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RESEARCH PAPERS

Genetic diversity of '*Candidatus* Phytoplasma mali' strains in Poland

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Summary. During 2010-2013, samples from 267 apple trees growing in six regions of Poland were tested for phytoplasma presence. '*Candidatus* Phytoplasma mali' was detected in 17 samples from phloem tissue of apple shoots showing typical apple proliferation symptoms. Molecular characterization of these strains was conducted using PCR-RFLP and sequence analyses of three regions: ribosomal DNA, ribosomal protein and non-ribosomal DNA fragment including nitroreductase and rhodanese-like protein genes. Fragments of 16S rDNA plus 16S-23S spacer region from the '*Ca.* P. mali' strains showed two restriction profiles: P-I and P-II when digested with *Hpa*II enzyme. Moreover, based on results of nitroreductase and rhodanese-like protein genes, these phytoplasmas were grouped into two subtypes: AP-15 and AT-1. The majority of the '*Ca.* P. mali' strains was classified to the AP-15 subtype. Two strains were grouped to the AT-1 subtype and another showed both AP-15 and AT-1 profiles. Based on results of analysis of *rpl22* and *rps3* ribosomal protein genes, most of the '*Ca.* P. mali' strains were identified as belonging to rpX-A. Only one strain was affiliated to rpX-B subgroup.

Key words: apple proliferation, multilocus gene analyses.

Introduction

Apple proliferation is one of the most economically important diseases causing significant losses in apple production. '*Candidatus* Phytoplasma mali' ('*Ca.* P. mali') (Seemüller & Schneider 2004), classified to the group 16SrX-A (Lee *et al.*, 1998), is the associated phytoplasma included in EPPO A2 list of pests recommended for regulation as quarantine organisms in Europe (OEPP/EPPO, 1978). Disease symptoms associated with the presence of this phytoplasma include the development of axillary buds resulting in proliferation of lateral shoots (witches' broom), premature reddening of the leaves, enlarged stipules and reductions in size and quality of apple fruits.

Apple proliferation was reported in many countries of Central and Southern Europe (Loi *et al.*, 1995; Del Serrone *et al.*, 1998; Fialova *et al.*, 2003; Blažek *et al.*, 2005; Delić *et al.*, 2005; Bertaccini *et al.*, 2008; Casati *et al.*, 2010; Fránová *et al.*, 2013). This disease commonly occurred in southern Poland in the 1960's and 70's (Kamińska and Zawadzka, 1970), and in recent years its presence has been observed in different areas of the country (Cieślińska and Kruczyńska, 2011; 2014).

The highly conserved 16S rDNA, while widely used for identification and classification of a broad range of phytoplasmas, is unable to differentiate closely related strains (Duduk *et al.*, 2009; Bertaccini *et al.*, 2014). Therefore the full-length 16S rDNA, 16S-23S rDNA intergenic region and partial sequence of 23S rDNA (Casati *et al.*, 2010; Paltrinieri *et al.*, 2010; Fránová *et al.*, 2013; Cieślińska and Kruczyńska, 2014), and other molecular markers, are required for the finer differentiation of '*Ca.* P. mali' strains. These markers include the ribosomal protein genes *rpl22* and *rps3*, nitroreductase and rhodanese-like protein genes or *aceF*, *pnp*, *imp*, *hflB* genes (Danet *et al.*, 2007; Martini *et al.*, 2008; Schneider and Seemüller, 2009; Casati *et al.*, 2010; Paltrinieri *et al.*, 2010; Fránová *et*

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al., 2013). Analyses of nitroreductase-like gene in particular distinguishes at least three different AP phytoplasma subtypes: AT-1, AT-2, and AP-15 (Jarausch *et al*., 1994; 2000), and characterization of the genes coding ribosomal proteins L22 and S3 differentiated four subgroups: rpX-A, rpX-B, rpX-C, and rpX-D (Martini *et al*., 2005; 2008).

The aim of the present study was to determine the association of phytoplasmas with symptoms of shoot proliferation in apple trees and to evaluate possible genetic diversity of phytoplasma strains found in Poland, using finer differentiation methods.

Materials and methods

During 2010–2013, samples consisting of six to ten shoots were collected from 17 symptomatic and 250 asymptomatic apple trees randomly selected from Golden Delicious, Red Boskoop, Jonagold, Mutsu, Cox's Orange, Šampion, Gloster, Idared, Lobo, Cortland, Elstar, Gala, and unknown cultivars, as well as from M 26 rootstock. The shoots from each sample were stored at 4ºC, and were then tested for presence of phytoplasmas. The symptomatic trees prematurely developed buds during spring as well as shoot proliferation, and produced small, malformed fruits. The selected trees were grown in commercial orchards, private gardens and natural environments located in six provinces of Central and South Poland: Lódzkie, Mazowieckie, Lubelskie, Malopolskie, Dolnośląskie, and Podkarpackie.

Extraction of nucleic acids and PCR analyses

DNeasy Plant Mini Kit (Qiagen) was used for extraction of total DNA from fresh phloem tissue of apple shoots grinded in liquid nitrogen. DNA from each sample was subjected to a nested PCR assay with the universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) for amplification of a 1.8 kbp product of the 16S ribosomal DNA, the spacer region between the 16S and 23S rDNA genes and the 5' of the 23S rDNA of the phytoplasmas. P1/P7 amplicons, diluted 1: 29 with sterile water were used as templates for the second round of PCR with the universal primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). The positive samples were used for further molecular characterization of '*Ca*. P. mali' strains. The P1/P7 amplicons were re-amplified with 16SrX-group-specific primers R16(X)F1/R1 (Lee *et al*., 1995), and with universal primers F1/B6 (Davis and Lee, 1993; Padovan *et al*., 1995), to confirm phytoplasma identity and to study the 16S ribosomal DNA and the 16S-23S rDNA spacer region sequence polymorphism. Non-ribosomal DNA fragments including nitroreductase-like gene were amplified in nested PCR assays with primers pairs AP13/AP10 (Jarausch *et al*., 2000) followed by AP14/AP15 (Casati *et al*., 2010). The *rpl22* and *rps3* gene sequences encoding ribosomal protein (rp) S10 were amplified in nested PCR using primers pairs rpAP15f2/rp(I)R1A followed by rpAP15f/rpAP15r (Martini *et al*., 2008).

All PCR assays were performed with a thermal cycler PTC-200 (MJ Research), and 7 μL of the amplification products were separated in 1% agarose gel in 0.5 × TBE buffer, followed by staining in ethidium bromide and visualization of DNA bands using UV transilluminator (Syngen). The molecular weight of the PCR products was estimated by comparison with 100 bp DNA ladder (Fermentas). DNA of a sample from an asymptomatic apple tree was included in each PCR assay as negative control, and positive control was the apple proliferation strain AP-15 (Bertaccini, 2014).

Restriction fragment length polymorphism analyses

The characterization of '*Ca*. P. mali' strains was carried out on the three phytoplasma genome fragments using the Restriction Fragment Length Polymorphism (RFLP) technique (Table 1). Five μL of the PCR products were separately digested with appropriate restriction endonucleases (Fermentas).

The generated restriction patterns were analyzed by electrophoresis in 8% polyacrylamide gels in 1% TBE buffer and compared with the profiles of the reference strain AP-15. They were also compared with previously published RFLP patterns of '*Ca*. P. mali' strains (Lee *et al*., 1998; Casati *et al*., 2010; 2011; Paltrinieri *et al*., 2010; Fránová *et al*., 2013).

Sequence analyses

The partial sequence of '*Ca*. P. mali' strains including 16S rDNA (16S rRNA gene) and 16S-23S rDNA spacer region, amplified with primer pairs F1/B6, the non-ribosomal region (nitroreductase and rhodanese-like protein genes), amplified with AP14/AP15, and the S10 rp operon (*rpl*22 and *rps*3 genes), amplified with rpAP15f/rpAP15r, were purified from the

Table 1. Primer pairs and restriction enzymes used in PCR-RFLP analyses of '*Candidatus* Phytoplasma mali' strains from a survey of apple trees in Central and Southern Poland during 2010-2013.

Genome segment	Approx. size (bp)	Primer pair	Reference	Restriction enzyme
16S-23S rDNA	1800	P1/P7[a]	Deng & Hiruki, 1991; Schneider *et al*., 1995	nd
	1700	F1/B6[a]	Davis & Lee, 1993; Padovan *et al*., 1995	*Hpa*II, *Smu*I
16S rDNA	1200	R16F2n/R2[a]	Gundersen & Lee, 1996	*Alu*I, *Hha*I, *Hpa*II, *Mse*I, *Rsa*I, *Ssp*I
	1100	R16(X)F1/R1[b]	Lee *et al*., 1995	nd
S10 ribosomal protein operon	1036	rpAP15f2[a]/rp(I)R1A	Martini *et al*., 2008	nd
	920	rpAP15f/rpAP15r[b]	Martini *et al*., 2008	*Alu*I
Nitroreductase and rhodanese-like protein	776	AP13/AP10[b]	Jarausch *et al*., 1994	nd
	717	AP14/AP15[b]	Casati *et al*., 2010	*Hinc*II, *Pag*I

[a] Universal primers.
[b] Primers specific for 16SrX group.
nd: not determined.

gels using QIAquick® Gel Extraction Kit (Qiagen). The partial sequences were obtained in both strains using the same primers employed for amplification and aligned using ClustalW of the DNASTAR's Lasergene software (DNASTAR Inc). The consensus sequences were compared with sequences available in GenBank using the BLAST algorithm (http://ncbi.nlm.nih.gov/BLAST/). The genetic relationships of phytoplasma strains were determined by phylogenetic analysis using the neighbour-joining method with the Tamura 3-parameter model and MEGA 4.0 program (Tamura *et al*., 2007). Bootstrap analysis (1,000 replicates) was performed for statistical significance estimation (values below 60% were collapsed).

Results

Detection and identification of '*Ca*. P. mali'

Direct PCR with the P1/P7 primers did not result in amplification of the expected length products from DNA templates extracted from phloem tissue of surveyed apple trees. Only the AP-15 reference strains was amplified. Seventeen samples collected from symptomatic trees (Table 2) were positive in nested PCR assays with the universal primers R16F2n/R16R2, F1/B6 and the primer pair R16(X)F1/R1, specific for phytoplasmas in the apple proliferation group (16SrX). RFLP analyses of R16F2n/R16R2 amplicons identified '*Ca*. P. mali' presence in all samples, since the patterns obtained after digestion with *Rsa*I, *Alu*I, *Mse*I, *Hpa*II, *Hha*I, and *Ssp*I enzymes were indistinguishable from those of the reference strain AP-15 and from AT strain of '*Ca*. P. mali', 16SrX-A (Lee *et al*., 1998) (data not shown). In turn, the PCR products amplified with F1/B6 primers revealed three different profiles after digestion with *Hpa*II (Figure 1). Seven samples from the cultivars listed in Table 2 (Kotlar, Mut, Przel, GDel, Oz, Kijak, and SzamP), showed the P-I profile. The other nine samples (from Głowno, GoP, RBoC, Bor, GlP, JoC, GoC, J4020, and J8148) showed the P-II profile, while the strain from Tward strain presented both P-I and P-II profiles (Table 2). When the *Smu*I enzyme was used, the restriction patterns for all analyzed samples was indistinguishable (data not shown).

Results of RFLP analyses of the nitroreductase and rhodanese-like protein gene with *Hinc*II enzyme distinguished the Polish strains of '*Ca.* P. mali' into AP-15 and AT-1 subtypes. The restriction profiles of 14 samples were indistinguishable from one another and from AP-15 strain, while the AT-1 subtype was obtained in 'J4020' and 'J8148' samples, and the 'GoP' strain showed a pattern referable to both the

Table 2. Results of multigene RFLP analyses of '*Candidatus* Phytoplasma mali' strains detected from the survey of apple trees in Central and Southern Poland during 2010-2013.

Sample code	Cultivar	Province	16S-23S rDNA (Profile)	Nitroreductase and rhodanese-like protein (AP subtype)	S10 ribosomal protein (rp subgroup)	Lineage
Oz	unknown	Łódzkie	P-I	AP-15	X-A	1
Głowno	unknown	Łódzkie	P-II	AP-15	X-A	2
Bor	unknown	Łódzkie	P-II	AP-15	X-A	2
GDel	Golden Delicious	Łódzkie	P-I	AP-15	X-A	1
Kijak	unknown	Łódzkie	P-I	AP-15	X-A	1
GoC	Golden Delicious	Mazowieckie	P-II	AP-15	X-A	2
RBoC	Red Boskoop	Mazowieckie	P-II	AP-15	X-A	2
JoC	Jonagold	Mazowieckie	P-II	AP-15	X-A	2
Mut	Mutsu	Mazowieckie	P-I	AP-15	X-A	1
Tward	unknown	Małopolskie	P-I+P-II	AP-15	X-A	3
J8148	M 26 rootstock	Małopolskie	P-II	AT-1	X-A	4
J4020	Cox's Orange	Małopolskie	P-II	AT-1	X-B	5
GoP	Golden Delicious	Dolnośląskie	P-II	AP-15+AT-1	X-A	6
SzamP	Šampion	Dolnośląskie	P-I	AP-15	X-A	2
GlP	Gloster	Dolnośląskie	P-II	AP-15	X-A	1
Kotlar	unknown	Lubleskie	P-I	AP-15	X-A	2
Przel	unknown	Podkarpackie	P-I	AP-15	X-A	2

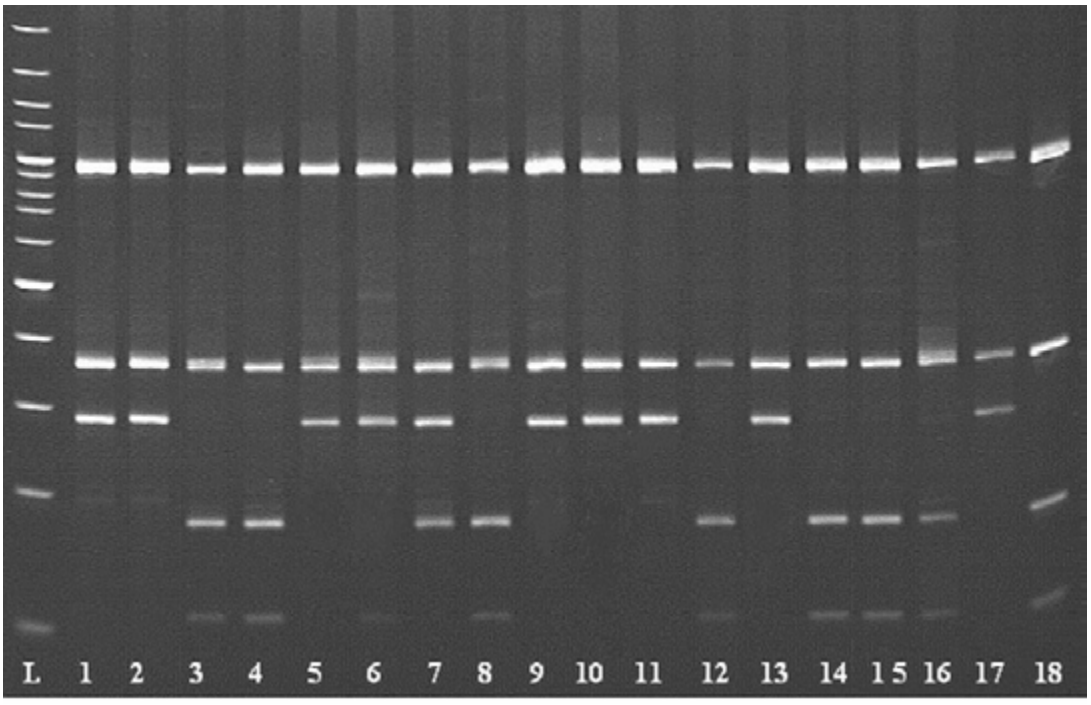

Figure 1. Polyacrylamide gel showing the RFLP patterns of phytoplasma ribosomal DNA fragment amplified with F1/B6 primers digested with *Hpa*II restriction enzyme of apple samples from the survey of apple trees in Central and Southern Poland during 2010-2013. Lanes: L – molecular marker 100 bp. 1. J8148, 2. J4020, 3. Przel, 4. SzamP, 5. GoC, 6. GoP, 7. Tward, 8. Oz, 9. RBoC, 10. Bor, 11. JoC , 12. Mut, 13. GlP, 14. Kotlar, 15. Kijak, 16. GDel, 17. Głowno, 18. AP-15 reference strain.

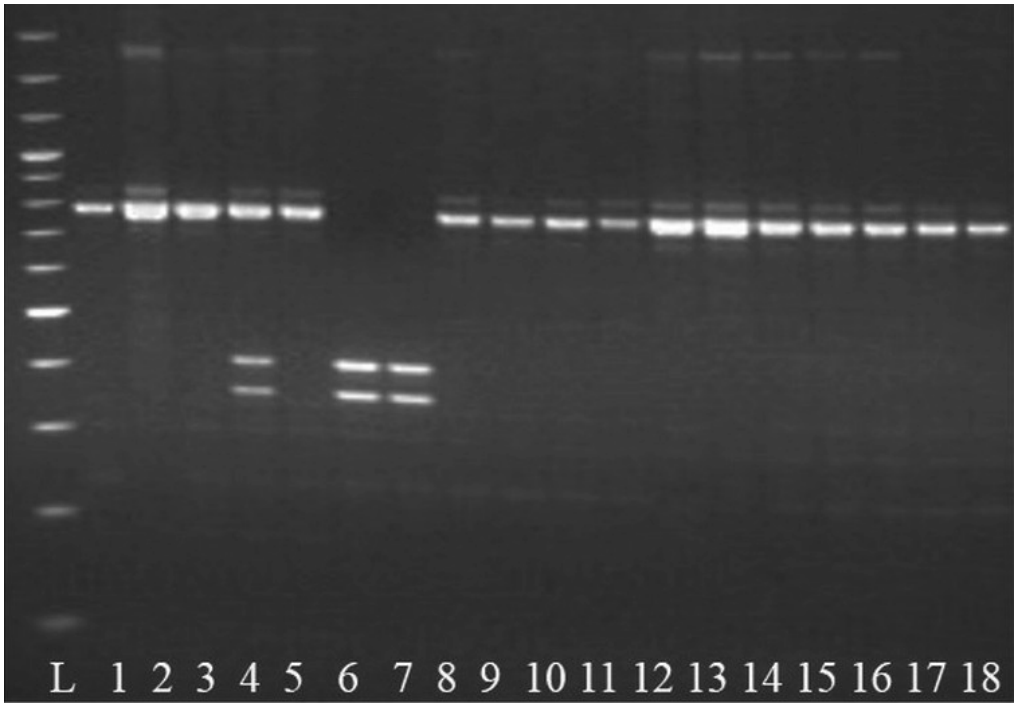

Figure 2. Polyacrylamide gel showing the RFLP patterns of phytoplasma nitroreductase-like gene amplified with AP14/AP15 primers digested with *Hinc*II restriction enzyme of apple samples from the survey on apple trees in Central and Southern Poland during 2010-2013. Lanes: L – molecular marker 100 bp. 1. Przel, 2. SzamP, 3. GoC, 4. GoP, 5. Tward, 6. J4020, 7. J8148, 8. Oz, 9. RBoC, 10. Bor, 11. JoC , 12. Mut, 13. GlP, 14. Kotlar, 15. Kijak, 16. GDel, 17. Głowno 18. AP-15 reference strain.

AP-15 and AT-1 subtypes (Figure 2, Table 2). After digestion with *Pag*I enzyme, identical restriction patterns were observed for all 17 '*Ca*. P. mali' samples and the AP-15 reference strain (data not shown).

The restriction profiles of the ribosomal protein (rp) genes (*rpl22* and *rps3*) digested with *Alu*I enzyme were the same for 16 Polish strains of *'Ca.* P. mali', as well as for the reference strain AP15 indicating that they belong to subgroup rpX-A (Figure 3, Table 2). However, the restriction pattern for this region of the strain J4020 was different and characteristic for the profile of AT type strains classified to the rpX-B subgroup (Paltrinieri *et al*., 2010; Casati *et al*., 2011).

Sequence analyses

Nucleotide sequences of the 16S rDNA and 16S-23S spacer region of rDNA of eleven '*Ca*. P. mali' strains (GlP, Bor, J4020, J8148, Kotlar, GDel, Oz, JoC, Przel, SzamP, and Głowno) were aligned with sequences of the three subtype strains: AP-15 (acc. no.: AJ542541, EF392655, and JN555596), AT-1 (acc. no.: AJ542542 and JN555595), and AT-2 (acc. no. JN555597), as well as AT (acc. no. X68375) available from the GenBank database. Sequence analysis of this region showed 99.9–100% identity of 16S rDNA and 16S-23S spacer region among these strains. Sequences of the same fragment from strains showing the P-I profile (SzamP, Kotlar, Oz and Przel) were indistinguishable from the sequences of the strains AT1/93 (acc. no. AJ542542), T16 (acc. no. EF392655), AT (acc. no. X68375) and 113/2010 (acc. no. JN555597). Phylogenetic analysis of 16S rDNA and 16S-23S spacer region sequences showed that these strains of '*Ca*. P. mali' were closely related and formed a well-supported clade (Figure 4). The sequence of GDel strain showing P-I profile was identical to the sequence of AP-15 reference strain (acc. no. AJ542541) and the other AP-15 subtype strain 116/2010 from the Czech Republic (acc. no. JN555596, Fránová *et al*., 2013). Nucleotide sequences of strains: Głowno, Bor, JoC J4020, J8148, and GlP as well as the reference P-II-

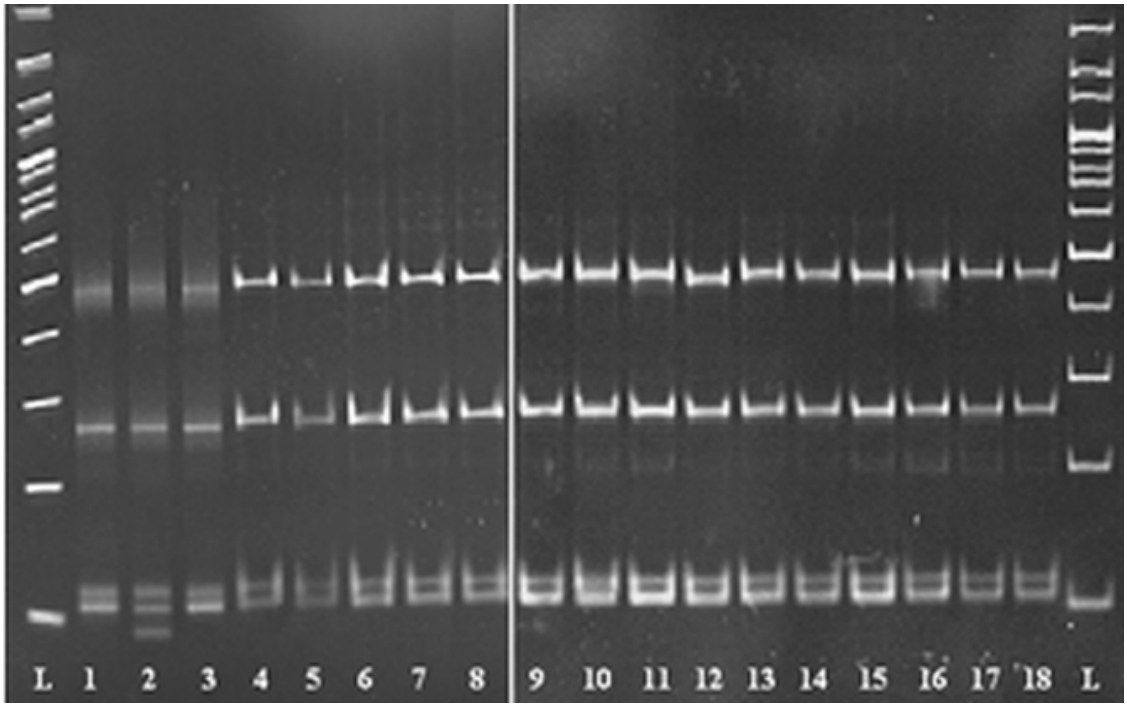

Figure 3. Polyacrylamide gel showing the RFLP patterns of phytoplasma ribosomal protein genes *rpl22* and *rps3* amplified with rpAP15f/rpAP15r primers digested with *Alu*I restriction enzyme. Lanes: L – molecular marker 100 bp. 1. J8148, 2. J4020, 3. Przel, 4. SzamP, 5. GoC, 6. GoP, 7. Tward, 8. Oz, 9. RBoC, 10. Bor, 11. JoC , 12. Mut, 13. GlP, 14. Kotlar, 15. Kijak, 16. GDel, 17. Głowno, 18. AP-15 reference strain.

profile strains 147 of '*Ca*. P. mali' (acc. no. EF392656) and 139 Prostejov Tistin 5 (acc. no. JN555595) were identical and these strains clustered together in the phylogenetic tree (Figure 4). Nucleotide sequences of the 16S rDNA and 16S-23S spacer region of the eight selected Polish strains: GDel, GlP, Bor, Kotlar, J8148, Oz, Przel, and J4020 were deposited in GenBank under accession numbers HG423135-423141 and HG798799.

Fragments of the nucleotide sequence of the nitroreductase-like protein gene of ten strains (Mut, GlP, Bor, J4020, J8148, GDel, Kotlar, Przel, Oz, and Głowno) were compared with sequences of the following strains deposited in GenBank: AT (acc. no. L22217, AT type), 116/2010 (acc. no. JN642116, AP-15 subtype), 139 Prostejov Tistin 5 (acc. no. JN642115, AT-1 subtype), 113/2010 (acc. no. JN642113, AT-2 subtype), and 201/2010 Borsov nad Vltavou (acc. no. JN642117, AP-15 subtype). The sequences of the great majority of these strains showed high similarity (99.9–100%) with the strain 201/2010 (acc. no. JN642117). The phylogenetic analysis grouped these strains close to the other AP-15 subtype strains (Figure 5); the analyses of the partial sequences of this region of the J4020 and J8148 strains showed their identity with sequences of AT strain (acc. no. L22217) and AT-1 subtype strain 139/2010 Prostejov Tistin 5 (acc. no. JN642115), and grouped these strains in the same branch of the phylogenetic tree. Sequences of nitroreductase and rhodanese-like protein gene of the strains J8148, GDel, GlP, Oz, and J4020 were deposited in GenBank under accession numbers HG423131-423134 and HG798805.

Nucleotide sequences of the ribosomal protein (rp) genes (*rpl22* and *rps3*) of eleven '*Ca*. P. mali' strains (GlP, Mut, Kijak, JoC, SzamP, GoP, J8148, J4020, Kotlar, GDel, and Oz) were aligned with sequences of the three subtypes' strains AP-15, rpX-A subgroup (acc. no.: EF193366, JN606863, and JN606864), AT-1, rpX-B subgroup (acc. no: JN606862), AT-2, rpX-A subgroup (acc. no. JN606865), and AT, rpX-B subgroup (acc. no. EF193367). The sequence of the rp region of ten Polish samples was identical and also homologous to the sequences of the three reference strains of AP-15

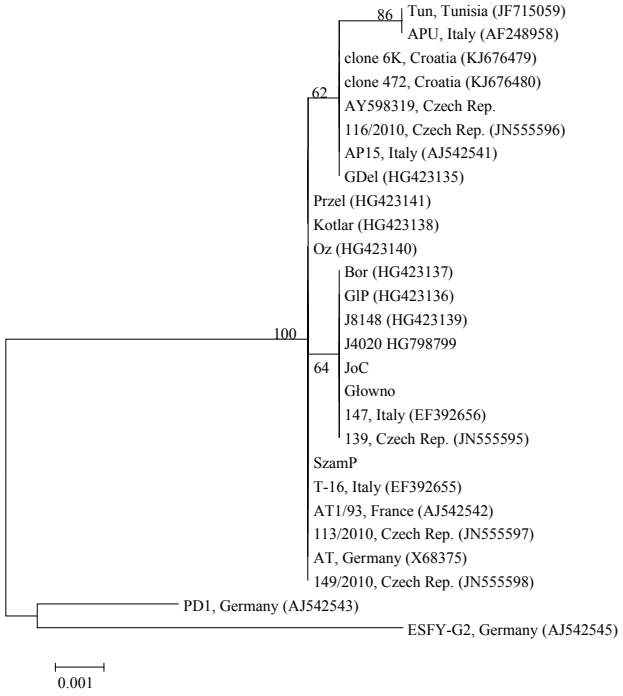

Figure 4. Phylogenic analysis of the 16S rDNA and 16S-23S spacer region sequences of 11 '*Ca*. P. mali' strains detected from a survey of apple trees in Central and Southern Poland during 2010-2013 and apple proliferation strains available in GenBank. PD1 strain of '*Ca*. P. pyri' (AJ542543) and ESFY-G2 strain of '*Ca*. P. prunorum' (AJ542545) were included as outgroups. Phylogenetic trees were constructed by the neighbor-joining algorithm (MEGA 4.0) and the Kimura 3-parameter nucleotide substitution model in using 1,000 bootstrap replicates. Only bootstrap values higher than 60 are shown. The bar indicates the genetic distance as Kimura units.

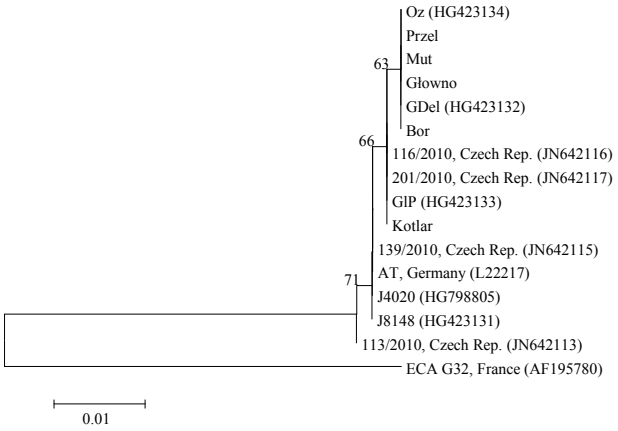

Figure 5. Phylogenic analysis of the nitroreductase and rhodanese-like protein sequences of 10 '*Ca.* P. mali' strains detected from a survey of apple trees in Central and Southern Poland during 2010-2013 and strains available in GenBank. ECA G32 strain of '*Ca*. P. prunorum' (AF195780) was included as outgroup. Phylogenetic trees were constructed by the neighbour-joining algorithm (MEGA 4.0) and the Kimura 3-parameter nucleotide substitution model in using 1,000 bootstrap replicates. Only bootstrap values higher than 60 are shown. The bar indicates the genetic distance as Kimura units.

subtype and AT-2 subtype strain (all of them classified to subgroup rpX-A). Phylogenetic analysis of the *rpl22* and *rps3* gene sequences showed close relationship among these strains (Figure 6). The only exception was a Single Nucleotide Polymorphism (SNP) in position 312 (T→C) in the sequence of the reference strain AP-15, rpX-A subgroup (acc. no. EF193366). RFLP analysis confirmed that these Polish strains belonged to the subgroup rpX-A of the '*Ca*. P. mali'. Contrary, in the ribosomal protein gene sequences of J4020 strain, a 12-nucleotide long sequence (AAGAAATTAAAG) deletion was detected in positions 446-457. The sequence of this fragment from the J4020 strain was identical to the sequence of the rp gene of the strains 139/2010 Prostejov Tistin 5 (acc. no. JN606862) from the Czech Republic and AT (acc. no. EF193367) from Germany, both of which classified to rpX-B subgroup. This result was confirmed by phylogenetic analysis, as the J4020 strain clustered with both reference strains. Sequences of ribosomal protein *rpl22* and *rps3* genes of strains GDel, GlP, Oz, J8148, and J4020 were deposited in GenBank under accession numbers HG798800-798804.

Discussion

In spite of the large number of apple tree samples tested in this study, only in 17 symptomatic trees the presence of apple proliferation (AP) phytoplasma was confirmed. This indicates that the apple proliferation disease is currently in a non-epidemic situation in the surveyed areas of Poland, since these single infected trees were scattered throughout the surveyed orchards.

It was confirmed that PCR-RFLP analysis on multiple phytoplasma genes is a useful method for determining genetic diversity of closely related AP

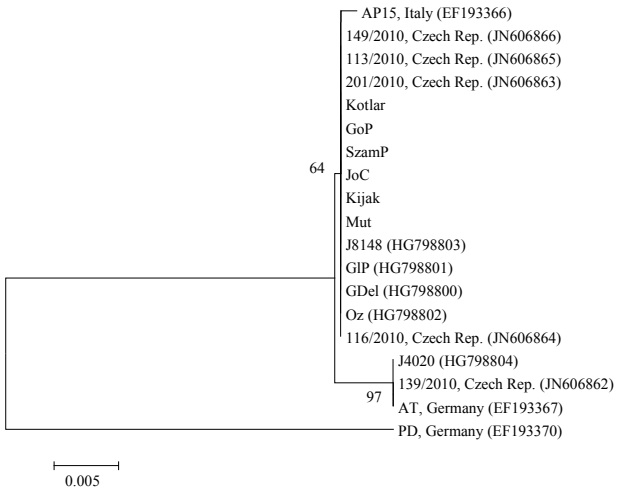

Figure 6. Phylogenic analysis of the S10 ribosomal protein sequences of 11 '*Ca.* P. mali' strains detected from a survey of apple trees in Central and Southern Poland during 2010-2013 and the strains available in GenBank. PD strain of '*Ca*. P. pyri' (EF193370) was included as outgroup. Phylogenetic trees were constructed by the neighbour-joining algorithm (MEGA 4.0) and the Kimura 3-parameter nucleotide substitution model in using 1,000 bootstrap replicates. Only bootstrap values higher than 60 are shown. The bar indicates the genetic distance as Kimura units.

phytoplasma strains. The 16S rDNA has been widely applied for identification and classification of phytoplasmas. However, analysis of 16S-23S rDNA intergenic region and other regions than 16S rRNA gene were recommended for finer molecular characterization of phytoplasmas (Lee *et al*., 2006, Duduk *et al*., 2009), and genetic diversity of '*Ca*. P. mali' strains was confirmed in several European countries (Casati *et al*., 2010; Paltrinieri *et al*., 2010; Fránová *et al*., 2013; Cieślińska and Kruczyńska, 2014).

In the present study, the SNP C→T in position 1589 of the 16S-23S spacer region resulted in missing of an additional *Hpa*II restriction site in phytoplasma strains Głowno, Bor, GoC, RBoC, JoC, J4020, J8148, GoP, and GlP. Although this polymorphism is not considered relevant for classification, it confirmed the consistence of P-I and P-II restriction patterns previously reported in '*Ca*. P. mali' strains from other European countries (Martini *et al*., 2007; Casati *et al*., 2010; 2011; Paltrinieri *et al*., 2010; Fránová *et al*., 2013). Only the *Hpa*II restriction profile showed polymorphism in the present study, while further molecular diversity of this sequence in some Italian AP strains was revealed by restriction with *Smu*I and *Hpy*CH4V enzymes (Casati *et al*., 2011).

Sequence analysis of nitroreductase and rhodanese-like protein genes allowed the grouping of ten Polish AP strains in the AP-15 subtype. The single nucleotide polymorphism C→G in position 1401 of this region in J4020 and J8148 strains resulted in the *Hinc*II-restriction site which was not present in the sequence of the other Polish strains. In turn, cytosine (C) was present at position 1365 of the nitroreductase-like gene sequence of the all analyzed Polish strains including J4020 and J8148. The SNP A→C at this position is a restriction site for the *Pag*I enzyme, and allows distinction of the AP-15 and AT-1 subtype strains (Fránová *et al*., 2013). Based on these findings, it can be concluded that J4020 and J8148 strains belong to the AT-1 subtype. Results of RFLP and sequence analyses enabled the detection of only two of the reported '*Ca*. P. mali' subtypes among the three (AT-1, AT-2 and AP-15) reported in apple orchards in Germany, France, Italy and in the Czech Republic (Cainelli *et al*., 2004; Jarausch *et al*., 2004; Paltrinieri *et al*., 2010; Casati *et al*., 2011; Fránová *et al*., 2013). Although only 17 strains were detected and characterized, the AP-15 subtype was the predominant strain in Poland, similar to the situation reported in France (Jarausch *et al*., 2000) and in the Friuli Venezia Giulia, region of northeastern Italy (Martini *et al*., 2008). In turn, the AT-2 subtype of '*Ca*. P. mali' was mostly found in Trentino Alto-Adige region (North Italy) (Cainelli and Grando, 2005; Bertaccini *et al*., 2008) and AT-1 strains were identified most frequently in Lombardia, Piemonte and Valle d'Aosta, regions of northwestern Italy (Casati *et al*., 2011) and Germany (Jarausch *et al*., 2000). Casati *et al*. (2010) reported the presence of at least two phytoplasma genetic lineages, designated AT-1a and AT-1b, among the AP phytoplasma strains of the AT-1 subtype.

Analyses of 16S-23S spacer region have shown that the strains belonging to all three subtypes (AP-15, AT-1, AT-2) gave the P-I profile, while P-II patterns were obtained for strains classified to AP-15 and AT-1 (Paltrinieri *et al*., 2010; Fránová *et al*., 2013). The present study revealed the presence equal proportions of P-I and P-II profiles in Polish strains, but both in subtype AP-15. J4020 and J8148 strains classified as AT-1 subtype each showed a P-II profile.

Generally, only a single subtype of '*Ca*. P. mali' (AP-15, AT-1 or AT-2) was detected in particular infected apple trees (Jarausch *et al*., 2000; Cainelli and Grando, 2005; Martini *et al*., 2008; Casati *et al*., 2010, 2011; Paltrinieri *et al*., 2010). However, simultaneous occurrence of two or three subtype strains of this phytoplasma have also been detected in Trentino Alto-Adige region of Italy and in the Czech Republic (Bertaccini *et al*., 2008; Fránová *et al*., 2013). Results of the present study showed that only the GoP sample was infected by AP-15 and AT-1 subtypes. This is the first time in which these two subtypes have been reported in mixed infection while AT-1 + AT-2 were simultaneously detected in Italy and in the Czech Republic (Bertaccini *et al*., 2008; Fránová *et al*., 2013), and a mixed infection with AT-1 + AT-2 + AP-15 subtypes was found in the Czech Republic (Fránová *et al*., 2013).

The sequence analysis of the *rpl22* and *rps3* genes encoding ribosomal protein showed that independently of 16S-23S restriction profile (P-I or P-II), the majority of Polish strains were classified to the rpX-A subgroup, in agreement with reports from the Czech Republic (Fránová *et al*., 2013). However, in Italy only AP type strains with P-I profile were present in rpX-A subgroup (Paltrinieri *et al*., 2010; Casati *et al*., 2011; Fránová *et al*., 2013), while in samples showing P-II profile other rpX subgroups (-B, -C, -D) were identified (Paltrinieri *et al*., 2010). The strains with restriction patterns characteristic for AT-1 after digestion of nitroreductase-like gene with *Hinc*II and *Pag*I were also enclosed to other rpX subgroups in Italy (Paltrinieri *et al*., 2010; Casati *et al*., 2011). In samples from the Czech Republic, it was demonstrated that the strains of AT-1 or AT-2 subtypes with P-II or P-I + P-II profiles always belonged to rpX-B subgroup, and strains of the AP-15, AT-1 and AT-2 subtypes showing P-I profile were classified to rpX-A subgroup (Fránová *et al*., 2013). In the present study two strains with P-II restriction profile were enclosed within the AT-1 subtype. However, different profiles after digestion of ribosomal protein genes enabled the classification of J8148 to the rpX-A subgroup and J4020 - to the rpX-B subgroup.

This survey has shown the presence of genetic diversity among '*Ca*. P. mali' strains found in Central and Southern Poland, and has confirmed their genetic heterogeneity compared with the strains identified in other European countries. RFLP and sequence analyses of three genomic segments allowed six '*Ca*. P. mali' lineages to be distinguished, and to be detected in Poland for the first time. These lineages were: PI/AP-15/rpX-A, PII/AP-15/rpX-A, PI+PII/AP-15/rpX-A, PII/AT-1/rpX-A, PII/AT-1/rpX-B, PII/AP-15+AT-1/rpX-A. While the lineages are different there is some homogeneity in the genes that are coding non-ribosomal fragments (nitroreductase and rhodanese-like protein). As reported in other studies, there was no direct correlation among these molecular markers and AP epidemic outbreaks. However, considering the low number of infected plants and this particular genetic heterogeneity, we hypothesize some molecular confirmation of the non-epidemic situation of the disease in Poland. There remains the possibility of new AP strains emerging, since individuals of *Cacopsylla picta* and *C. melanoneura,* that were reported to be infected with '*Ca*. P. mali' (Cieślińska *et al*., 2012), could increase the risk of disease spread. Large scale exchange of nursery material could also be involved in the appearance of epidemic strains in apple production areas where apple proliferation has not been previously observed.

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