Detection and molecular characterization of phytoplasmas infecting apple trees in Poland

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Abstract

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During 2010–2012, samples from 225 apple trees growing in six regions of Poland were tested for phytoplasmas. 16S rRNA gene and 16S-23S spacer region sequences were amplified from total DNAs prepared from phloem tissue of apple shoots. According to the results of PCR-RFLP and sequence analyses, apple trees were infected by *Candidatus* Phytoplasma mali and *Ca*. P. asteris. Fragments of 16S rDNA plus 16S-23S spacer region of the *Ca*. P. mali isolates digested with *Hpa*II enzyme showed two restriction profiles: P-I and P-II. Multiple alignments of 16S rRNA gene fragments revealed that the isolates of *Ca*. P. mali shared 100% sequence identity among themselves as well as with reference strains AT and AP-15 of apple proliferation phytoplasma. The nucleotide sequence of the same region of *Ca*. P. asteris isolates confirmed the phylogenetic relationship with reference strains OAY (MIAY) and AY1 of aster yellows phytoplasma PCR-RFLP analysis of ribosomal protein (*rpl22* and *rpS3*), *sec*Y, and *tuf* genes did not show the sequence diversity of the isolates of aster yellows phytoplasma.

Keywords: identification; PCR-RFLP; sequencing; phylogenetic analysis

Apple proliferation (AP) is a quarantine disease widespread in Europe leading to serious economic losses in apple production. These symptoms include proliferation of affected shoots, premature reddening of the leaves, lower quality of fruits, and enlarged stipules. The causal agent of AP is *Candidatus* Phytoplasma mali (*Ca*. P. mali), classified to the taxonomic group 16SrX, subgroup A (Lee et al. 1998; Seemüller, Schneider 2004). Apple proliferation occurs in many European countries and was reported to cause the biggest losses in apple orchards in northern Italy and south-eastern Germany (Bliefernicht, Krczal 1995; Loi et al. 1995). The disease commonly occurred in southern Poland in the 60' and 70' of the last century (Kamińska, Zawadzka 1970) and recently, apple proliferation was recorded in our country (Cieślińska, Kruczyńska 2011; Cieślińska et al. 2012). Based on the results of the PCR-RFLP analyses the isolates of *Candidatus* Phytoplasma mali can be classified into three subtypes: AP, AP-1 and AT-2 (Jarausch et al. 2000). *Ca*. P. mali is transmitted in a persistent manner by *Cacopsylla picta* (Carraro et al. 2001) and *C. melanoneura* (Tedeschi et al. 2002). The role of leafhopper *Fieberiella flori* as a vector of this phytoplasma was also discussed (Krczal et al. 1989; Tedeschi, Alma 2006).

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Ca. P. asteris (Lee et al. 2004), classified to aster yellows group (AY, 16SrI) incidentally occurs in apple orchards inducing various symptoms including leaf yellowing, shoot proliferation, branch twisting, and rubbery wood. *Ca*. P. asteris is transmitted by leafhoppers.

The aim of this study was to evaluate genetic diversity of phytoplasma isolates based on molecular characterization of several regions of their genome.

MATERIAL AND METHODS

During 2010–2012 the apple trees growing in orchards, home gardens and natural environments in six regions (voivodeships) of Poland were surveyed. Some of the trees showed witches' broom symptoms and produced small and malformed fruits. Shoots were collected from 225 symptomatic and asymptomatic apple trees and fresh phloem tissue was ground in liquid nitrogen. Total DNA extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was subjected to PCR with P1/P7 primers universal for 16S/23S rDNA region of phytoplasmas. The PCR products were diluted 1:29 with sterile deionized water and used as templates in nested PCRs primed by the second universal primer pairs F1/B6 or R16F2n/R16R2 (Table 1) as well as primers specific for apple proliferation phytoplasma (16SrX) and for aster yellows phytoplasma (16SrI) groups. Moreover, ribosomal protein L22 (*rpl22*) and ribosomal protein S3 (*rps3*), *sec*Y, and *tuf* genes of the *Ca*. P. asteris isolates were amplified with primer pairs targeting these regions.

PCRs were carried out using Fast Star Taq DNA Polymerase (Roche, Warsaw, Poland) in the final volume of 25 µl reaction mix (2.5 µl 10X PCR buffer, 2.0mM $MgCl_2$, 0.2 µl *Taq* polymerase, 0.2mM dNTPs, and 0.4µM each primer). The following conditions of PCRs were programmed: 4 min at 95°C for initial denaturation followed by 35 cycles of denaturation 30 s at 95°C, annealing 30 s (annealing temperatures listed in Table 1) and 2 min for primer extension at 72°C. The last step of all PCR's was final elongation for 7 min at 72°C. All reactions were performed with a thermocycler PTC-200 (MJ Research, Watertown, USA) and the amplification products (7 µl) were separated in 1% agarose gel in 0.5 × TBE buffer, followed by staining in ethidium bromide and visualization of DNA bands using UVi-Tec transilluminator (Syngen, Cambridge, UK). The molecular weight of the PCR products was estimated using comparison with 100 bp DNA ladder (Fermentas, Vilnius, Lithuania). DNA from sample of healthy apple tree was included in the PCRs as a negative control. AP-15 (16SrX-A) and AY2192 (16SrI-B) strains kindly provided by Prof. A. Bertaccini (Institute of Plant Pathology, University of Bologna, Italy) were subjected to this study as the reference strains.

Characterization of *Ca*. P. mali and *Ca*. P. asteris isolates was carried out using Restriction Frag-

Table 1. Primer pairs and restriction enzymes used in PCR-RFLP analyses of *Ca*. P. mali and *Ca*. P. asteris isolates from apple trees

Genome segment	Approx. size (kb)	Primer pair	Temperature of annealing (°C)	References	Restriction enzymes
16S/23S rDNA	1.8	P1/P7[1]	50	Deng, Hiruki (1991), Schneider et al. (1995)	nd
16S/23S rDNA	1.7	F1/B6[1]	50	Davis, Lee (1993), Padovan et al. (1995)	*Hpa*II, *Mse*I
16S rDNA	1.2	R16F2n/R2[1]	55	Lee et al. (1993)	*Rsa*I, *Hha*I, *Mse*I, *Ssp*I
16S rDNA	1.1	R16(X)F1/R1[2]	55	Lee et al. (1995)	nd
16S rDNA	1.1	R16(I)F1/R1[3]	55	Lee et al. (1994)	nd
secY	1.4	AYsecYF1/AYsecYR1[3]	55	Lee et al. (2006)	*Alu*I, *Rsa*I, *Hha*I, *Hpa*II
rpl22, rps3	1.2	rpF1/rpR1[1]	50	Lim, Sears (1992), Lee et al. (2003)	nd
	1.2	rp(I)F1A/rp(I)R1A[3]	50		*Alu*I
tuf	1.0	fTuf1/rTuf1[1]	45	Lee et al. (2003), Schneider, Gibb (1997)	nd
	0.9	fTufAY/rTufAY[3]	53		*Alu*I, *Hpa*II

[1]universal primers; [2]primers specific for apple proliferation group; [3]primers specific for aster yellows group; nd – not determined

ment Length Polymorphism (RFLP) technique. PCR products were digested with selected enzymes (Fermentas, Vilnius, Lithuania) listed in Table 1 and were analysed by electrophoresis in 8% polyacrylamide gels in 1% Tris/Borate/EDTA (TBE) buffer. The restriction patterns were compared with the profiles for the reference strains (Lee et al. 1998).

PCR products of selected isolates were purified from the gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced from both directions. The nucleotide sequence analyses of the genome fragments of phytoplasmas were compared with sequences available in GenBank using the blast algorithm (http://ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments were performed with ClustalW of the DNASTAR Lasergene software (DNASTAR, Madison, USA), and phylogenetic and molecular evolutionary analyses were conducted using the neighbour-joining method of MEGA, version 4.02 (Tamura et al. 2007).

RESULTS AND DISCUSSION

No PCR products were obtained after direct-PCR with the P1/P7 universal primers. Nested PCR primed by R16F2n/R16R2 yielded products of the expected size (~1.2 kb) from 19 apple samples and from the reference strains AP-15 and AY2192 (data not shown). Fifteen samples (SzamP, Mut, GoP, GoC, J629, GlP, JoC, RBoC, Kotl, J8148, Przel, J4020, Oz, Bor, and Kijak) were positively tested in nested PCR with primers R16(X)F1/R1 specific for phytoplasmas from apple proliferation group (16SrX). Nested PCR with primers pair R16(I)F1/R1 specific for aster yellows group phytoplasma (16SrI) resulted in products for other four samples (Pin, Evel, DKII/4, and DKII/6). No products were amplified from DNAs of healthy plants. The infected apple trees were grown in orchards, home gardens and natural environments located in all six surveyed voivodeships of Poland, which suggests that phytoplasmal diseases are distributed in most of the apple production regions.

Results of the restriction fragment length polymorphism and nucleotide sequence analyses of 16S rDNA amplicons allowed identifying *Ca.* P. mali and *Ca*. P. asteris in apple trees. The RFLP analysis on R16F2n/R16R2 using *Hha*I, *Mse*I, *Ssp*I, and *Rsa*I enzymes showed that the restriction patterns obtained for samples Kotl, J8148, SzamP, Mut, GoP, Przel, GlP, and JoC were characteristic for *Ca*.

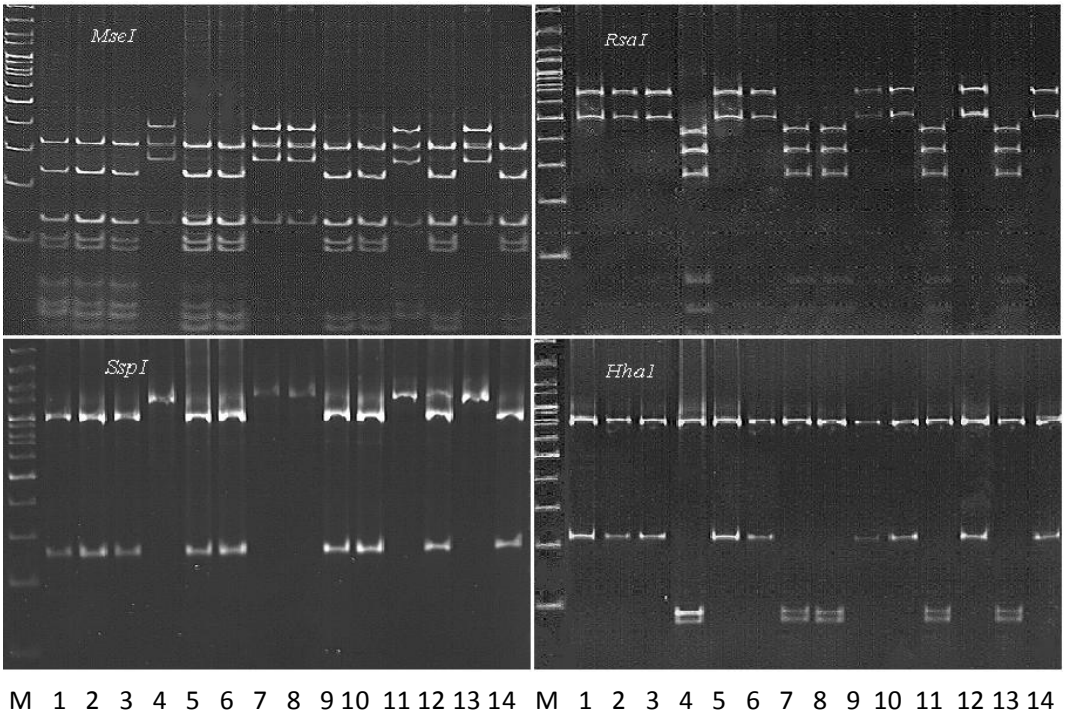

Fig. 1. RFLP profiles of phytoplasmas from samples of apple trees of ~1.2 kb rDNA fragments amplified with primers R16F2n/R16R2 analysed with *Mse*I, *Rsa*I, *Ssp*I, and *Hha*I restriction enzymes

Lanes: M – 100 bp DNA Ladder (Fermentas, Lithuania); 1 – Kotl; 2 – J8148; 3 – SzamP; 4 – Evel; 5 – Mut; 6 – GoP; 7 – Pin; 8 – DKII/4; 9 – Przel; 10 – GlP; 11 – DKII/6; 12 – JoC; 13 – AY2192 reference strain; 14 – AP-15 reference strain

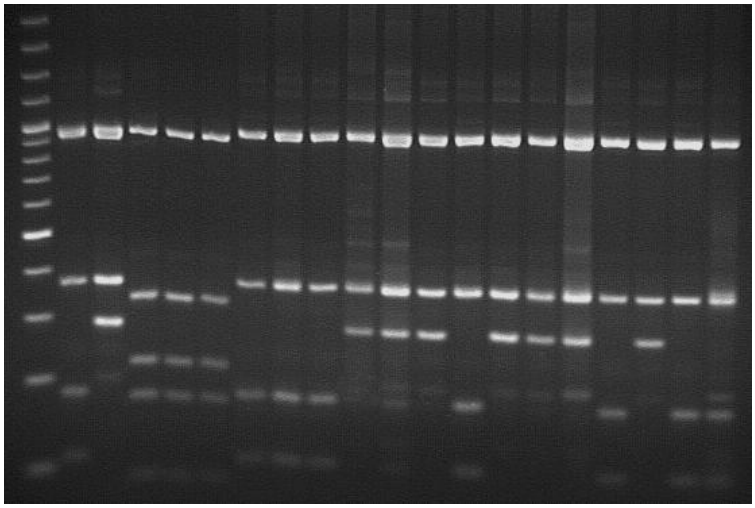

Fig. 2. RFLP profiles of phytoplasmas from samples of apple trees of ~1.7 kb rDNA fragments amplified with primers F1/B6 analysed with *Hpa*II restriction enzyme
Lanes: M – 100 bp DNA Ladder (Fermentas, Vilnius, Lithuania); 1 – Kotl; 2 – J8148; 3 – Evel; 4 – DKII/4; 5 – AY2192 reference strain; 6 – SzamP; 7 – Mut; 8 – Przel; 9 – GoP; 10 – J4020; 11 – GlP; 12 – J629; 13 – JoC; 14 – RBoC; 15 – GoC; 16 – Oz; 17 – Bor; 18 – Kijak; 19 – AP-15 reference strain

P. mali (Fig. 1; samples 1, 2, 3, 5, 6, 9, 10, and 12) but based on this result it was not possible to discriminate the isolates of this phytoplasma. It has been indicated that sequence analysis of 16S rDNA and 16S-23S spacer region led to finer differentiation of the *Ca*. P. mali strains (Casati et al. 2010, 2011; Paltrinieri et al. 2010; Fránová et al. 2013). It was also confirmed in our study as the PCR prod-

Table 2. Origin of *Candidatus* Phytoplasma mali and *Candidatus* Phytoplasma asteris isolates identified in apple trees

Cultivar	Phytoplasma isolate	Geographic location (voivodeship)	16Sr group	16SrX profile
Unknown	Kotl	lubelskie	X-A	P-I
Unknown	J8148	małopolskie	X-A	P-II
Shampion	SzamP	dolnośląskie	X-A	P-I
Mutsu	Mut	mazowieckie	X-A	P-I
Unknown	Przel	podkarpackie	X-A	P-I
Golden Delicious	GoP	dolnośląskie	X-A	P-II
Unknown	J4020	małopolskie	X-A	P-II
Gloster	GlP	dolnośląskie	X-A	P-II
Golden Delicious	J629	łódzkie	X-A	P-I
Jonagold	JoC	mazowieckie	X-A	P-II
Red boskoop	RBoC	mazowieckie	X-A	P-II
Golden Delicious	GoC	mazowieckie	X-A	P-II
Unknown	Oz	łódzkie	X-A	P-I
Unknown	Bor	łódzkie	X-A	P-II
Unknown	Kijak	łódzkie	X-A	P-I
Evelina	Evel	mazowieckie	I-B	nd
Pinova	Pin	mazowieckie	I-B	nd
Unknown	DKII/4	podkarpackie	I-B	nd
Unknown	DKII/6	podkarpackie	I-B	nd
Ref. Strain	AP-15	nd	X-A	P-I
Ref. Strain	AY2192	nd	I-B	nd

nd – not determined

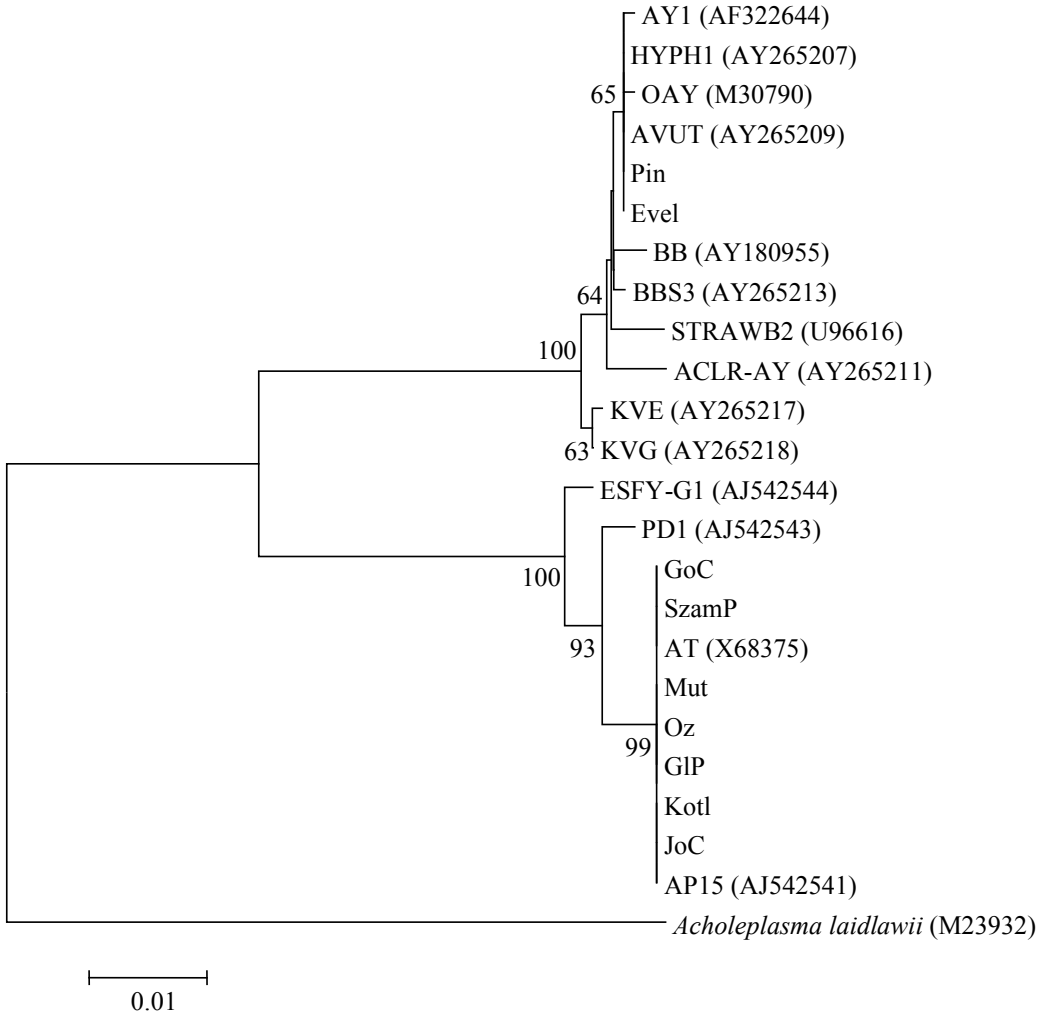

Fig. 3. Phylogenetic tree constructed with partial nucleotide sequences of the 16S rDNA from phytoplasmas infecting apple trees and phytoplasma reference strains of the aster yellows and apple proliferation groups available in GenBank. *Acholeplasma laidlawii* (M23932) is included as an outgroup

Constructed using the neighbour-joining method (MEGA 4.02). Bootstrap values (% replication) are showed by each branch node. For Phytoplasma isolates description see Table 2

ucts amplified with F1/B6 primers revealed different restriction profiles after digestion with *Hpa*II enzyme (Fig. 2, Table 2).

In turn, Evel, Pin, DKII/4, and DKII/6 isolates showed profiles indistinguishable from the restriction patterns for the reference strains of *Ca*. P. asteris (16SrI-B) (Lee et al. 1998) after digestion of PCR products amplified with R16F2n/R16R2 (Fig. 1; samples 4, 7, 8, and 11). The only one pattern, characteristic for *Ca*. P. asteris, was also obtained for F1/B6 amplicons of Evel and DKII/4 samples digested with *Hpa*II enzyme (Fig. 2., Table 2).

The sequence analyses of the *rpl22-rps3*, *sec*Y, and *tuf* genes allowed to distinguish *Ca*. P. asteris isolates (Marcone et al. 2000; Lee et al. 2004, 2006). Although the results of our PCR-RFLP analyses on these regions confirmed that apple trees cvs Evelina (Evel) and Pinova (Pin) and two trees of unknown cultivars (DKII/4 and DKII/6) were infected by *Ca*. P. asteris, the restriction profiles of these isolates were indistinguishable from each other and the reference strain AY2192. This is the first report of PCR-RFLP analysis of *rpl22-rps3*, *sec*Y, and *tuf* genes of *Ca*. P. asteris isolates found in apple trees.

Multiple alignments of partial 16S rDNA sequences revealed that the phytoplasma isolates detected in seven apple trees (SzamP, Mut, GoC, Oz, GlP, Kotl, and JoC) shared 100% sequence iden-

tity among themselves as well as with *Ca*. P. mali reference strains AT from Germany (GenBank ID: X68375) and AP-15 from Italy (GenBank ID: AJ542541). The phylogenetic analysis grouped the detected phytoplasma close to the other members of the apple proliferation group, 16Sr-XA (Fig. 3). The nucleotide sequences of the same region of two *Ca*. P. asteris isolates (cvs Evel and Pin) were identical and confirmed their phylogenetic relationship with reference strains OAY (MIAY) (GenBank ID: M30790) and AY1 (GenBank ID: AF322644) as well as with the other members of subgroup B of aster yellows phytoplasma group.

As apple is widely cultivated in Poland, the occurrence of the both phytoplasmas in orchards has a considerable significance for fruit production. There is a risk of spreading of *Ca*. P. mali by *Cacopsylla picta* and *C. melanoneura* which were found in our country. The preliminary study showed the presence of apple proliferation phytoplasma in the both species of psylla (Cieślińska et al. 2012).

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