

## A survey of ‘*Candidatus Phytoplasma pyri*’ isolates in the Czech Republic based on *imp* gene genotyping

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### Abstract

‘*Candidatus Phytoplasma* spp.’ are pathogenic bacteria that infect many plant species. ‘*Candidatus Phytoplasma pyri*’, one of the members of the 16SrX group causes pear decline disease that adversely affects pear crops. To describe the prevalence of ‘*Ca. P. pyri*’ genotypes in the Czech Republic, 143 pear samples were collected from 41 locations including commercial orchards as well as trees along roads. Phytoplasma was detected by PCR in 115 samples, and it was possible to determine *imp* gene genotype in 84 samples. The most frequent genotypes were A1, B1, and C, which were identified in 71% of phytoplasma positive samples. ‘*Ca. P. pyri*’ was present either alone or as a mix of two populations in 88% of genotyped samples, and in another 6% of samples it was found in a mixed infection with ‘*Ca. Phytoplasma mali*’. A sole infection with ‘*Ca. Phytoplasma mali*’ was observed in 6% of samples. As for symptoms, 19% of symptomatic samples were found to be phytoplasma negative, and 74% of asymptomatic samples proved to be phytoplasma positive; leaf roll was more often observed in phytoplasma positive samples, while leaf narrowing rather indicated the absence of phytoplasma. The mildest symptoms were observed in samples infected with ‘*Ca. P. pyri*’ of the A1 *imp* genotype.

**Keywords:** 16SrX group phytoplasma; genetic variability; immunodominant membrane protein; pear decline symptoms

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## Introduction

'*Candidatus* Phytoplasma spp.' are small bacteria with a diameter of less than 1  $\mu\text{m}$ , and belong to the class Mollicutes that is characterized by having no cell wall. Phytoplasmas are obligate parasites infecting the phloem tissue of a plethora of plant species. They are transmitted by sap-sucking insect vectors, especially from the families Cicadellidae, Fulgoridae and Psyllidae, or by vegetative propagation.

Based on their 16S rDNA sequence, phytoplasmas have been divided into 33 groups (Zhao and Davis, 2016). For fruit-tree growers, phytoplasmas of the apple proliferation group 16SrX are the most important, including '*Ca. P. pyri*' infecting pear trees and causing pear decline; '*Ca. Phytoplasma mali*' infecting apple trees and causing apple proliferation; and '*Ca. Phytoplasma prunorum*' infecting stone fruit trees and causing European stone fruit yellows (Seemüller and Schneider, 2004).

Pear decline occurs worldwide, and '*Ca. P. pyri*' is a regulated organism in the European Union and in many other countries. Slow and quick forms of decline can be distinguished (Seemüller *et al.*, 2011). Slow decline is often followed by quick decline, which is usually initiated by stress conditions and is characterized by the sudden wilt and death of the tree within a week(s). The typical symptoms of slow decline include purple leaves or premature leaf reddening, altered leaf morphology (leaf narrowing and/or rolling, reduced size), the slow dieback of branches, reduced fruit set and size, and a generally shorter habitus with progressive weakening of the tree (Seemüller *et al.*, 2011; Bertaccini *et al.*, 2014). In Europe, the main vectors of '*Ca. P. pyri*' are naturally occurring *Cacopsylla pyri* L., *C. pyricola* (Förster) and *Cacopsylla pyrisuga* (Förster) (Seemüller *et al.*, 2011). There are currently no treatments for control of the disease, only prevention.

To describe the diversity and evolutionary relationships between and within '*Candidatus* Phytoplasma' species, various polymorphic genes have been used, e.g., *aceF*, *pnp*, *secY* and *imp* (Danet *et al.*, 2011). In our previous study, we focused on the '*Ca. P. pyri*' *imp* gene that codes for the immunodominant membrane protein (Imp); we defined 17 distinct *imp* genotypes labelled A-K, and developed an RFLP method for quick *imp* genotype determination (Bohunická *et al.*, 2018). In the present follow-up study, we report the prevalence and geographical distribution of '*Ca. P. pyri*' genotypes in the Czech Republic, thus complementing studies dealing with '*Ca. P. mali*' in the Czech Republic (Fránová *et al.*, 2013; Rejlová *et al.*, 2019), and those describing the molecular diversity of '*Ca. Phytoplasma mali*' and '*Ca. P. prunorum*' in Slovenia (Dermastia *et al.*, 2018), and other European countries (Danet *et al.*, 2011).

Imp proteins play an important role in phytoplasma pathogenesis (Berg *et al.*, 1999; Kakizawa *et al.*, 2009), likely modulating pathogen-host interactions and the defense response of the host during infection (Zhang and Wise, 1996). Recently, the role of Imp proteins in phytoplasma-insect vector interactions was studied in Flavescence dorée phytoplasma, where it was shown to specifically bind to vector proteins that were most likely located in guts, supporting the role of Imp in transmission (Trivellone *et al.*, 2019). Likewise, Imp proteins of wheat blue dwarf phytoplasma were shown to promote transmission by directly interacting with  $\alpha$ -tubulin in leafhoppers (Ding *et al.*, 2022). Moreover, '*Ca. P. mali*' Imp proteins were demonstrated to bind actin, suggesting its role in phytoplasma motility within the plant (Boonrod *et al.*, 2012). Another aim of the present work was therefore to assess possible associations of *imp* genotypes and symptoms, in order to better understand '*Ca. P. pyri*' epidemiology and pear decline etiology.

## Materials and Methods

### *Sample collection*

Pear samples (*Pyrus communis* L.) were collected throughout the Czech Republic between 2015 and 2016. Pear trees were selected randomly with a focus on predominantly symptomatic trees, but asymptomatic trees were sampled as well. Typically, four shoots were sampled for each tree. For analyses, trees were selected along roads, in old orchards, municipalities, gardens and in production orchards; the variety and rootstock

information were seldom available. If possible, symptoms were described in two categories: habitus/vitality (growth depression; withering) and leaves (purple leaves; leaf reddening; chlorosis; leaf narrowing; leaf roll) (Table 1). When information about the tree age was not available, it was estimated. For each symptom, its presence (on a scale from “absence” to “the whole tree” affected) and intensity (on a scale from a “none” to a “very strong” manifestation) were evaluated. Fisher’s exact test was used for statistical analyses.

**Table 1.** Symptom descriptions

Leaves	Description
Purple leaves	The basic leaf color (green to yellowish) is covered by a purple tint of various intensity; the basic color is clearly visible.
Leaf reddening	The basic leaf color is red of various intensity.
Chlorosis	Leaf yellowing due to chlorophyll disintegration.
Leaf narrowing	The middle leaf width is distinguishably shorter in comparison with asymptomatic leaves.
Leaf roll	Leaf has its upper sides rolled together with various intensity.
Habitus, vitality	Description
Growth depression	Weaker branches and shorter habitus in comparison with asymptomatic trees of the same age.
Withering	Branches without leaves, the tree declines and dies back.
Symptom presence	Symptom intensity
0: absence	0: none
1: one branch	1: slight (signs of a symptom)
2: less than half the tree	2: medium (a symptom is noticeable)
3: more than half the tree	3: strong (clear manifestation of a symptom)
4: the whole tree	4: very strong (full manifestation of a symptom)

#### *DNA isolation, PCR, sequencing*

In all samples, total DNA was isolated using an Exgene Plant SV mini kit (GeneAll Biotechnology) according to the manufacturer’s instructions, using 100 mg of phloem tissue from shoots after homogenization in liquid nitrogen. The quality of isolated DNA was assessed by a Nanodrop Lite (ThermoFisher Scientific). To analyze the presence of 16SrX phytoplasma in a sample, the method and primers fU5 and rU3 targeted to the 16S rDNA sequence described by Lorenz *et al.* (1995) were used for PCR. Amplicons were separated on a 2% agarose gel, PCR products were cut out, purified by an Expin Combo GP kit (GeneAll Biotechnology), and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and Genetic Analyzer 3500 (ThermoFisher Scientific). Sequences were then compared with GenBank records using BLAST (blast.ncbi.nlm.nih.gov) to determine phytoplasma species.

#### *Imp genotype determination*

‘*Ca. P. pyri*’ *imp* genotypes were determined as a part of results in Bohunická *et al.* (2018) either by RFLP or by direct *imp* gene sequencing if RFLP results were inconclusive. Briefly, an *imp* gene fragment of an expected length 171 bp – 191 bp was PCR amplified using an equimolar mixture of primers:

forward 01: 5'-CCAAAATTACTGAAGGCGATAAAA-3';

forward 02: 5'-AACCAAACACAGAACCCTAAAGAAGA-3';

reverse 01: 5'-ACAATTATCAATAATAGCTTTTATTTCA-3';

reverse 02: 5' ACATTCATCAACGATAATTTTATTTCA-3' at a final concentration of 250 nM each. PCR reactions were carried out in 20 µL using a 2x Combi PPP Master Mix (Top-Bio), using the following PCR protocol: initial denaturation 95 °C/5 min; 40 cycles consisting of: denaturation 95 °C/20 s, annealing 55 °C/20 s, elongation 72 °C/45 s; and then final elongation 72 °C/1 min. In the first step, 10 µL of

a PCR product was digested with the *AluI* restriction enzyme for 1 hour following the manufacturer's protocol (ThermoFisher Scientific; all restriction enzymes used). In the second step, based on the observed *AluI* restriction pattern, restrictases *AluI* + *SfaNI* + *SfiI*; *MwoI* + *BbsI*; or *HinI* were used to determine *imp* genotypes A2, D and F1; B1, B2, and B3; or H and I. Genotypes A1, C, and E1 could be readily distinguished after the *AluI* restriction.

In case 16S rDNA sequencing indicated the presence of '*Ca. P. mali*' in the sample, the specific PCR for the '*Ca. P. mali*' *imp* gene amplification was used to determine the *imp* genotype. PCR reactions were carried out in 20  $\mu$ L using a 2x Combi PPP Master Mix (Top-Bio), each primer at a 250 nM final concentration

(forward: 5'-CTTATAGGTGTTGGTTCAGTTGTTGG-3';

reverse: 5'-CTGTGGCTTTATTAGTGTCTGCTTTC-3'),

using the PCR protocol described above. Resulting amplicons of anticipated length 400 bp were separated on a 2% agarose gel, bands were cut out, purified by an Expin Combo GP kit (GeneAll Biotechnology), and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and Genetic Analyzer 3500 (ThermoFisher Scientific). Sequences were then compared with GenBank records using BLAST to determine the '*Ca. P. mali*' *imp* genotype.

## Results

### *An overview of symptoms in pear trees*

In total, 143 pear samples were collected, of which 115 (80.4%) were phytoplasma positive. An *imp* genotype was obtained for 84 samples (73% of positive samples). Symptom descriptions were available for 126 samples: 74 phytoplasma positive samples with a determined *imp* genotype, 26 phytoplasma positive samples with an undetermined *imp* genotype, and 26 phytoplasma negative samples, respectively (Table 2). As for symptoms, 74.3% of asymptomatic samples were phytoplasma positive, and 18.7% of symptomatic samples were phytoplasma negative. On the contrary, a quarter (26%) of phytoplasma positive samples exhibited no symptoms.

In the phytoplasma positive group, more samples were symptomatic in comparison with the phytoplasma negative group (74% and 65.4%, respectively; not significant). The differences in symptom manifestations between those two categories were minimal, within a 6-percentage point range (not significant) for all symptoms with the exception of leaf narrowing and leaf roll. While leaf narrowing was more common in phytoplasma negative samples (47.1% versus 28.4% in positive samples), leaf roll was more prevalent in phytoplasma positive samples (54.1% versus 41.2%), however, the differences were not significant.

One or two symptoms in combination were observed in 13.2% of samples; on average, four symptoms were present in phytoplasma positive and negative pear trees, respectively. There was no particular combination of symptoms that could be attributed to the presence of phytoplasma – the most prevalent combination of symptoms in both groups was the presence of chlorosis and leaf narrowing combined with growth depression and withering. In general, if more than three symptoms were manifested, growth depression and/or withering were usually also present (89.4% and 81.1% in phytoplasma positive and negative samples, respectively).

Sample grouping according to tree age showed no clear effect on the presence of leaf symptoms or their intensity regardless of the presence of phytoplasma. Growth depression in both phytoplasma positive and negative trees culminated around a tree age of 15 years and then declined whereas withering gradually rose, being highest in the oldest trees 30+ years (data not shown).

**Table 2.** Symptom distribution in phytoplasma positive and negative samples

Symptoms	Phytoplasma '+'	Phytoplasma '-'	All in the category
Samples for symptom studies (n)	100 [79.4%]	26 [20.6%]	126
Asymptomatic samples (n)	26 (26%) [74.3%]	9 (34.6%) [25.7%]	35 (27.8%)
Symptomatic samples (n)	74 (74%) [81.3%]	17 (65.4%) [18.7%]	91 (72.2%)
	↓	↓	↓
<b>Symptoms in symptomatic samples (n)</b>	74	17	91
Purple leaves (n)	56 (75.7%)	13 (76.5%)	69 (75.8%)
Leaf reddening (n)	39 (52.7%)	8 (47.1%)	47 (51.6%)
Chlorosis (n)	56 (75.7%)	13 (76.5%)	69 (75.8%)
Leaf narrowing (n)	21 (28.4%)	8 (47.1%)	29 (31.9%)
Leaf roll (n)	40 (54.1%)	7 (41.2%)	47 (51.6%)
Growth depression (n)	39 (52.7%)	8 (47.1%)	47 (51.6%)
Withering (n)	30 (40.5%)	7 (41.2%)	37 (40.7%)
<b>Distribution of symptoms (n)</b>	74	17	91
<b>One symptom only (n)</b>	<b>7 (9.5%)</b>	<b>1 (5.9%)</b>	<b>8 (8.8%)</b>
<b>2 symptoms (n)</b>	<b>2 (2.7%)</b>	<b>2 (11.8%)</b>	<b>4 (4.4%)</b>
<b>3 symptoms (n)</b>	<b>18 (24.3%)</b>	<b>3 (17.6%)</b>	<b>21 (23.1%)</b>
Of them the most prevalent combination	PL RE CH	PL CH GD PL RE CH PL RE LN	
(n)	6 (33.3)	1 (33.3%) each	
<b>4 symptoms (n)</b>	<b>26 (35.1%)</b>	<b>7 (41.2%)</b>	<b>33 (36.3%)</b>
Of them the most prevalent combination	CH LN GD WI	CH LN GD WI	
(n)	8 (30.8%)	3 (42.9%)	
<b>5 symptoms (n)</b>	<b>15 (20.3%)</b>	<b>2 (11.8%)</b>	<b>17 (18.7%)</b>
Of them the most prevalent combination	PL RE CH RO WI PL RE CH RO GD	PL CH LN RO WI PL RE LN RO GD	
(n)	4 (26.7) each	1 (50.0%) each	
<b>6 symptoms (n)</b>	<b>5 (6.8%)</b>	<b>2 (11.8%)</b>	<b>7 (7.7%)</b>
<b>All 7 symptoms (n)</b>	<b>1 (1.4%)</b>	<b>0</b>	<b>1 (1.1%)</b>

Numbers in square brackets indicate the percentage of all samples in a category. Abbreviations: PL: purple leaves; RE: leaf reddening; CH: chlorosis; LN: leaf narrowing; RO: leaf roll; GD: growth depression; WI: Withering.

#### *Phytoplasma genotypes identified in pear trees in the Czech Republic*

An *imp* genotype was available for 84 samples (Table 3). In 68 samples (81%), '*Ca. P. pyri*' of one genotype was detected, while in six samples (7.1%) two distinct '*Ca. P. pyri*' isolates were detected. In total, 88% of *imp* genotypes were of '*Ca. P. pyri*' origin. In five samples (6%) only '*Ca. P. mali*' was identified, and in another five samples (6%) a coinfection of '*Ca. P. pyri*' and '*Ca. P. mali*' was observed. The most frequent genotypes were B1, A1 and C, accounting for 38.1%, 13.1% and 8.3% of samples infected with one '*Ca. P. pyri*' isolate, respectively. Genotypes B1 and B3 were also present in 50% and 67% of samples with two distinct '*Ca. P. pyri*' isolates, respectively. In addition, both A1 and B1 *imp* genotypes were also detected in two of five samples with a '*Ca. P. pyri*' and '*Ca. P. mali*' coinfection. Counted together, three *imp* genotypes – A1, B1, and C – were present in 71.4% of all findings.

All ten '*Ca. Phytoplasma mali*' findings, either in mixed infections or standalone, were of three genotypes already described in GenBank/NCBI under accession numbers FN600730, FN600731, and FN600732, the first being the most abundant (in 5/10 samples).

#### *The geographical distribution of phytoplasma imp genotypes*

Most samples (98) were collected from orchards (68.5%), 24.5% of trees (35) were grown along roads, and the remaining ten samples (7%) were from gardens and municipalities. The *imp* genotype was determined in 63 orchard samples, 20 road samples, and one garden sample. While the B1 *imp* genotype was dominant in orchard samples and was found in nearly half of them, the A1 *imp* genotype prevailed in road samples representing 25% (Table 4).

**Table 3.** Overview of '*Ca. Phytoplasma spp.*' genotypes found in pear trees in the Czech Republic

<i>'Ca. P. pyri'</i> one isolate only				<i>'Ca. P. pyri'</i> two isolates identified			
<i>imp</i> genotype	n	% Category	% Total	<i>imp</i> genotypes	n	% Category	% Total
<b>A1</b> [MF374924]	11	16.2%	13.1%	<b>A1+D</b>	1	16.7%	1.19%
<b>A2</b> [MF374925]	1	1.5%	1.2%	<b>B1+H</b>	1	16.7%	1.19%
<b>B1</b> [MF374926]	32	47.1%	38.1%	<b>B1+B3</b>	2	33.3%	2.38%
<b>B2</b> [MF374927]	1	1.5%	1.2%	<b>B3+C</b>	2	33.3%	2.38%
<b>B3</b> [MF374928]	3	4.4%	3.6%				
<b>C</b> [MF374929]	7	10.3%	8.3%				
<b>D</b> [MF374930]	4	5.9%	4.8%				
<b>E1</b> [MF374931]	2	2.9%	2.4%				
<b>F1</b> [MF374932]	5	7.4%	6.0%				
<b>G</b> [MF374933]	0	0.0%	0.0%				
<b>H</b> [MF374934]	1	1.5%	1.2%				
<b>I</b> [MF374935]	1	1.5%	1.2%				
<b>Sum</b>	<b>68</b>	<b>100%</b>	<b>81.0%</b>	<b>Sum</b>	<b>6</b>	<b>100%</b>	<b>7.1%</b>
<i>'Ca. P. pyri'</i> + <i>'Ca. P. mali'</i>				<i>'Ca. P. mali'</i> only			
<i>imp</i> genotype	n	% Category	% Total	<i>imp</i> genotype	n	% Category	% Total
<b>A1+</b> [FN600730]	1	20.0%	1.19%	[FN600730]	3	60.0%	3.57%
<b>A1+</b> [FN600732]	1	20.0%	1.19%	[FN600731]	1	20.0%	1.19%
<b>B1+</b> [FN600730]	1	20.0%	1.19%	[FN600732]	1	20.0%	1.19%
<b>B1+</b> [FN600732]	1	20.0%	1.19%				
<b>G +</b> [FN600731]	1	20.0%	1.19%				
<b>Sum</b>	<b>5</b>	<b>100%</b>	<b>5.95%</b>	<b>Sum</b>	<b>5</b>	<b>100%</b>	<b>5.95%</b>
<b>Samples total</b>	143						
<b>Phytoplasma positive</b>	115		80.4% of total				
<b><i>Imp</i> genotypes</b>	<b>84</b>		73.0% of positive samples				

*Imp* genotype names were adapted from Bohunická *et al.* (2018). GenBank accession numbers are given in square brackets

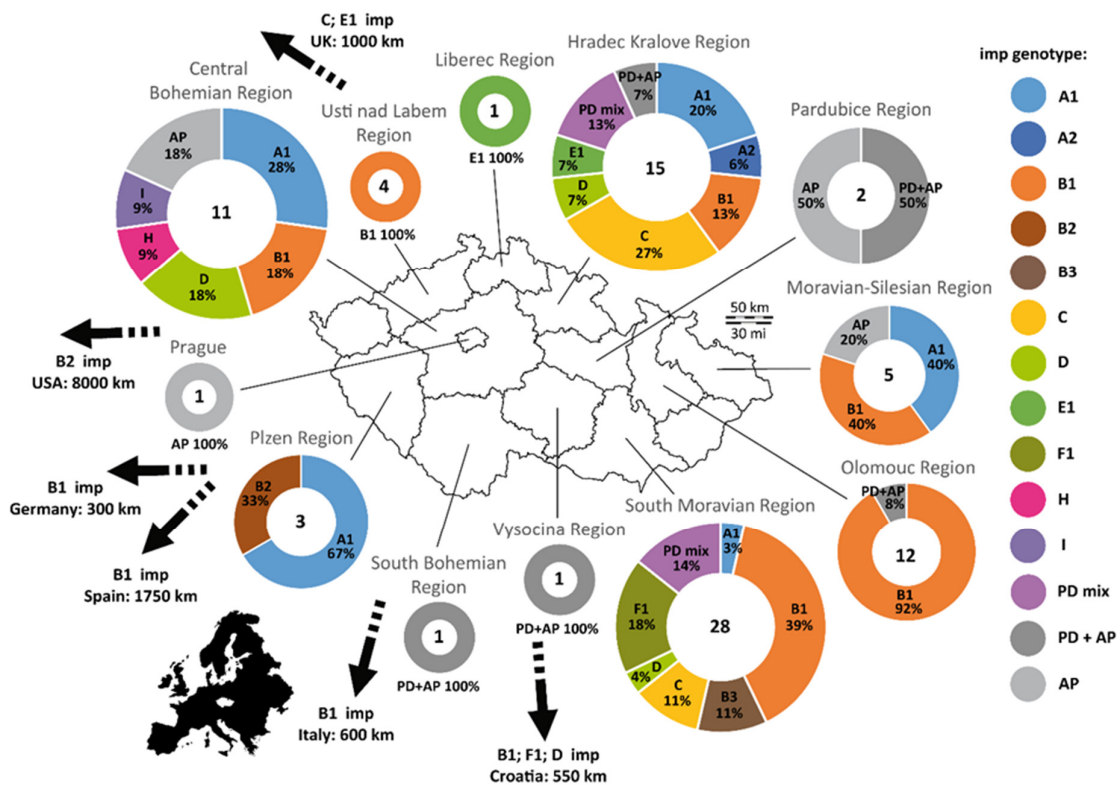
**Table 4.** Distribution of '*Ca. Phytoplasma pyri*' *imp* genotypes identified in samples collected in orchards and along roads

<i>imp</i> genotype	Orchard				Roads				Others	All
	Samples		Locations		Samples		Locations			
	n	%	n	%	n	%	n	%		
<b>A1</b>	6	9.5%	5	12.2%	5	25.0%	3	7.3%		11
<b>A2</b>	1	1.6%	1	2.4%						1
<b>B1</b>	30	47.6%	8	19.5%	2	10.0%	1	2.4%		32
<b>B2</b>					1	5.0%	1	2.4%		1
<b>B3</b>	3	4.8%	1	2.4%						3
<b>C</b>	6	9.5%	3	7.3%	1	5.0%	1	2.4%		7
<b>D</b>	2	3.2%	2	4.9%	2	10.0%	2			4
<b>E1</b>	1	1.6%	1	2.4%	1	5.0%	1	2.4%		2

F1	5	7.9%	1	2.4%						5
H					1	5.0%	1	2.4%		1
I					1	5.0%	1	2.4%		1
' <i>Ca. P. pyri</i> ' 2 genotypes	5	7.9%	2	4.9%	1	5.0%	1	2.4%		6
' <i>Ca. P. pyri</i> ' + ' <i>Ca. P. mali</i> '	3	4.8%	3	7.3%	2	10.0%	2	4.9%		5
' <i>Ca. P. mali</i> '	1	1.6%	1	2.4%	3	15.0%	2	4.9%	1	5
<b>Sum</b> [% of all]	<b>63</b> [75%]	100%			<b>20</b> [23.8%]	100%			<b>1</b> [1.2%]	<b>84</b>

Total number of locations: n=41.

The Czech Republic is divided into 14 administrative regions (European Union NUTS 3 level). At least two samples were collected in 10 regions at a total of 41 locations. Samples were not collected in two regions. The geographical distribution of *imp* genotypes is shown in Figure 1. While the B1 *imp* genotype was present in six regions at eight locations, *imp* genotypes A2, B2, B3, F1, H, and I were each found at only one location in one sample except for genotypes B3 and F1 that were found in 3 and 5 samples, respectively. The B3 *imp* genotype was also found in four samples with a coinfection of two different '*Ca. P. pyri*' isolates.



**Figure 1.** Geographical distribution of '*Ca. Phytoplasma spp.*' isolates based on *imp* gene genotyping in pear trees in the Czech Republic

Arrows show the direction and distance to other countries with a finding of the respective *imp* genotype. Numbers in circles indicate the number of samples with a determined *imp* genotype that were collected in the region. For simplicity, "PD" stands for '*Ca. P. pyri*', and "AP" for '*Ca. Phytoplasma mali*'.

*Symptom associations*

Only three *imp* genotype groups (A1, B1 and C) contained more than five symptomatic samples (Table 5), making a thorough statistical analysis inconclusive. Neither a clear association between symptom manifestations nor a specific combination of symptoms could be associated with a particular *imp* genotype. The only remarkable difference noted was that trees infected with the A1 *imp* genotype tended to have more leaf reddening and purple leaves, while chlorosis was least common (not significant compared to *imp* B1 and C). In addition, tree vitality did not seem to be affected – no growth depression (significant compared to both *imp* B1 ( $p=0.02$ ) and C ( $p=0.01$ )) or withering (not significant compared to *imp* B1 and C) was observed for the A1 *imp* genotype group.

**Table 5.** Symptom distribution in the phytoplasma particular *imp* genotype

Symptoms	<i>Imp</i> A1	<i>Imp</i> B1	<i>Imp</i> B3	<i>Imp</i> C	<i>Imp</i> D	<i>Imp</i> F1
Samples for symptom studies (n)	8	32	3	7	3	5
Asymptomatic samples (n)	2 (25.0%)	9 (28.1%)	2 (66.7%)	1 (14.3%)	1 (33.3%)	2 (40.0%)
Symptomatic samples (n)	6 (75.0%)	23 (71.9%)	1 (33.3%)	6 (85.7%)	2 (66.7%)	3 (60.0%)
	↓	↓	↓	↓	↓	↓
<b>Symptoms in symptomatic samples (n)</b>	6	23	1	6	2	3
Purple leaves (n)	6 (100%)	16 (69.6%)	1 (100%)	5 (83.3%)	2 (100%)	2 (66.7%)
Leaf reddening (n)	5 (83.3%)	10 (43.5%)	0	3 (50.0%)	2 (100%)	2 (66.7%)
Chlorosis (n)	2 (33.3%)	18 (78.3%)	1 (100%)	4 (66.7%)	1 (50.0%)	3 (100%)
Leaf narrowing (n)	1 (16.7%)	9 (39.1%)	0	1 (16.7%)	0	2 (66.7%)
Leaf roll (n)	3 (50.0%)	11 (47.8%)	1 (100%)	4 (66.7%)	2 (100%)	3 (100%)
Growth depression (n)	0	13 (56.5%)	1 (100%)	5 (83.3%)	1 (50.0%)	2 (66.7%)
Withering (n)	0	10 (43.5%)	0	1 (16.7%)	1 (50.0%)	2 (66.7%)
<b>Distribution of symptoms (n)</b>	6	23	1	6	2	3
<b>One symptom only (n)</b>	0	3 (13.0%)	0	0	0	0
Imp B1: PL: 2 (66.7%); GD: 1 (33.3%)						
<b>2 symptoms (n)</b>	2 (33.3%)	0	0	0	0	0
Imp A1: PL RE: 23 (100%)						
<b>3 symptoms (n)</b>	3 (50%)	5 (21.7%)	0	4 (66.7%)	0	0
Imp A1: PL RE RO: 2 (66.7%); PL RE CH: 1 (33.3%)						
Imp B1: PL RE CH: 2 (40%); CH RO GD: 2 (40%); PL CH WI: 1 (20%)						
Imp C: PL RE RO: 1 (25%); PL RE GD: 1 (25%); PL CH GD: 1 (25%); CH RO GD: 1 (25%)						
<b>4 symptoms (n)</b>	1 (16.7%)	8 (34.8%)	1 (100%)	0	1 (50.0%)	1 (33.3%)
Imp A1: PL CH LN RO: 1 (100%)						
Imp B1: CH LN GD WI: 4 (50%); PL RE RO GD: 2 (25%); PL RE CH WI: 1 (12.5%); PL CH RO GD: 1 (12.5%)						
Imp B3: PL CH RO GD: 1 (100%)						
Imp D: PL RE RO GD: 1 (100%)						
Imp F1: CH RO GD WI: 1 (100%)						
<b>5 symptoms (n)</b>	0	5 (21.7%)	0	1 (16.7%)	1 (50.0%)	1 (33.3%)
Imp B1: PL RE CH LN RO: 2 (40%); PL RE CH RO WI: 1 (20%); PL RE CH GD WI: 1 (20%); PL CH LN RO GD: 1 (20%)						
Imp C: PL RE CH RO GD: 1 (100%)						
Imp D: PL RE CH RO WI: 1 (100%)						
Imp F1: PL RE CH LN RO: 1 (100%)						
<b>6 symptoms (n)</b>	0	2 (8.7%)	0	1 (16.7%)	0	0
Imp B1: PL CH LN RO GD WI: 1 (50%); PL RE CH LN RO WI: 1 (50%)						
Imp C: PL CH LN RO GD WI: 1 (100%)						
<b>All 7 symptoms (n)</b>	0	0	0	0	0	1 (33.3%)
Imp F1: PL RE CH LN RO GD WI: 1 (100%)						

Abbreviations: PL: purple leaves; RE: leaf reddening; CH: chlorosis; LN: leaf narrowing; RO: leaf roll; GD: growth depression; WI: withering.

## Discussion

In the present work we studied the symptoms and spatial distribution of ‘*Ca. P. pyri*’ genotypes in pear trees in the Czech Republic. First, the presence of phytoplasma was evaluated by PCR, and symptoms were compared between phytoplasma negative and positive samples. Though narrow leaves were observed more often in phytoplasma negative samples, and leaf roll more frequently in phytoplasma positive samples, there were otherwise no obvious differences between positive and negative samples (not significant for all symptoms). For asymptomatic samples, 74% were phytoplasma positive, and on the contrary, 19% of symptomatic samples were phytoplasma negative, confirming that a visual control is not sufficient to diagnose or exclude phytoplasmosis in pears. Interestingly, 26% of phytoplasma-positive samples were asymptomatic, which is almost the same as that reported for ‘*Ca. P. prunorum*’ where 25% of infected trees showed no symptoms (Nečas *et al.*, 2018).

Next, *imp* genotyping was performed in phytoplasma positive samples. An *imp* genotype was obtained for 84 samples but could not be resolved in the remaining samples, likely because the *imp* gene is A/T rich, making it difficult to amplify in weakly positive samples (Bohunická *et al.*, 2018). In the collection of resolved phytoplasma *imp* sequences, 74 (88%) were of ‘*Ca. P. pyri*’ origin; the remaining 12% were represented by ‘*Ca. P. mali*’ infections (5 cases) or ‘*Ca. P. pyri*’ and ‘*Ca. P. mali*’ coinfections (5 cases). ‘*Ca. P. mali*’ was found in pears at various locations in the Czech Republic, indicating that this trans-host infection may be common. Agarose gel analyses showed that ‘*Ca. P. mali*’ was found in low concentrations in standalone infections as well as in ‘*Ca. P. pyri*’ and ‘*Ca. P. mali*’ coinfections, where ‘*Ca. P. pyri*’ clearly dominated, implying that though ‘*Ca. P. mali*’ can infect pears there are other factors prohibiting it from thriving and causing major harm. Indeed, two of three ‘*Ca. P. mali*’ positive pear samples for which symptoms were recorded were asymptomatic, and the third one showed only average leaf symptoms and no habitus/vitality symptoms. In six samples, two ‘*Ca. P. pyri*’ different isolates cohabitating the same niche at various concentrations were identified, corroborating the results of Seemüller *et al.* (2010), who described this phenomenon for ‘*Ca. P. mali*’. Whether a coinfection of two strains has a more detrimental effect on a tree health status remains to be resolved.

In total, twelve ‘*Ca. P. pyri*’ *imp* genotypes were identified (Bohunická *et al.*, 2018). Surprisingly, all *imp* B3 and F1 phytoplasma genotypes were found in 12-year-old pear trees at the same location in only one orchard in the South Moravian region. Phytoplasmas of the F1 *imp* genotype were detected in “Hortenzia”, “Bosc”, and “Williams” pear varieties, and the B3 *imp* genotype phytoplasmas were observed in “Hortenzia”, “Packham triumph”, and “Williams” varieties; quince was used as a rootstock in all cases. The B3 *imp* genotype has not been described elsewhere so far; the occurrence of the F1 *imp* genotype has also been confirmed in Croatia (Danet *et al.*, 2011; GenBank No. FN600728), and subtype F2 (differing in one amino acid) also in Croatia, Azerbaijan (Danet *et al.*, 2011; GenBank No. FN600723) and Iran (Hashemi-Tameh *et al.*, 2014; GenBank No. KF360366). Therefore, it would be interesting to perform larger scale genotyping in other European countries to unravel possible spreading routes of different ‘*Ca. P. pyri*’ variants, particularly of the F1 *imp* genotype.

The most common Czech *imp* genotype B1 has also been observed in other European countries – Germany, Spain, Croatia, and Italy (Danet *et al.*, 2011; GenBank No. FN600726; Sabaté *et al.*, 2014; GenBank No. HG737344). Interestingly, the second most frequent genotype A1 (17%) has so far only been identified in the Czech Republic.

In the next step, symptom manifestations were assessed in relation to *imp* genotypes. However, due to the limited number of samples only the top three *imp* genotypes, A1, B1 and C, could be compared. The only remarkable difference noted was that trees infected with the ‘*Ca. P. pyri*’ A1 *imp* genotype tended, though not significantly, to have more leaf reddening and purple leaves, while chlorosis was least common. In addition, tree vitality did not seem to be affected – no growth depression or withering was observed for the A1 *imp* genotype group. Interestingly, the A1 *imp* genotype was the most frequent genotype found in samples collected from trees growing along roads, which were on average 25-30 years old (in contrast, orchard trees were 10-15 years

old on average), indicating that ‘*Ca. P. pyri*’ of the A1 *imp* genotype might generally lead to mild symptoms. Indeed, the absence of growth depression was the only symptom significantly correlated to the A1 *imp* genotype. However, these results must be taken with caution since only a limited number of particular genotypes could be analyzed, and more work is needed to exclude the possibility that mild or absent symptoms are not simply due, for instance, to a very recent and not-fully established infection of the tree. On the other hand, the most frequent ‘*Ca. P. pyri*’ *imp* genotype B1 as well as B2 have also been shown to infect peaches, causing peach yellow leaf roll, a disease characterized by early reddening, leaf curling, decline, abnormal fruits, and in some cases chlorosis and tree death (Sabaté *et al.*, 2014; Morton *et al.*, 2003; GenBank No. AF400589 corresponds to *imp* genotype B2), suggesting that ‘*Ca. P. pyri*’ *imp* B genotypes may be more virulent.

Interestingly, A1 Imp protein shares only 71% identity with B1 Imp protein and is one amino acid shorter than B1 protein (compared from Ile24 to the C-end using sequences GeneBank/NCBI Acc. No. ASN77139 and ASN77141). Since it is presumed that Imp proteins play an important role in phytoplasma pathogenesis (Berg *et al.*, 1999; Kakizawa *et al.*, 2009), likely modulating pathogen-insect vector and/or pathogen-host plant interactions during infection (e.g., Boonrod *et al.*, 2012; Ding *et al.*, 2022), it is tempting to speculate that the differences between A1 and B1 Imp proteins underlie these phytoplasma genotypes’ pathogenicity, fitness, and/or spreading. Nonetheless, further research is clearly needed to confirm this hypothesis.

## Conclusions

The prevalence of ‘*Ca. P. pyri*’ variants in the Czech Republic was assessed by employing *imp* gene genotyping. At 41 locations twelve ‘*Ca. P. pyri*’ *imp* genotypes were found, of which genotypes A1, B1, and C were present in 71% of findings. While the B1 genotype is common in Europe, the second most prevalent *imp* genotype A1 has only been observed in the Czech Republic so far. Trees infected with ‘*Ca. P. pyri*’ carrying the *imp* genotype A1 showed generally milder symptoms in comparison with the ‘*Ca. P. pyri*’ B1 or C genotype-infected trees. Otherwise, no clear associations between symptoms and various criteria were observed except for leaf roll, which was more often observed in phytoplasma positive samples.

## Authors’ Contributions

Conceptualization: RC, LV, MB and JS; Data curation: RC, LV, MB, JS and JC; Formal analysis: RC, MB and JC; Funding acquisition: JS and TN; Investigation: MB, LV and MR; Methodology: RC; Resources: JS, TN, AE and TK; Writing - original draft: RC; Writing - review and editing: MB, JC, AE, TK and TN.

All authors read and approved the final manuscript.

## Ethical approval (for researches involving animals or humans)

Not applicable.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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