et al.*, 2003a, respectively.

RESULTS

Potential insect vectors identification

The potential insect vectors captured in the plots were classified and analyzed to determine if they were carriers of '*Ca*. P. pyri' (Table 1). The only species captured that was carrier of this phytoplasma was *C. pyri*. Other insect species captured and known to be phytoplasma transmitters such as *Neoaliturus fenestratus, Laodelphax striatellus* or *Opsius stactogalus*, presented very low populations and were not infected by '*Ca*. P. pyri'. *Empoasca sp*. was captured in higher number but only very few individuals were positive of '*Ca*. P. pyri' (Table 1). The phytoplasma concentration determined by real-time PCR in *Empoasca sp.* was very low in comparison to the concentration found in *C. pyri* individuals (results not shown). A total of 1928 individuals of *C. pyri* were captured in the two peach plots surveyed and the percentage of phytoplasma carriers in these plots was around 49% (Table 1).

Population dynamics of *C. pyri* in the studied plots

The dynamic population of *C. pyri* adults in peach and pear plots was determined from June 2015 to February 2016. Figure 1 shows the total number of individuals captured in different months in pear and peach orchards. Results showed three flight peaks taking place in July, September and November. Figure 2 shows the individuals captured only in

peach plots, where a lower population peak was obtained in July and a higher peak in November. In the two peach plots (Figure 2), a higher number of individuals was captured in the traps placed inside the plots, showing that *C. pyri* was present inside the peach plots especially in the flight peak of November. No *C. pyri* individuals were captured in the months of December and January on any host. Hibernant individuals were captured again in February 2016 in the pear plots but not in peach plots (Figures 1 and 2).

Frequency of 'Ca. P. pyri' positive psyllids in peach and pear plots.

In figure 3 and figure 4 the percentage of *C. pyri* specimens infected with '*Ca*. P. pyri' phytoplasma in pear and peach plots along the year and the percentage of the infected individuals captured only in peach plots are respectively shown.

The mean of individuals which are carriers of the phytoplasma in pear orchards was 69% (Figure 3), whereas concerning peach plots the mean of carriers was 49 % (Figure 4). Considering the total of *C.pyri* individuals, the mean that are carriers of the phytoplasma was 62 % (Table 1), twenty five % of them were strong positives (Ct below 27 cycles) and 37% were weak positives (Ct between 27 and 33 cycles). The rest 38% were non-infected individuals.

Looking at the gender, the strong positives were 48% females and 52% males. A bifactorial ANOVA was used to calculate the influence of gender and of the month of sampling on the percentage of individuals infected with the phytoplasma. Results indicated that sampling time was significantly different (*p-value* < 0.001***) while gender was not (*p-value = 0.2878*). Then *C. pyri* was infected independently of the captured insect's gender.

‘*Ca*. P. pyri’ transmission to insect feeding medium

To confirm that the *C. pyri* individuals captured were capable to transmit ‘*Ca*. P. pyri’ and to determine the transmission efficiency by males and females, transmission trials to an insect feeding medium were conducted. The phytoplasma detection in the artificial feeding medium was more sensitive when it was done after a previous extraction of DNA from the medium than when the PCR was done using the medium directly, therefore only the first methodology was used.

Figure 6 shows the percentage of transmission of ‘*Ca.* P. pyri’ through *C. pyri* captured on studied plots to a feeding medium linked to gender and to the capture time. The total percentage of transmission obtained was 52.9% for males and 67.6% for females. Results obtained by bifactorial ANOVA considering results obtained by Real-time PCR show that capture time (*p-value <0.001****) and *C. pyri* gender (*p-value =0.04**), both had a significant effect.

Transmission to healthy peach plants

Results of PCR analyses indicated that all psyllid groups used in the transmission experiment were PD phytoplasma positive. All the peach trees exposed to *C. pyri* in 2014 (10 trees) were also ‘*Ca*. P. pyri’ positive by real-time PCR one year after exposure. The 65% of the peach trees exposed to *C. pyri* in 2015 (40 trees) were ‘*Ca*. P. pyri’ infected one year later. No amplification was obtained from healthy peach trees DNA. The infected trees showed typical symptoms of the diseases as leaf roll, early reddening and in some cases, collapse of the tree in spring.

Molecular characterization based on genes *imp, pnp, ace*F, *sec*Y and 16S rDNA of isolates obtained from peach, pear and *C. pyri*.

Table 2 shows the results obtained after the molecular characterization of phytoplasma isolates from peach and pear infected samples and from individuals of *C. pyri* captured in peach and pear orchards. Results obtained in the study of 16SrDNA genes indicated that all isolates belonged to '*Ca*. P. pyri' with a 100% of homology with the reference isolate PD1 (GenBank Accession No. AJ542543), confirming that this phytoplasma is capable to infect peach causing PYLR. Concerning the other genes, usually the same genotypes were obtained in samples of pear trees and in the psyllids collected in pear orchards (Table 2). Equal results occur between the sequences obtained in the phytoplasma isolated in peach and in the psyllids captured in the peach orchards. This is very clear in gene *pnp*, as in pear samples and in the psyllids from pear orchards matches with strain P8 in the nomenclature of Danet *et al*., (2011), whereas in peach and psyllid from peach orchards the main isolate was genotype P3 (Table 2). For gene *secY* sequences from infected psyllids in peach analyzed were S8 and S7 while on peach trees S8 was not detected and S7 was the main isolate, being S2 also identified in a peach sample (Table 2). The peach sample with S2 profile shows a recombination between the genome of '*Ca*. P. pyri' and '*Ca*. P. prunorum'. The marker profile combination of this sample is S2, I19, A10 and P7. The sequentiation of 16SrDNA gene (fragment obtained with the ribosomic primer R16F2n/R16R2) showed 100% homology with 'Ca. P. pyri' profile (Table 2).

DISCUSSION

It has been demonstrated that *C. pyri* is a vector of '*Ca*. P. pyri' in both peach and pear, causing PYLR and PD diseases in Spain. The Phytoplasma transmission with *C. pyri* was demonstrated in both experiments, transmission to healthy peach plants and transmission to an artificial feeding medium. In fact all phytoplasmas belonging to 16SrX group are transmitted by psyllids (Carraro *et al*., 1998 a; Weintraub & Jones, 2010), confirming the idea that the transmission of this phytoplasma in peach is effective also by *C. pyri*, the only psyllid collected in peach affected orchards. Similar results have been reported in California where the phytoplasma was transmitted from pear to peach through the vector of PD in USA, the psyllid *Cacopsylla pyricola*, causing similar symptoms to those observed in peach in Spain (Blomquist & Kirkpatrick, 2002). Moreover, it is especially during autumn, when a high number of individuals enter in peach orchards, and the highest number of individuals carrying the phytoplasma is found. Results show that *C. pyri* returns to match and lie eggs in February exclusively on pear trees. On the other hand, *C. pyri* individuals used in the transmission trials to peach trees, were all dead during the week of transmisión trial, confirming that they cannot feed on peach trees exclusively, but they transmitted the fitoplasma. Consequently, we consider that adults of *C. pyri* only feed occasionally on peach, especially in autumn when the insects fly out to overwinter and stopped temporary in the close peach trees. Other potential vectors have been discarded because they were found in very low numbers or not infected by this phytoplasma, including *C. pruni* that has not been captured in this study and previous in the area (Sabaté *et al*., 2016). The percentage of pear psylla carrying the PD phytoplasma showed a seasonal trend through the year, which coincides with the seasonal detection of '*Ca.* P. pyri' in the trees (Garcia-Chapa *et al*., 2005). In autumn, *C. pyri* can acquire the phytoplasma more easily because, at this time of the year, the colonization of the

phytoplasma in the tree is the highest (Garcia-Chapa *et al*., 2003; Seemüller *et al*., 1984), consequently the highest infection rate was recorded in this season. The percentage of pear psylla carrying the PD phytoplasma was high in September but slightly decreased in October, most possibly because the new winter forms of *C. pyri* adults were emerging. In November the highest fly peak and an increase of the positives insects were determined in peach orchards due to the fact that they have more time to multiply the phytoplasma. (Garcia-Chapa *et al*., 2003b). These results indicated that autumn could be the most dangerous period for the infection of peach trees by '*Ca.* P. pyri'. The control of the *C. pyri* populations in pear orchards could be a useful tool to minimize PYLR incidence in close peach orchards. The control in autumn is especially important in the early varieties, because the farmers normally stop the treatments after harvest, causing an increase of the *C. pyri* populations.

The transmission of the phytoplasma from individuals of *C. pyri* to artificial feeding medium has been confirmed. Furthermore, the transmission by sap-sucking insect vectors to an artificial medium appears as an efficient alternative method to test phytoplasma transmission, and allows taking disease control measures earlier than other classical methods. This alternative method also showed that the females have the ability to transmit the phytoplasma more efficiently. Similar results were obtained before with psyllids collected in pear orchards (Garcia-Chapa *et al*., 2005). The molecular characterization and MLST have demonstrated that '*Ca*. P. pyri' is the phytoplasma causing PYLR disease, and the genotypes obtained show a great diversity of '*Ca*. P. pyri' phytoplasma in the area, and support a high capability of recombination between phytoplasmas. The results obtained in this study show that some genotypes are preferentially determined in peach, as this is the case of P3. Concerning gene *Ace*F, for which two genotypes were determined (A10 and A12), the two strains were identified in pear trees and in psyllids

captured in both pear and peach orchards, whereas in peach trees only A10 was determined. According to the results obtained an isolate with the haplotype A10 (PD isolate), I19 (PD isolate), P3 (PD isolate), S7 (PD isoalte), appears as the main haplotype of '*Ca*. P. pyri' capable to infect peach in this area. According to Danet *et al*. (2011) PD isolate has been characterized in samples from Germany, France, Italy, Spain, Croatia and is one of the main isolates in Europe and Asia. The coexistence of different isolates in the area, allows the multiple infection of the hosts (plants or insects) and the exchange of genes between them. A recombination in the gene SecY was determined in a sample of peach that presents the profile S2 (ESFY isoalte), A10 (PD isolate) I 19 (PD isolate), P7 (PD36 Az isolate) . The recombination of the genomes of '*Ca*. P. pyri' and '*Ca*. P. prunorum' was described before in the gene *Imp* with some samples of the same region of Spain (Danet et al. 2011; Brathislava et al. 2012), but this is the first time that this recombination was found in the gene Sec. The alternation of peach and pear orchards and the abundance of the *C .pyri* favors this recombination. More samples of pear, peach and vectors in both plots and surrounding orchards, have to be sequenced with non-ribosomic genes to increase the number of samples and genes studied. In parallel, these isolates identification has to be extended to other PYLR infected orchards and to '*Ca*. P. pyri' on pear trees in areas without PYLR in the Lleida province. Further work is needed to determine which is the dominant isolate in peach affected trees, and whether '*Ca*. P. pyri' performs a specific adaptation to infect peach hosts. These data will increase the understanding of virulence and host specificity in the group X of phytoplasmas, providing knowledge for a better control of PYLR and the other phytoplasmas within this group.

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Table 1 Potential insect vector species collected in traps placed in two pear and peach orchards. Identification of species that are phytoplasma carriers.

Insect species	Pear Total collected	Peach Total collected	No. analysed	'*Candidatus* Phytoplasma pyri' positive		
				Strong	Weak	Total
Cicadella sp.	1	0	1	0	0	0
Neoaliturus fenestratus	13	1	14	0	0	0
Empoasca sp.	0	3200	250	0	4	4 (1.6%)
Laodelphax striatellus	0	2	2	0	0	0
Opsius stactogalus	0	2	2	0	0	0
Cacopsylla pyri	9138	1928	4212	1053 (25%)	1558 (37%)	2611 (62%)

Table 2 Genotypes identified in the different samples of *Cacopsylla pyri* collected in pear and peach orchards, and in samples of affected pear and peach trees.

	No. of samples of each genotype									
	secY			*imp*		*aceF*		*pnp*		
Sample origin	S8	S7	S2	I19	I18	A10	A12	P8	P3	P7
C. pyri from pear	4	1	0	4	1	3	2	5	0	0
Pear trees	5	0	0	4	1	3	2	5	0	0
C. pyri from peach	3	2	0	5	0	3	2	0	5	0
Peach trees	0	4	1	5	0	5	0	0	4	1

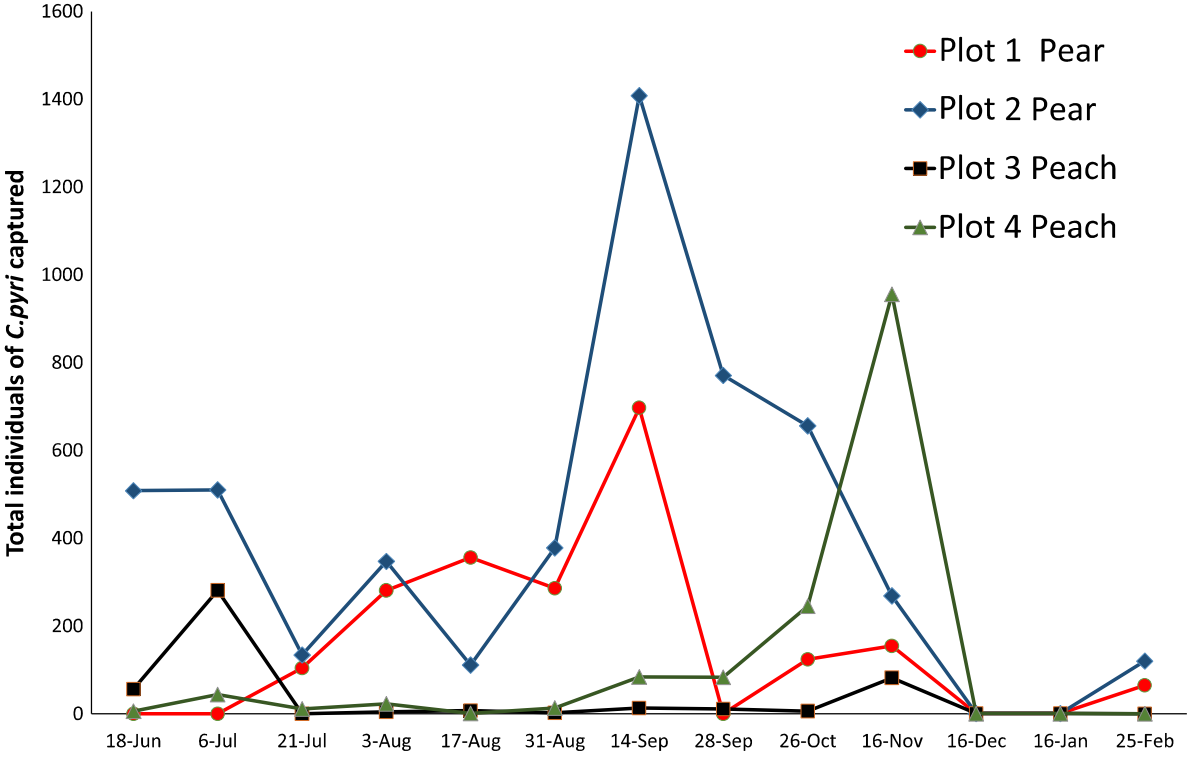

Figure 1 Flight activity of *Cacopsylla pyri* adults in two pear and two peach orchards. Total number of individuals captured in four yellow traps per orchard.

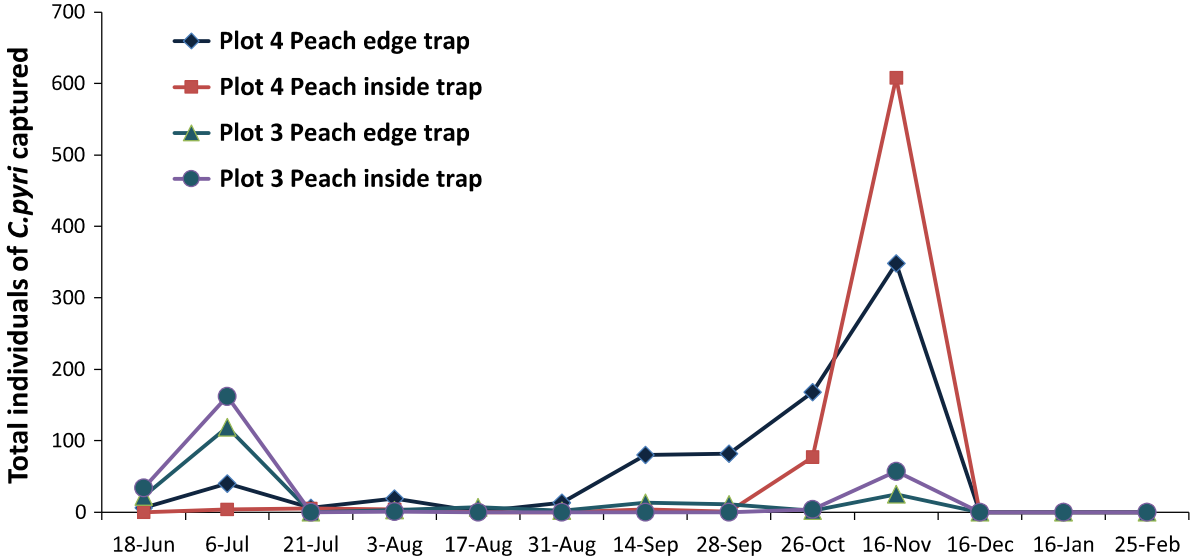

Figure 2 Flight activity of *Cacopsylla pyri* in two peach orchards. Total number of individuals captured in two yellow traps placed at the edges of the orchards and in two traps placed inside the orchards.

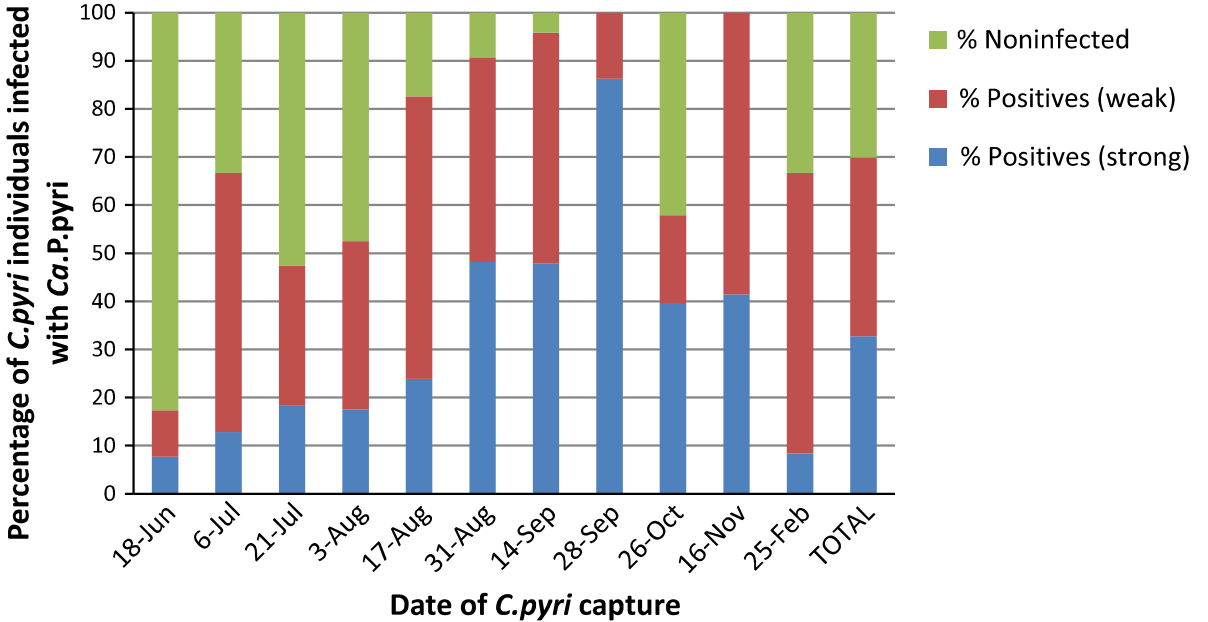

Figure 3 Percentage of *Cacopsylla pyri* individuals infected with '*Candidatus* Phytoplasma pyri' in two pear orchards. Strong positives (C_t in real-time PCR < 27); weak positives (C_t 27–33).

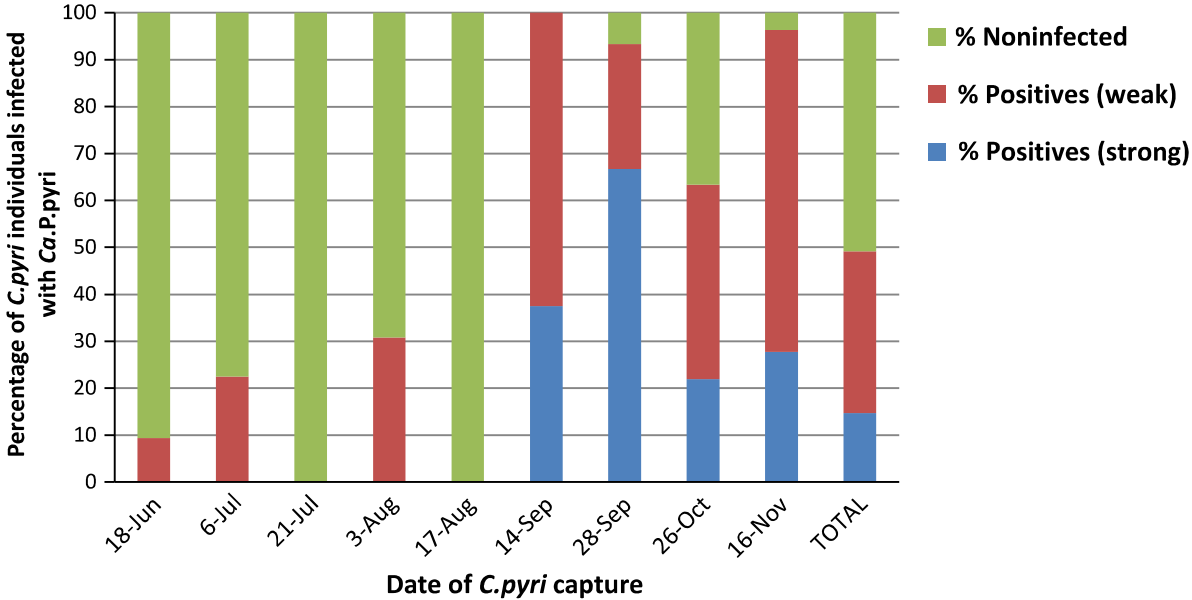

Figure 4 Percentage of *Cacopsylla pyri* individuals infected with '*Candidatus* Phytoplasma pyri' in two peach orchards. Strong positives (C_t in real-time PCR < 27); weak positives (C_t 27–33).

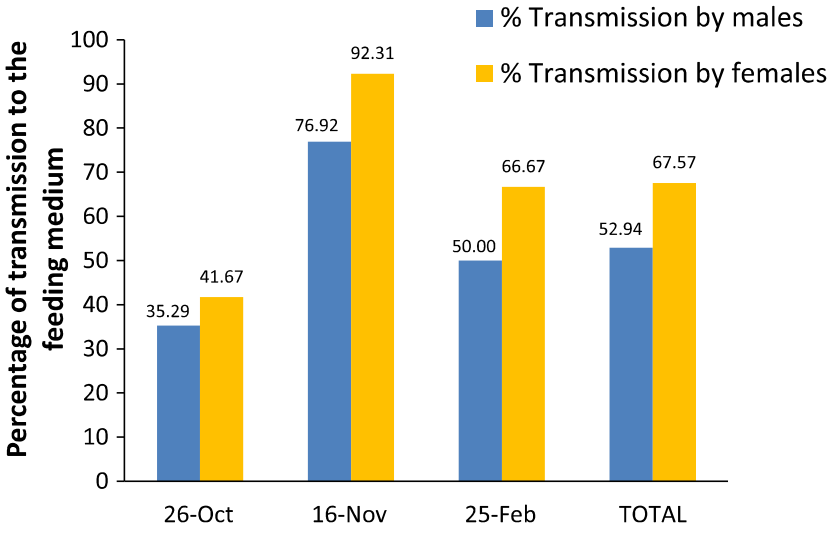

Figure 5 Percentage of individuals (males and females) infected by '*Candidatus* Phytoplasma pyri' that transmit it to the insect feeding medium.