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Molecular characterization of 'Candidatus *Phytoplasma mali*' strains in outbreaks of apple proliferation in north eastern Italy, Hungary, and Serbia

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Abstract

During 2005-2008 apple plants of different varieties showing proliferation symptoms were observed in diverse areas of north eastern Italy, Hungary and Serbia. PCR/RFLP analyses showed that all the samples were infected with 'Candidatus *Phytoplasma mali*'. In the 16S plus spacer region two phytoplasma profiles (P-I and P-II) were distinguished. P-I profile was detected in reference strains AP, AT1, AT2, in samples from Serbia, and in the majority of samples from Trentino; the P-II profile was prevalent in samples from Veneto; both profiles were identified in samples from Hungary, in some cases together in single samples. The analyses of rpl22-s3 genes allow the identification, in all the samples showing a P-I profile, the presence of phytoplasmas belonging to rpX-A subgroup, while in the samples showing a P-II profile it was possible to distinguish the other three reported rpX subgroups. In the majority of samples from the Veneto region phytoplasmas belonging to rpX-D subgroup were identified, while rpX-B and rpX-C subgroups were identified only in a few samples from Trentino and Veneto regions, respectively. Further RFLP analyses on AP13/AP10 amplicons differentiate among strains belonging to the rpX-A subgroup: the samples from Serbia show AP profiles, while those from Trentino show AT-2 profiles. In the samples from Hungary the presence of AT1, AT2, and AP profiles was identified.

Keywords: Apple, 'Candidatus *Phytoplasma mali*', phytoplasma strains, PCR/RFLP analyses, epidemiology

Introduction

Apple Proliferation (AP) is a phytoplasma disease found only in Europe, reported for the first time in Veneto in the fifties (Rui, 1950).Its major impact on agriculture is that the infected plants continue to vegetate producing small and unmarketable fruits. Affected apple cultivars are almost all those present in the main apple growing areas of Europe (Refatti and Ciferri, 1954; Bovey, 1961; Brcak et al., 1972; Minoiu and Craciun, 1983; Bliefernicht and Krczal, 1995, Marcone et al., 1996a), like Golden Delicious, and Renetta of Canada grafted on different rootstocks. Recently the disease was also reported in Hungary (Del Serrone et al., 1998) and in Serbia (Duduk et al., 2008). AP is one of the most important phytoplasma diseases of apple, affecting almost all cultivars, reducing size (by about 50 %), weight (by 63-74 %) and quality of fruit, as well as reducing tree vigour and increasing susceptibility of the plants to powdery mildew.

Apple is the main host of 'Candidatus *Phytoplasma mali*', the agent of AP (Seemüller and Schneider, 2004). The disease can be observed on cultivars or on rootstocks, as well as on wild and ornamental Malus. 'Ca. *P. mali*' was found in hazelnut (*Corylus* spp.) (Marcone et al., 1996b), cherry (*Prunus avium*), apricot (*P. armeniaca*) and plum (*P. domestica*) (Mehle et al., 2007).

Two insect species, *Cacopsylla melanoneura* and *C. picta*, have been reported as AP vectors in Italy (Frisinghelli et al., 2000; Tedeschi et al., 2002). Whereas *C. picta* appears to be the main vector in Germany (Jarausch et al., 2003; Mayer at al., 2008). Another leafhopper, *Fieberiella florii* Stal (Homoptera: Cicadellidae), has been implicated as a vector of AP in Germany (Krczal et al., 1989) and recently, also in Italy (Tedeschi and Alma, 2006).

Although in Europe AP disease affects most or all varieties of apple trees, it is caused by a relatively homogeneous pathogen in which strains or subtypes were identified by PCR/RFLP of AP10 and rpS3 gene (Jarausch et al., 2000; Martini et al., 2008).

To further evaluate the possibility to correlate molecular polymorphism with geographical distribution of AP strains three regions of the AP genome; the 16S rDNA, spacer region and beginning of 23S (Khan et al., 2002; Casati et al., 2007), the ribosomal protein (rp) gene sequences *rpl22* and *rps3,* and the nitroreductase gene (Martini et al., 2008; Bertaccini et al., 2008; Jarausch et al., 2000; 2004) were studied in selected strains from different geographic areas.

Material and methods

During 2005-2008 apple plants belonging to diverse varieties and showing proliferation symptoms were observed in different areas of northeastern Italy, Hungary and Serbia. Selected plants were employed for sampling (Table 1) in particular: six from Hungary collected in a small field destroyed by AP, near to the Austrian border (samples H-1 trough H-6). Two from Serbia (RS-135 and RS-151) collected in Bela Crkva where the disease was only observed in a few plants. Fourteen samples were collected in Italy, three in different areas of the Trentino region (I-TN1 to I-TN3) where the disease is epidemically present for more than 15 years, and another 11 samples from Veneto region (Table 1) in areas where the disease was observed in 2007. As reference strains the three AP strains reported in the literature as AP-15, AT-1 and AT-2 were employed; AT-2 was a strain isolated from Golden Delicious apple kindly provided by S. Grando (E. Mach Foundation, S. Michele all'Adige, TN; Italy) while AP-15, AT-1 are from the phytoplasma collection at DiSTA (Bertaccini, 2003).

Tab. 1 Results of RFLP analyses and the diverse phytoplasma genomic sequences to characterize AP strains from different geographic origin.

Strain	Primers F1/B6			Primers AP13/AP10			Primers rpAP15f/rpAP15r	
acronyms	HpaII	FauI	Group	RcaI	HincII	Group	AluI	Group
H-1	-	-	nd	A	A	AT2	A	rpX-A
H-2	A+B	A	PI+PII	-	-	nd	A	rpX-A
H-3	B	A	PII	B	B	AP	A	rpX-A
H-4	A+B	A	PI+PII	B	A	AT1	A	rpX-A
H-5	A+B	A	PI+PII	B	B	AP	A	rpX-A
H-6	A	A	PI	B	B	AP	A	rpX-A
RS-135	A	A	PI	B	B	AP	A	rpX-A
RS-151	A	A	PI	B	B	AP	A	rpX-A
I-VE11	B	A	PII	-	-	nd	C	rpX-C
I-VE12	A	A	PI	-	-	nd	A	rpX-A
I-VE14	A	A	PI	A	A	AT2	A	rpX-A
I-VE16	B	A	PII	B	A	AT1	C	rpX-C
I-VE22	B	A	PII	-	-	nd	D	rpX-D
I-VE27	B	A	PII	B	A	AT1	D	rpX-D
I-VE28	B	A	PII	-	-	nd	D	rpX-D
I-VE30	B	A	PII	-	-	nd	D	rpX-D
I-VE31	B	A	PII	-	-	nd	D	rpX-D
I-VE32	B	A	PII	-	-	nd	D	rpX-D
I-VE34	B	A	PII	-	-	nd	D	rpX-D
I-TN1	A	A	PI	A	A	AT2	A	rpX-A
I-TN2	A	A	PI	-	-	nd	A	rpX-A
I-TN3	B	A	PII	-	B	nd	B	rpX-B
AP-15	A	A	PI	B	B	AP	A	rpX-A
AT-1	A	A	PI	B	A	AT1	B	rpX-B
AT-2	A	A	PI	A	A	AT2	A	rpX-A

nd, group not determined

Leaves and young apple shoots were collected from June to October and nucleic acid was extracted from fresh leaf midribs and phloem by a chloroform/phenol procedure (Prince et al., 1993) or by a CTAB procedure (Angelini et al., 2001).

16S ribosomal DNA plus spacer region: Direct PCR amplification with P1/P7 universal phytoplasma primer pair (Deng and Hiruki, 1991; Schneider et al., 1995), amplifying 16S rDNA, the spacer region between 16S and 23S rDNA and the 5' portion of 23S rDNA, was performed. Nested PCR amplification was carried out on P1/P7 amplicons diluted 1:30 in sterile distilled water with R16F2/R2 (Lee et al., 1995) and F1/B6 (Davis and Lee, 1993; Padovan et al., 1995) primer pairs. Each 25 µl PCR reaction mix contained 2.5 µl 10X PCR buffer, 0.8 U of *Taq* polymerase, 0.2 mM dNTPs, 1.5 mM MgCl2 and 0.4 µM each primer. PCR conditions were: 35 cycles (Biometra, Uno Thermoblock, Gottingen, Germany as thermal cycler), 1 min (2 min for the first cycle) denaturation step at 94 °C, 2 min for annealing at 50 °C and 3 min (10 min for the last cycle) for primer extension at 72 °C. Samples with the reaction mixture lacking a DNA template were included in each experiment as negative controls. PCR products were subjected to electrophoresis in a 1 % agarose gel and visualized by staining with ethidium bromide and UV illumination. Three µl of PCR product was digested using *Ssp*I and *Rsa*I restriction enzymes for R16F2/R16R2 amplicons following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). Further strain characterization was carried out using RFLP analyses with

*Hpa*II and *Fau*I restriction enzymes on F1/B6 amplicons. The comparison of restriction patterns obtained with those of control strains was carried out after electrophoresis through a 5 % polyacrylamide gel in 1X TBE buffer followed by staining with ethidium bromide and visualization under an UV transilluminator.

Ribosomal protein (rp) gene sequences *rpl22* and *rps3*: *RpS3* gene was amplified in direct PCR reactions using primers rpAP15f/rpAP15r. This detection method is specific for 'Ca. *P. mali*' and can distinguish up to four different RFLP-subtypes (rpX-A, B, C and D) (Martini et al., 2008). PCR conditions were as follows: initial denaturation for 2 min at 94 °C; 40 cycles (1 min at 94 °C, 45 s at 55 °C and 90 s at 72 °C); final extension for 8 min at 72 °C. Negative control and visualization of results were carried out as described above. Three µl of PCR products were digested using *Alu*I restriction enzyme following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction patterns were then observed as described above.

Non ribosomal DNA fragment: For the amplification of the non-ribosomal DNA fragment (nitroreductase–like gene, Jarausch et al., 2000) a semi-nested PCR assay was employed using as a template the PCR product of the initial amplification diluted 1:30 with primers AP8/AP10 followed by primers AP13/AP10. The PCR cycle was as follows: 95°C for 1 min, followed by 45 cycles with 94 °C for 1 min, 53 °C for 45 sec, 72 °C for 1.30 min, the extension of last cycle was at 72 °C for 8 min. Three µl of PCR products were digested using enzymes *Rca*I and *Hinc*I following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction patterns were then compared as described above.

Results

Nested PCR amplification with R16F2/R2 primers followed by RFLP analyses with *Rsa*I and *Ssp*I allow to confirm that all samples analyzed were infected with '*Ca*. P. mali' (data not shown). Also primers F1/B6 amplified all tested samples in nested PCR assays and RFLP analyses on these amplicons distinguished two phytoplasma profiles (P-I and P-II) (Figure 1). P-I profile was detected in reference strains AP, AT1, AT2, in samples from Serbia, in one sample from Hungary, in two out of the three samples from Trentino, and in two out of the 11 samples from Veneto. The P-II profile was detected in the majority of samples from Veneto and in samples from Hungary, however the majority of the latter samples show both profiles together (Table 1).

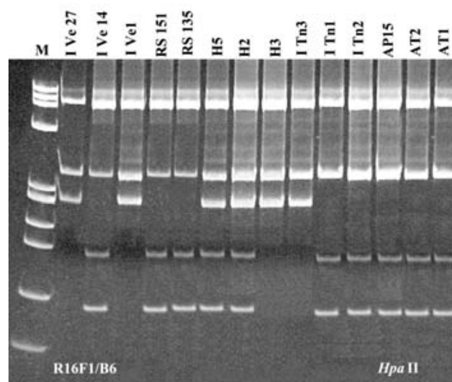

Fig. 1 Polyacrylamide gel showing RFLP profiles with *Hpa*II of nested-PCR amplicons amplified from selected samples from Serbia, Hungary and Italy (see list in Table 1) with primers F1/B6. Control samples AP15, AT1 and AT2, apple proliferation strains from Germany and from Italy (16SrX-A). M, marker phiX174 *Hae*III digested, fragment sizes in base pairs (top to bottom): 1,353; 1078; 872; 603; 310; 281; 271; 234; and 194.

The RFLP analyses of *rpl22-s3* genes allowed identification, in all the samples showing P-I profile the presence, of phytoplasmas belonging to rpX-A subgroup, while in samples showing P-II profile it was possible to distinguish the other described rp subgroups. The rpX-B and rpX-C subgroups were identified in one of the samples from Trentino and in the two samples from Veneto respectively, while in the majority of samples from Veneto subgroup rpX-D was identified (Figure 2, and Table 1).

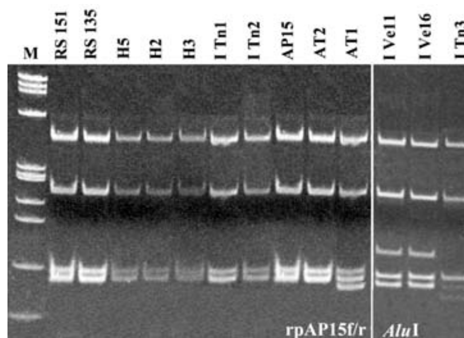

Fig. 2 Polyacrylamide gel electrophoresis of RFLP results from selected positive samples (see Table 1) obtained with *Alu*I restriction enzyme on amplicons from rp primers. Control samples AP15, AT1 and AT2, apple proliferation strains from Germany and from Italy (16SrX-A). M, marker phiX174 *Hae*III digested, fragment sizes in base pairs (top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, and 194.

Further RFLP characterization on AP13/AP10 amplicons differentiates among strains belonging to rpX-A subgroup: the two samples from Serbia showed AP profiles, while those from Trentino and the only positive among the two samples from Veneto showed AT-2 profiles. In the samples from Hungary the presence of AT1, AT2, and AP profiles was confirmed without the rp strain being identified. (Table 1).

Discussion

The combined use of the three molecular markers employed in this study allows the differentiation of '*Ca*. P. mali' strains according to geographical and, in some cases, also with epidemic distribution. The polymorphism detected in the 16S ribosomal region plus spacer region was shown to be related to a restriction site located inside the spacer region in agreement with a recent finding (Casati et al., 2009), therefore not relevant to phytoplasma classification but reliable for AP strain characterization. It was shown that in the Trentino areas where the disease is at epidemic levels, these three molecular markers show a high homogeneity in their RFLP profiles (Bertaccini et al., 2008) indicating a possible link of the epidemic to one phytoplasma strain. Strain variability was detected in samples from Veneto, and the presence of mixed AP strains was observed in the Hungarian samples. Strains showing rpX-D profile were found in several apple growing areas of Veneto region, surrounding areas where the disease is starting to show epidemic tendencies. The combined use of these molecular markers allows differentiating 'Ca. *P. mali*' strains according to their geographical and epidemic distribution.

In several orchards of Veneto vector monitoring by yellow sticky traps was carried out and *C. melanoneura* was consistently detected, while *F. florii* was erratically found, and only one specimen of *C. picta* was captured. Work is in progress to further detect and differentiate these phytoplasma strains in insect vectors and alternative host plants and relate these findings to the epidemiology of the phytoplasma diseases.

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