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MOLECULAR CHARACTERIZATION OF '*Candidatus* Phytoplasma mali' STRAINS FROM BULGARIA AND POLAND

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ABSTRACT

During 2015, samples from 22 apple trees showing proliferation symptoms were collected in southwest Bulgaria and Central and South Poland and tested for phytoplasma presence. '*Candidatus* Phytoplasma mali' was identified in 18 samples based on results of restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene amplified in nested PCR using primer pair P1/P7 followed by R16F2n/R16R2 and F1/B6 primer pairs. The nitroreductase and rhodonase-like genes and ribosomal protein genes *rpl22* and *rps3* were then analyzed using PCR-RFLP technique to study the genetic variability of the phytoplasma strains. Two restriction profiles, P-I or P-II, were obtained from fragments of 16S rDNA plus 16S-23S spacer region digested with *Hpa*II enzyme. Restriction fragment length polymorphism analysis of nitroreductase and rhodonase-like genes using digestion with *Hinc*II endonuclease revealed that all '*Ca*. P. mali' strains belonged to the subtype AP-15. Analysis of *rpl22* and *rps3* ribosomal protein genes digested with *Alu*I enzyme resulted in classification of detected phytoplasma strains to rpX-A subgroup.

Key words: apple proliferation, PCR, RFLP, multilocus gene analyses

INTRODUCTION

Apple proliferation is one of the most important diseases leading to significant losses in apple production. The agent of the disease is '*Candidatus* Phytoplasma mali' classified to the subgroup A of 16SrX group. It is specified in EPPO A2 list of pests recommended for regulation as quarantine organisms in Europe. The symptoms associated with the presence of '*Ca*. P. mali' include proliferation of lateral shoots (witches' broom), enlarged stipules and reductions in size and quality of apple fruits. The susceptibility of the infected trees to powdery mildew is increased.

In Bulgaria, apple proliferation symptoms were observed in early 1960's [Trifonov 1965]. After several decades the study on detection and identification of '*Ca*. P. mali' in different regions of Bulgaria was conducted [Milusheva and Terziev 2013, Etropolska et. al. 2015, Borisova and Kamenova 2016, Fránová et al. 2018]. Apple proliferation commonly occurred in southern Poland in the 1960's and 70's [Kamińska and Zawadzka 1970]. Over the last few years the disease symptoms were observed in apple trees growing in different areas of the country and molecular characterization of the detected strains of '*Ca*. P. mali' was conducted [Cieślińska et al. 2012, 2014, 2015].

The highly conserved 16S rDNA region is widely applied for identification and classification of a broad range of phytoplasmas, however its usefulness for differentiation of the closely related strains is limit-

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ed [Bertaccini et al. 2014]. The analysis of different molecular markers was conducted for characterization of '*Ca*. P. mali' strains including full-length 16S rDNA, 16S-23S rDNA intergenic region and partial sequence of 23S rDNA, the ribosomal protein genes *rpl22* and *rpS3*, nitroreductase and rhodanese-like protein genes or *aceF*, *pnp*, *imp*, *hflB*, and *tuf* genes [Danet et al. 2007, Martini et al. 2008, Schneider and Seemüller 2009, Casati et al. 2010, Paltrinieri et al. 2010, Etropolska 2012, Makarova et al. 2012, Fránová et al. 2013, Cieślińska et al. 2014, 2015].

The analysis of a non-ribosomal DNA fragment including nitroreductase-like gene resulted in distinguishing AT-1, AT-2, and AP-15 subtypes of '*Ca*. P. mali' [Jarausch et al. 1994, 2000]. Molecular characterization of the genes coding the ribosomal proteins L22 and S3 revealed the presence of higher genetic heterogeneity within strains of the apple proliferation phytoplasma and led to the proposal of four subtypes: rpX-A, rpX-B, rpX-C, and rpX-D [Martini et al. 2008].

The aim of this study was to determine the molecular variability of '*Ca*. P. mali' strains in symptomatic apple plants from Bulgaria and Poland based on results of PCR-RFLP analysis of three genome fragments.

MATERIALS AND METHODS

Plant samples

During the late summer of 2015, shoots from 10 symptomatic apple trees were collected in Kyustendil region, southwestern Bulgaria. Some of the trees were previously positively tested for phytoplasma presence [Borisova and Kamenova 2016]. Samples from 12 apple trees collected in three provinces of Central and South Poland were also enclosed in this study including positive control which was the Polish strain GoC [Cieślińska et al. 2015]. The symptoms observed in the the trees included shoot proliferation (Fig. 1), small leaves and fruits and in some cases also enlarged stipules. Phloem tissue from shoot samples collected form the apple trees were analyzed for phytoplasma presence. Phytoplasma-free P14 rootstock sample was used as a negative control.

Extraction of nucleic acids, PCR analysis

Total DNA was extracted from phloem tissue of apple shoots using DNeasy Plant Mini Kit (Qiagen, Germany). P1/P7 primer pair was used in direct PCR for amplification of a 1,800- nucleotide (nt) genome fragment containing the 16S ribosomal DNA, the spacer region between the 16S and 23S rDNA genes and the 5' of the 23S rDNA (Tab. 1). P1/P7 amplicons, diluted 1:29 with sterile water were employed as templates in a nested PCR using primers R16F2n/R16R2 and, only for the positive samples, also the F1/B6 primer pair. The amplification of the ribosomal protein genes *rpl22* and *rps3* and nitroreductase-like and rhodanese-like protein genes was also performed by nested PCR using the universal pair of primers rpAP15f2/rp(I)R1A followed by the primers rpAP15f/rpAP15r specific for '*Ca*. P. mali'. Non-ribosomal DNA fragments including nitroreductase-like and rhodanese-like protein genes were amplified in nested PCR assays using the primers pairs AP13/AP10 followed by AP14/AP15.

All PCR assays were performed with a thermocycler PTC-200 (MJ Research), and 7 µl of the amplification products were separated in 1% agarose gel in 0.5 X 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 (Thermo Scientific). DNA from a sample of an asymptomatic apple trees was included in each PCR assay as a negative control. The positive control was the '*Ca*. P. mali' strain AP-15 [Bertaccini 2014].

Restriction fragment length polymorphism (RFLP)

PCR products primed with F1/B6 primer pair were separately digested by *Hpa*II, and *Mse*I enzymes (ThermoFisher Scientific) to conduct the restriction fragment length polymorphism (RFLP) analysis of 16S ribosomal DNA, 16S-23S rDNA spacer region and 23S rDNA fragment. Polish strain GoC classified to P-II type [Cieślińska et al. 2015] was the positive control. RFLP analysis was also conducted after digestion of ribosomal protein and nitroreductase and rhodanese-like protein DNA fragments with *Alu*I and *Hinc*II enzymes, respectively.

The generated restriction patterns were analyzed by electrophoresis in 8% polyacrylamide gels in 1% TBE buffer and compared with the previously published RFLP profiles of the apple proliferation strains of phytoplasmas [Casati et al. 2010, Paltrinieri et al. 2010, Fránová et al. 2013, Cieślińska et al. 2015].

Fig. 1. Shoot proliferation on 'Golden Delicious' apple tree infected by '*Candidatus* Phytoplasma mali'

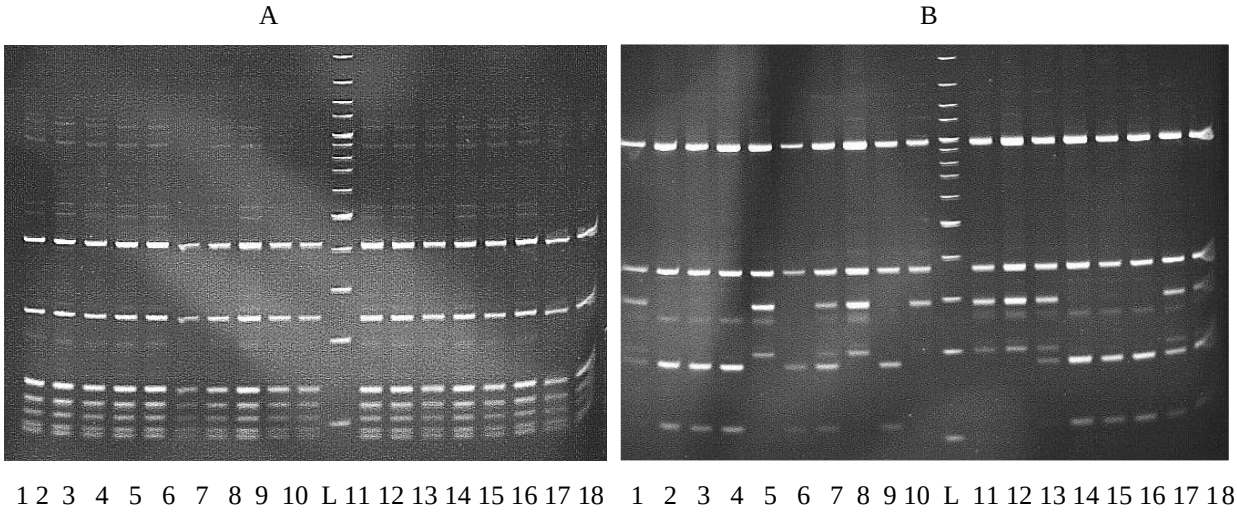


Fig. 2. Polyacrylamide gel showing the RFLP patterns of phytoplasmas ribosomal DNA fragment amplified with F1/B6 primers digested with *Mse*I (A) and *Hpa*II (B) restriction enzymes of apple samples collected in 2015 in Bulgaria and Poland. Lanes: L – Thermo Scientific GeneRuler 100 bp Plus DNA Ladder, fragment sizes in base pairs (from top to bottom): 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100. Samples: 1. GS, 2. Flo, 3. Pr1, 4. Co10, 5. Fre, 6. GoD, 7. Lib, 8. Pr2, 9. V144, 10. GoC (positive control), 11. Top, 12. Raj, 13. Pr4, 14. Mel, 15. Glo, 16. McI, 17. Ev1, 18. Ev2

Table 1. Primer pairs and restriction enzymes used in PCR-RFLP analyses of '*Candidatus* Phytoplasma mali' strains detected in apple trees in Bulgaria and Poland in 2015

Genome segment	Approx. size (bp)	Primer pair	Reference	Restriction enzyme
16S-23S rDNA	1800	P1/P7*	Deng and Hiruki [1991], Schneider et al. [1995]	nd
16S rDNA	1700	F1/B6*	Davis and Lee [1993], Padovan et al. [1995]	*Hpa*II, *Mse*I
	1200	R16F2n/R16R2*	Gundersen and Lee [1996]	nd
S10 ribosomal protein operon	1036	rpAP15f2**/rp(I)R1A	Martini et al. [2008]	nd
	920	rpAP15f/rpAP15r**	Martini et al. [2008]	*Alu*I
nitroreductase and rhodanese-like protein	776	AP13/AP10**	Jarausch et al. [1994]	nd
	717	AP14/AP15**	Casati et al. [2010]	*Hinc*II

*universal primers; **primers specific for 16SrX group; nd – not determined

Table 2. Results of multigene RFLP analyses of '*Candidatus* Phytoplasma mali' strains detected in apple trees in Bulgaria and Poland in 2015

Sample codes	Cultivar	Country*/ Province	PCR result	16S-23S rDNA	Nitroreductase and rhodanese-like protein	S10 ribosomal protein
				profile	AP subtype	rp subgroup
GS	Granny Smith	BG/Kyustendil	+	P-II	AP-15	X-A
Unk	unknown	BG/Kyustendil	–	na	na	na
Flo	Florina	BG/Kyustendil	+	P-I	AP-15	X-A
Pr1	Prima	BG/Kyustendil	+	P-I	AP-15	X-A
Co10	Coop10	BG/Kyustendil	+	P-I	AP-15	X-A
Fre	Freedom	BG/Kyustendil	+	P-II	AP-15	X-A
GoD	Golden Delicious	BG/Kyustendil	+	P-I	AP-15	X-A
Lib	Liberty	BG/Kyustendil	+	P-I+P-II	AP-15	X-A
Pr2	Prima	BG/Kyustendil	+	P-II	AP-15	X-A
V144	hybrid V144	BG/Kyustendil	+	P-I	AP-15	X-A
GoC**	Golden Delicious	PL/mazowieckie	+	P-II	AP-15	X-A
Pr3	Prima	PL/dolnośląskie	–	na	na	na
Top	Topaz	PL/lubelskie	+	P-II	AP-15	X-A
Raj	Rajka	PL/lubelskie	+	P-II	AP-15	X-A
GoS	Gold Star	PL/lubelskie	–	na	na	na
Pr4	Prima	PL/dolnośląskie	+	P-II	AP-15	X-A
Jon	Jonagold	PL/dolnośląskie	–	na	na	na
Mel	Melrose	PL/dolnośląskie	+	P-I	AP-15	X-A
Glo	Gloster	PL/dolnośląskie	+	P-I	AP-15	X-A
McI	McIntosh	PL/mazowieckie	+	P-I	AP-15	X-A
Ev1	Evelina	PL/mazowieckie	+	P-I+P-II	AP-15	X-A
Ev2	Evelina	PL/mazowieckie	+	P-I+P-II	AP-15	X-A

*BG – Bulgaria; PL – Poland, **positive control, na – not applicable

RESULTS AND DISCUSSION

Direct PCR with primer pair P1/P7 did not result in amplification. Eighteen out of 22 samples collected from symptomatic trees (Tab. 2) were positive in nested PCR with universal primers R16F2n/R16R2 (data not shown).

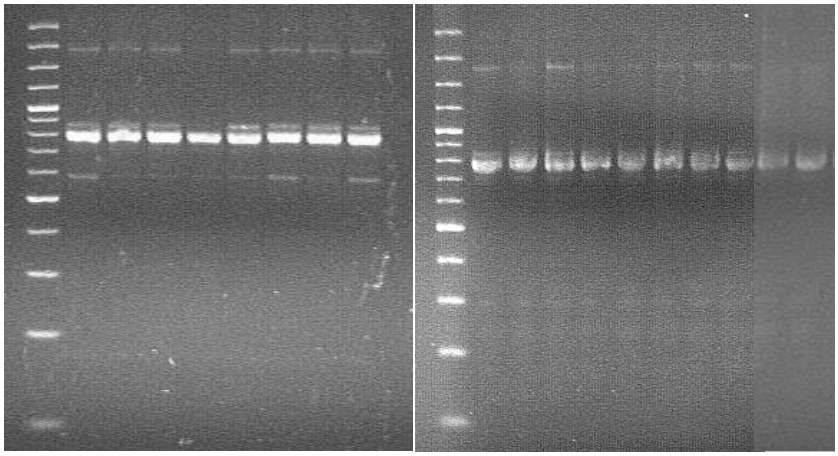
The restriction pattern after digestion of F1/B6 amplicons by *Mse*I enzyme was indistinguishable for all analyzed samples (Fig. 2A). However, RFLP analyses of this DNA fragment with *Hpa*II restriction enzyme revealed three different profiles characteristic for '*Ca*. P. mali' (Fig. 2B, Tab. 2). Five samples from Bulgaria (Flo, Pr1, Co10, GoD, V144) and three from Poland (Mel, Glo, McI) showed the P-I restriction pattern [Martini et al. 2007, Casati et al. 2010, Paltrinieri et al. 2010, Fránová et al. 2013, Cieślińska et al. 2015]. The other three '*Ca*. P. mali' strains from Bulgaria (GrS, Free, Pr1) and three from Poland (Top, Raj, Pr4) presented the P-II profile indistinguishable from the pattern obtained for the Polish reference strain GoC [Cieślińska et al. 2015]. Interestingly, the strains Lib, Ev1 and Ev2 showed both, P-I and P-II profiles. Similar results were also obtained during the studies on molecular characterization of '*Ca*. P. mali' strains in Italy, the Czech Republic and Poland [Paltrinieri et al. 2010, Fránová et al. 2013, Cieślińska et al. 2015].

Results of the PCR-RFLP analyses confirmed the usefulness of 16S-23S rDNA for determining genetic diversity of closely related AP phytoplasma strains. Although this fragment of rDNA is widely applied for classification of phytoplasmas, the analysis of other regions is recommended for their finer molecular characterization [Lee et al. 2006]. The nitroreductase and rhodanese-like protein genes RFLP analysis, however, showed restriction profiles of the 18 samples as indistinguishable from one another after digestion with *Hinc*II. Based on this result all the Bulgarian and Polish strains of '*Ca*. P. mali' were classified to AP-15 subtype (Fig. 3, Tab. 2). This results was not in agreement with the results of a previous study indicating the presence of molecular diversity in '*Ca*. P. mali' strains found in the three different and geographically isolated regions of Bulgaria where it was shown that almost all the tested Bulgarian strains belonged to AT-1 subtype [Etropolska 2012]. The study conducted during 2010–2013 revealed that most strains of '*Ca*. P. mali' found in Poland were grouped into AP-15 subtype whereas only one of them showed AT-1 or both AP-15 and AT-1 profiles [Cieślińska et al. 2015]. During the present study all the detected strains were classified to AP-15 subtype of '*Ca*. P. mali'. A small number of analyzed samples and the origin of the Bulgarian strains from only one region (Kyustendil) could be the reasons for finding only one subtype of this phytoplasma. Depending on the regions, different types of '*Ca*. P. mali' were identified in Italy. AP-15 type was detected in the Friuli Venezia Giulia, region of northeastern Italy [Martini et al. 2008], the AT-2 subtype was mostly found in Trentino Alto-Adige region, north Italy [Cainelli et al. 2004] and AT-1 strains were identified most frequently in Lombardia, Piemonte and Valle d'Aosta, regions of northwestern Italy [Casati et al. 2010]. The detection of all three types of '*Ca*. P. mali' strains, AT-1, AT-2 and AP-15, were also reported in Germany, France, and the Czech Republic [Jarausch et al. 2000, Paltrinieri et al. 2010, Fránová et al. 2013].

Further study showed that after digestion with *Alu*I of the ribosomal protein genes *rpl22* and *rpS3*, identical restriction pattern referable to subgroup rpX-A was obtained for the reference strain GoC and for all the strains of the '*Ca*. P. mali' regardless the profiles, P-I or P-II (Fig. 4, Tab. 2). Similar results were obtained during the study conducted in the Czech Republic [Fránová et al. 2013] and in Poland [Cieślińska et al. 2015]. In turn, Paltrinieri et al. [2010] reported that strains with P-I profile found in Italy, Serbia and Hungary were classified to rpX-A subgroup while samples showing P-II profile – to -B, -C, -D subgroups of rpX group. The strains belonging to the rpX-A subgroup from Serbia showed AP profile, while those from Trentino, North Italy – AT-2 profile. AT1, AT2, and AP profiles were characteristic for phytoplasma strains found in the samples from Hungary.

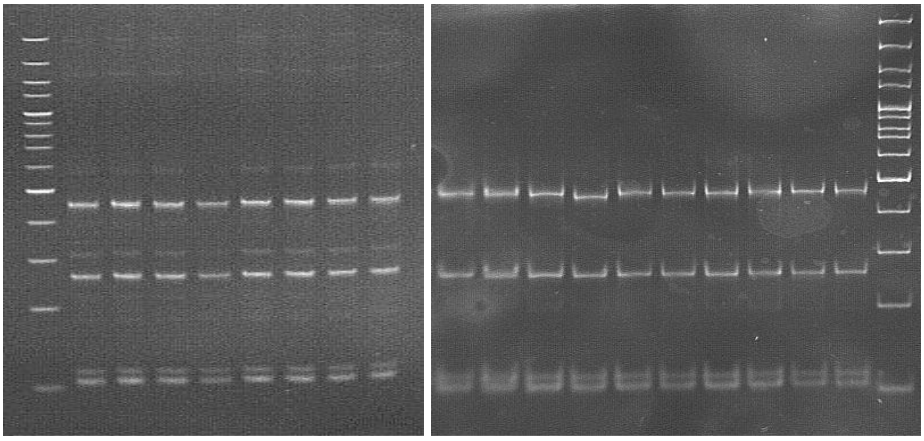
Even though 22 samples were collected from symptomatic apple trees, only 18 resulted positive for '*Ca*. P. mali' presence. This may mean that the proliferation symptoms are not always associated with phytoplasma infection or may indicate a false negative results of PCR due to the kwon uneven distribution of phytoplasmas in different parts of the infected trees [Olivier et al. 2014].

The question is about the reservoir of '*Ca*. P. mali' and the way of phytoplasma spread in both countries.



L 1 2 3 4 5 6 7 8 L 9 10 11 12 13 14 15 16 17 18

Fig. 3. Polyacrylamide gel showing the RFLP patterns of phytoplasmas nitroreductase-like gene amplified with AP14/AP15 primers digested with *Hinc*II restriction enzyme of apple samples collected in 2015 in Bulgaria and Poland. Lanes: L – Thermo Scientific GeneRuler 100 bp Plus DNA Ladder, fragment sizes in base pairs (from top to bottom): 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100. Samples: 1. GS, 2. Flo, 3. Pr1, 4. Co10, 5. Fre, 6. GoD, 7. Lib, 8. Pr2, 9. V144, 10. GoC (positive control), 11. Top, 12. Raj, 13. Pr4, 14. Mel, 15. Glo, 16. McI, 17. Ev1, 18. Ev2



L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig. 4. Polyacrylamide gel showing the RFLP patterns of phytoplasmas ribosomal protein genes (*rpl22* and *rpS3*) amplified with rpAP15f/rpAP15r primers digested with *Alu*I restriction enzyme of apple samples collected in 2015 in Bulgaria and Poland. Lanes: L – Thermo Scientific GeneRuler 100 bp Plus DNA Ladder, fragment sizes in base pairs (from top to bottom): 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100. Samples: 1. GS, 2. Flo, 3. Pr1, 4. Co10, 5. Fre, 6. GoD, 7. Lib, 8. Pr2, 9. V144, 10. GoC (positive control), 11. Top, 12. Raj, 13. Pr4, 14. Mel, 15. Glo, 16. McI, 17. Ev1, 18. Ev2

The old, phytoplasma-infected apple trees growing in neighborhood of newly-established orchard can be factors increasing the risk of the disease spread especially considering that '*Ca*. P. mali' insect vectors are present in both countries. *Cacopsylla picta* and single individuals of *C. melanoneura* were found in Poland and were infected by '*Ca*. P. mali' [Cieślińska et al. 2012]. Apple proliferation phytoplasma was also detected in single specimens of *C. picta* caught in Sofia and Kyustendil region, Bulgaria [Etropolska et al. 2015]. In turn, *C. melanoneura* was reported to have the most important role in transmission of apple proliferation phytoplasma in northwestern Italy [Tedeschi et al. 2002]. The presence of the insect vectors in combination with phytoplasma sources availability and uncontrolled exchange of nursery material could result in the appearance of epidemic outbreaks in apple production areas.

CONCLUSIONS

1. The present paper provides a comparative study on the molecular characterization of '*Candidatus* Phytoplasma mali' strains found in apple trees in Bulgaria and Poland. Although witches' broom, and small fruits were observed on 22 tested trees, '*Ca*. P. mali' presence was confirmed in 18 of them. It can mean that these symptoms were not related to the disease or may indicate a false negative PCR results due to uneven distribution of the phytoplasma in the different parts of the infected tree.

2. Restriction patterns obtained after digestion of 16S rRNA gene's fragment with *Hpa*II enzyme revealed the genetic diversity within rDNA region of the phytoplasma strains. Some of '*Ca*. P. mali' strains, both Bulgarian and Polish, showed P-I restriction profile while others had the pattern characteristic for P-II-type strains.

3. The detected strains of '*Ca*. P. mali' were grouped into subtype AP-15 based on the results of PCR-RFLP analysis of nitroreductase and rhodanese-like protein genes. Analysis of *rpl22* and *rps3* ribosomal protein genes resulted in classification of all of these strains to rpX-A subgroup.

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