Identification and quantification of '*Candidatus* Phytoplasma pyri' in declining trees of a Swiss cider pear orchard after incision treatment at the stem base

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Abstract

Orchards of 30 to >100 years old fruit trees used for cider production are endangered by an accumulation of abiotic and biotic stress factors. Among biotic stress factors, diseases such as pear decline (PD) caused by the bacterial pathogen 'Candidatus Phytoplasma pyri' contributes to a weakening and reduced life time of affected trees. Since direct treatment of this disease is not possible, approaches have gained attention, which might lead to an increased resilience against this pathogen, such as incisions of the cambium at the graft union at the stem base. Six 35 years old pear trees (Pyrus communis) of a Swiss cider production orchard, all affected by mild decline were chosen for this study. Four out of them were treated with 2-4 incisions per tree in February 2016. Symptoms were visually assessed during summer and autumn 2016 and 2017, respectively, and 'Ca. P. pyri' measured in branch samples with a newly developed duplex TaqMan qPCR assay. No effect could be determined within these two subsequent growing seasons after treatment. Both, visual assessment of symptoms and qPCR measurement of the pathogen in branches did not show any difference between treated and untreated trees. The sequencing of two marker genes of the pathogen detected in this orchard confirmed its identity as 'Ca. P. pyri' and revealed that it belongs to the major genotype present in Europe.

Keywords: *Pyrus communis*, pear decline (PD), incision treatment, duplex TaqMan qPCR

Introduction

In temperate Europe, orchards of up to more than 100 years old pear trees (beside other fruit trees) used for cider production are a valuable asset in terms of biological diversity and landscape aesthetics (Herzog, 1998). In recent years, however, they have been endangered more and more by an accumulation of different abiotic and biotic stress factors, such as prolonged drought periods related to climate change, and diseases, such as fire blight and pear decline (PD). Disease development and symptom expression of PD have been classified into quick and slow decline. Quick decline causes death of the tree within a few weeks due to complete blocking of sieve tubes, whereas slow decline is characterized by a progressive weakening of the tree which is fluctuating in severity from year to year (Seemüller, 1989). Reddening of the foliage in late summer or autumn is a mild form of slow decline (Jarausch & Jarausch, 2010).

PD caused by the bacterial pathogen '*Candidatus* Phytoplasma pyri' is wide-spread in Switzerland (Bünter & Schaerer, 2012) and Europe (Jarausch & Jarausch, 2010). This pathogen is mainly transmitted by grafting or by the insect vectors *Cacopsylla pyri* and *C. pyricola* (Jarausch & Jarausch, 2010). Direct, curative treatment of the disease is not possible, but indirect disease control targeting the insect vector is basically possible e.g. by application of kaolin (Pasqualini *et al.,* 2007). However, even though kaolin, in contrast to

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insecticides would be compatible with organic fruit production, it is rather difficult to be applied on trees of 10 m or more in height.

Recently, an alternative treatment was proposed to incise trees at the graft union at the stem base with vertical 1-3 cm deep cuttings with a motor saw (Weber 2010). The idea behind it is to provoke the tree to build new phloem connections in response to the wounding, bypassing the necrotic phloem observed at the graft union of PD symptomatic trees. Results presented by Weber (2010) were promising and incision treatments were reported having been related to a reduction of both symptoms and percentage of trees tested positive for phytoplasma by nested PCR.

The aim of this study was to investigate whether this incision treatment on PD symptomatic 35 years old pear trees (*Pyrus communis*) inhibits or slows down symptom development of the foliage and spread of the pathogen '*Ca*. P. pyri' in the phloem of the crown in the following growing seasons. To this end, a duplex TaqMan qPCR assay was developed for testing branch samples of the crown for presence of '*Ca*. P. pyri'. Furthermore, marker genes of one positive sample of this orchard were amplified and sequenced in order to identify and characterize the genotype of the pathogen according to Danet *et al*. (2011).

Materials and methods

Incision treatment and disease assessment. In a cider pear orchard (Steinmaur, Switzerland) of 35 years old pear trees (*Pyrus communis*; cultivar "Schweizer Wasserbirne" grafted on rootstock "Kirchensaller Mostbirne"), six trees were chosen for this study which showed PD symptoms (red leaves in late summer/early autumn) and which had been positively tested with nested PCR for presence of '*Ca.* P. pyri' in root samples before (M. Riedle-Bauer, BOKU Vienna, Austria; pers. communication). Four out of these six trees were treated with 2-4 vertical incisions at the graft union at the stem base on February 2016 (Table 2). The incisions were approx. 20 cm in length and of 2-3 cm in depth in the cambium layer. The disease was visually assessed during summer and autumn 2016 and 2017, respectively, at monthly intervals.

DNA extraction. From each of the six trees, ten two-years old branches were cut around symptomatic regions of the lower part of the crown. One of the trees, which was treated with three incisions under each of the three leader branches, was sampled with ten separate samples of each leader branch region of the lower crown (610/19a, b, c; Table 2). The bark firmly attached to the cambium was scraped off with a scalpel blade and then 0.2 - 0.3 g of thin cuttings of the green cambium layer homogenized with the homogenizer HOMEX 6 in extraction bags "Universal" (both from Bioreba, Reinach, Switzerland) containing 5 ml of CTAB extraction buffer (2% CTAB, 1% PVP K25, 100 mM Tris-HCl pH 8.0, 50 mM Na_2EDTA, 1.4 M NaCl, 0.1% 2-mercaptoethanol) (Oberhänsli *et al.* 2014). One ml of the suspension was incubated for 20 min at 70°C, and then mixed with 0.8 ml chloroform-isoamylalcohol (24:1). The suspension was vortexed for 3 x 2 s and centrifuged for 2 min at 20,000 g at RT. For DNA precipitation, 0.8 ml of the aqueous supernatant was mixed with 0.6 ml of isopropanol, incubated at -20°C over night and centrifuged for 2 min at 20,000 g. The pellet was washed once with 0.8 ml of 70% EtOH, dried and finally dissolved in 100 µl TE (10mM Tris, 1 mM Na_2EDTA, pH 8.0). DNA was stored at -20°C until further use.

Real-time PCR assays. Novel real-time primers (PD05_F/R) and a fluorogenic TaqMan probe (PD05_P) specific for '*Ca*. P. pyri' were designed matching the 3' end of the 16S rRNA gene of '*Ca.* P. pyri' strain PD1 (AJ542543) using Beacon Designer 8.1 (Fig 1). The probe PD05_P was labelled with 6-FAM Fluorescein (FAM) at the 5' and the black hole quencher

(BHQ1) at the 3' end. They were combined to a duplex assay with the previously described host-specific 18S rRNA gene TaqMan assay using Rhodamin X (ROX) as reporter dye and BHQ2 as quencher, respectively (Table 1). The duplex TaqMan qPCR assay was run totalling 15 µl using 1 µl of DNA, 7.5 µl of SsoAdvanced Universal Probes Supermix (BioRad), and 6.5 µl of primers and probes at final concentrations as indicated in Table 1. FAM and ROX signals were recorded after each annealing/elongation step real-time in a 72 well rotor (Rotorgene Q, Qiagen) during 45 cycles in a two-step protocol of 3 s at 95°C and 20 s at 60°C after an initial step of 3 min at 95°C for activation of the hot start DNA polymerase.

Table 1: Primers (forward/reverse; F/R) and probes (P) for duplex TaqMan qPCR assay for detection of the 16S rRNA gene of the bacterial pathogen '*Ca*. P. pyri' (this work) and the 18S rRNA gene of the host *Pyrus communis* (Md, Oberhänsli *et al*. 2014).

Primer/ probe	Sequence 5'-3'	Final conc.
PD05_F	CACACCGCCCGTCAAACC	300 nM
PD05_R	CAACCCTACCTTAGACAGTTCCC	300 nM
PD05_P	FAM-TGAAAGTTGACAATACCCGAAACCAGTAGCC-BHQ1	100 nM
Md_F	AGAGGGAGCCTGAGAAACGG	50 nM
Md_R	CAGACTCATAGAGCCCGGTATTG	50 nM
Md_P	ROX-CCACATCCAAGGAAGGCAGCAGGCG-BHQ2	50 nM

The specificity of the '*Ca.* P. pyri'-specific primers and probe was checked by direct sequencing of obtained PCR amplicons (by GATC Biotech, Konstanz, Germany) using the pathogen-specific primers for amplification of the PCR-product. Furthermore, the real-time PCR assay by Torres *et al.* (2005) was used to verify the duplex TaqMan PCR assay. Real-time PCR reactions consisted of 1-4 µl template DNA, SsoAdvanced Universal SYBR Green Supermix (BioRad) and 0.3 µM of each primer in a total volume of 25 µl were performed on a CFX96 Touch Real-Time PCR Detection System. Negative (no DNA added) and positive controls were included in all PCR reactions. The presence and size of amplicons were checked by electrophoresis in a 1.5% (w/v) agarose gel stained with Roti Gelstain (Carl Roth, Karlsruhe, Germany). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and directly sequenced using an ABI 3730xl DNA Analyzer systems (by GATC Biotech, Konstanz, Germany).

PCR for *aceF* and *pnp* genes. The PCR primers of Danet *et al.* (2011) were used to amplify the dihydrolipoamide acyltransferase (*aceF*) and the polynucleotide phosphorylase (*pnp*) genes of '*Ca*. P. pyri' strain PDH16-20 originated from pear tree 610/19c. PCR reactions and subsequent sequencing of PCR products were performed as described above.

Phylogenetic analysis. The program Geneious version 9.1.8 (http://www.geneious.com), and MEGA7 (Tamura *et al.*, 2013) were used for phylogenetic analysis. Databases for genes encoding *aceF* and *pnp* were established with representative sequences downloaded from the GenBank database (NCBI, https://www.ncbi.nlm.nih.gov/; query date: December 20, 2017) provided by the NCBI (National Centre for Biotechnology Information), and aligned using CLUSTAL W (Thompson *et al.*, 1994) implemented in Geneious. Phylogenetic trees were calculated using MrBayes 3.2.6 (Huelsenbeck *et al.*, 2001) and the distance method Neighbor-Joining (Jukes-Cantor correction, 1000 bootstrap replicates) implemented in

Geneious, and maximum likelihood (HKY85, 1000 replicates) (Guindon *et al.*, 2010) and maximum parsimony (1000 replicates) implemented in MEGA7.

Nucleotide sequence accession numbers

The obtained partial 16S rRNA gene sequences for '*Ca.* P. pyri' strain PDH16-20 (MG764531), '*Ca.* P. pyri' strain PDH17-127 (MG764532) and '*Ca.* P. pyri' strain PDH17-143 (MG764533), the *aceF* gene sequence for '*Ca.* P. pyri' strain PDH16-20 (MG764529) and the *pnp* gene sequence for '*Ca.* P. pyri' strain PDH16-20 (MG764530) were deposited at GenBank/EMBL/DDBJ databases.

Results

PD symptom development did not correlate with incision treatment. There was no correlation between incision treatments and delay of symptom development in terms of reddening of the foliage of the six trees. But symptom development differed between years: in 2017, symptoms appeared already beginning August and peaking at end of September when all six trees were visually assessed positive (Table 2). In summer and autumn of 2016, however, the first growing season after the incision treatment, first weak symptoms of foliage reddening developed only in September (data not shown). Any further progression of the disease symptoms was masked then by natural foliar discoloration starting at beginning of October.

Table 2: List of pear trees, treatments, qPCR results of branch samples, and visual assessments of symptoms in summer and autumn (- no symptoms, + slight/partial reddening of foliage, ++ strong reddening of foliage). *sampling of branches after leaf fall, n.d.: not determined.

Tree No.	Treatment	Symptoms (visual assessment)		qPCR (No. positives / total branches tested)		
		03 Aug '17	29 Sep '17	26 Oct '16*	29 Sept '17	07 Nov '17*
610/1	2 incisions	-	++	n.d.	2/6	6/10
610/3	untreated	+	++	0/3	0/6	3/10
610/4	4 incisions	+	++	1/3	0/6	4/10
611/4	3 incisions	+	++	1/3	2/6	2/10
610/20	untreated	-	+	n.d.	2/6	2/10
610/19a		-	++	3/3	0/6	2/10
610/19b	3 incisions	-	++	2/3	0/6	2/10
610/19c		-	++	2/3	0/6	3/10

Validation of the novel duplex TaqMan qPCR assay. This novel assay based on primer and probes listed in Table 1, had an amplification efficiency of 95-102% at an annealing/elongation temperature of 61°C for a 10-fold dilution series of DNA extracts of cambium of infected branches of pear. Cq values of positive pear cambium samples were similar (deltaCq<1.2) when tested samples with the PD05 reagents (Table 1) alone or duplex in combination with Md primers (Table 1) that react with pear 18S rRNA gene as well. The qPCR based on PD05 primer/probe amplifies a fragment of 87 base pairs which is specific for '*Ca*. P. pyri' based on similarities with homologous sequences in NCBI using BLAST (https://blast.ncbi.nlm.nih.gov). In addition, positively tested samples of the pear trees in this study with the new TaqMan qPCR assay, were confirmed with the Sybr assay employing the primer pair P1 / R16(X)F1r (Torres *et al.* 2005).

DNA extracts made from cambium peelings without preceding removal of the bark were brownish and had to be diluted up to 50x before qPCR analysis to get amplification. In contrast, DNA extracts of positive samples made from cambium peelings from branch sections where the bark had been scraped off produced also undiluted readily a signal.

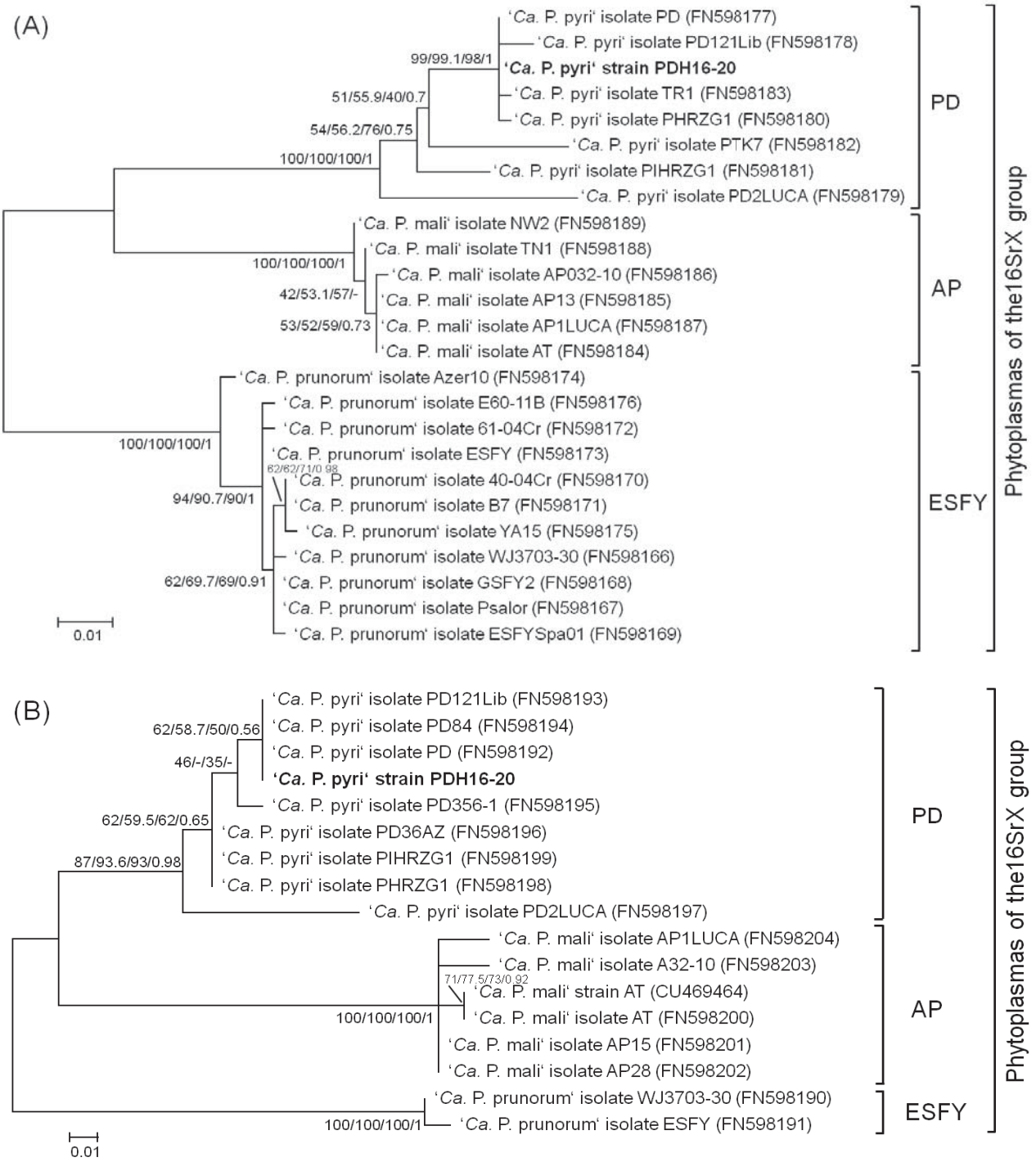

Figure 1: Phylogenetic position of '*Ca.* P. pyri' strain PDH16-20 within the phytoplasmas of the 16SrX group using maximum likelihood trees based on (A) the dihydrolipoamide acyltransferase (*aceF*) and (B) the polynucleotide phosphorylase (*pnp*) genes according to Danet *et al.* (2011). Maximum likelihood, neighbor joining and maximum parsimony bootstrap values (1000 replicates), and Bayesian posterior probabilities are indicated at the inner nodes. Select members of the '*Ca.* P. prunorum' were used as outgroup. GenBank/EMBL/DDBJ accession numbers are given in brackets. PD, pear decline; AP, apple proliferation; ESFY, European stone fruit yellows.

Detection rate of '*Ca*. P. pyri' did not correlate with incision treatments. Detection of '*Ca.* P. pyri' in branches with this duplex TaqMan qPCR assay, was best in samples taken in late October (2016) or beginning November (2017) with Cq values of 12-15 for the host gene and between 25 and 37 for the 16S rRNA gene of '*Ca.* P. pyri'. Highest and lowest concentration of the pathogens, or lowest and highest Cq values, respectively, were recorded both in tree 610/19 which had been treated with three incisions. Only 20-60 % of branch samples were positive for '*Ca.* P. pyri' (Table 2). Most of the samples taken in August and September were '*Ca.* P. pyri' negative, with exception of positive samples of trees 610/1, 611/4, and 610/19 taken end of September 2017 (Table 2).

The swiss '*Ca.* P. pyri' strain PDH16-20 represents a major genotype found in Europe. To position the phytoplasmas causing PD of the cider pear orchard in Steinmaur, Switzerland to other phytoplasmas of the 16SrX group, we amplified, sequenced and analyzed two marker genes - the dihydrolipoamide acyltransferase (*aceF)* and polynucleotide phosphorylase (*pnp)*. The sequences were obtained from '*Ca.* P. pyri' strain PDH16-20 originated from the pear tree 610/19c sampled in 2016. The *aceF* gene showed 100% similarity with the '*Ca.* P. pyri' isolate PD (FN598177) which represents a highly abundant genotype (A10) found in Germany, Azerbaijan, Croatia, France, UK, Italy and Turkey (Danet *et al*., 2011). The *pnp* gene showed 100% similarity with '*Ca.* P. pyri' isolate PD84 (FN598194) demonstrating a genotype (P5) detected in Italy and Lebanon, '*Ca.* P. pyri' isolate PD121Lib (FN598193) representing a genotype (P4) found in Lebanon, Azerbaijan, Croatia, UK and Spain, and '*Ca.* P. pyri' isolate PD (FN598192) reflecting a genotype (P2) found in Germany, France, Lebanon and Spain (Danet *et al*., 2011). Our phylogenetic analyses using published datasets (Danet *et al*., 2011) showed that the obtained *aceF* and *pnp* gene sequences of '*Ca.* P. pyri' strain PDH16-20 clustered together with other members of the well-supported '*Ca.* P. pyri' subgroup causing PD (Figure 1A und 1B).

Discussion

The results indicate no effect of the incision treatments on the development of the disease in the two subsequent growing seasons. Symptoms development differed between the two growing seasons 2016 and 2017, respectively, but not between trees treated or not with incisions. In contrast to Weber (2010) who treated young trees (approx. 5-10 years old pear trees), the pear trees involved in this study were 35 years old. In branch samples '*Ca.* P. pyri' was readily detectable with Cq values between 25 and 38 with this newly developed duplex TaqMan qPCR assay. The simultaneous amplification of the host gene in the same reaction was very helpful to rule out false negatives. This was indeed important by the fact that the detection of phytoplasma was with few exceptions only possible late in the season after leaf fall of mid-October at a rate of 20% to a maximum of 60% in pear branches even in highly symptomatic trees (Table 2). This uneven and seasonal distribution of the pathogen in the cambium tissue of pear trees has been reported before (Garcia-Chapa *et al*. 2003). This novel duplex TaqMan qPCR has proved to be suitable to determine '*Ca.* P. pyri' in cambium tissue of infected pears by circumstantial evidence: (i) it showed good amplification efficiency for pear tissue extracts containing '*Ca*. P. pyri' and absence of any cross-reaction with healthy plant tissue; (ii) the sequencing of the amplified product and the subsequent similarity search indicated amplification of DNA identical to that of known phytoplasmas, and (iii) the positive results were confirmed with the qPCR assay specific for fruit tree phytoplasmas by Torres et al. (2005). Although specific for '*Ca.* P. pyri' *in silico'* it cannot be ruled out, however, that the TaqMan qPCR based on PD05 primer/probe (Table 2), will cross-react with the closely related '*Ca.* P. mali' and '*Ca.* P. prunorum', respectively, since

they differ from probe PD05_P just with 1-2 mismatches in their homologous nucleotide sequence. As trees of the cultivar “Schweizer Wasserbirne” normally reach an age of 100 years, further efforts will be undertaken aiming to enhance the health status of the trees in this orchard. One such measure comprises the injection of plant growth promoting microorganisms into the rhizosphere of the pear trees to improve root growth and nutritional status. If this more conservatory means fails to be successful, cutting down of the most affected trees and replanting with grafts on PD robust or resistant root stocks will be another but ultimate option.

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References

Bünter, M. & Schaerer, S. (2012). Phytoplasmes en arboriculture. *Revue Suisse de Viticulture Arboriculture Horticulture* **44**: 21.

Danet, J.L., Balakishiyeva, G., Cimerman, A., Sauvion, N., Marie-Jeanne, V., Labonne, G., Laviña, A., Batlle, A., Križanac, I., Škorić, D., Ermacora, P., Serçe, Ç.U.¸, Çağlayan, K., Jarausch, W. & Foissac, X. (2011). Multilocus sequence analysis reveals the genetic diversity of European fruit tree phytoplasmas and supports the existence of inter-species recombination. *Microbiology* **157**: 438-450.

Garcia-Chapa, M., Medina, V., Viruel, M.A., Laviña, A. & Battle, A. (2003). Seasonal detection of pear decline phytoplasma by nested-PCR in different pear cultivars. *Plant Pathology* **52**: 513-520.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology* **59**: 307-21

Herzog, F. (1998). Streuobst: a traditional agroforestry system as a model for agroforestry development in temperate Europe. *Agroforestry Systems* **42**: 61-80.

Huelsenbeck, J.P. & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754-5.

Jarausch, B. & Jarauch, W. (2010). Psyllid vectors and their control. In *Phytoplasmas: Genomes, Plant Hosts and Vectors* (ed. Weintraub, P.G. & Jones, P.), pp. 250-271. CAB International.

Oberhänsli, T., Vorley, T., Tamm, L. & Schärer, H.-J. (2014). Development of a quantitative PCR for improved detection of *Marssonina coronaria* in field samples. In *Proceeding of the 16th International Conference on Organic Fruit-Growing*, Hohenheim, Germany, pp 187-190.

Pasqualini, E., Civolani, S. & Grappadelli, L.E. (2007). Particle film technology: approach for a biorational control of *Cacopsylla pyri* (*Rhynchota Psyllidae*) in Northern Italy. *Bulletin of Insectology* **55**: 39-42.

Seemüller, E. (1989). Pear decline. In *Virus and viruslike diseases of pome fruits and simulating noninfectious disorders* (ed. Fridlund, P.R.), pp. 188–201. Cooperative Extension College of Agriculture and Home Economics. Washington State University, Pullman, Washington.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725-2729.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

Torres, E., Bertolini, E., Cambra, M., Montón, C. & Martín M.P. (2005). Real-time PCR for simultaneous and quantitative detection of quarantine phytoplasmas from apple proliferation (16SrX) group. *Molecular and Cellular Probes* **19**: 334-340.

Weber H.-J. (2010). Der Birnenverfall – und dann?, DLR Rheinpfalz, Kompetenzzentrum Gartenbau, Rheinbach. In http://www.hortipendium.de/Birnenverfall.