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Molecular typing of '*Candidatus* Phytoplasma mali' and epidemic history tracing by a combined T-RFLP/VNTR analysis approach

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Abstract Apple proliferation caused by '*Candidatus* Phytoplasma mali' is a disease of apple trees gaining increasing importance in Europe. The present study describes a high-throughput method for simultaneous typing of '*Ca.* P. mali' at two genetic loci. This novel approach combines terminal restriction fragment length polymorphism (T-RFLP) analysis of a putative rhodanese-like protein gene and the analysis of the variable number of tandem repeats (VNTR) of the ribosomal protein L22 gene. The typing approach was applied to analyse a collection of DNA isolates from 310 apple trees tested positive for '*Ca*. P. mali'. Samples were taken between 2002 and 2010 in South Tyrol (Northern Italy). In addition, 15 samples of *Cacopsylla melanoneura* and 19 of *C. picta* were typed. Seven combined genetic profiles were found in the samples of infected apple trees: AT-2/rpX-A (81.0%), AT-1/rpX-D (8.4%), AT-1/rpX-E (4.2%), AT-1/rpX-A (3.2%), AT-1/rpX-B (1.6%), AT-1/rpX-C (1.0%) and AP/rpX-A (0.3%), and one mixed infection AP + AT-1/rpX-A + rpX-D (0.3%). Subtype rpX-E was discovered for the first time. In *C. melanoneura* samples the most frequent subtype was AT-1/rpX-E, followed by AT-1/rpX-D and AT-1/rpX-C. All *C. picta* samples displayed subtype AT-2/rpX-A. Analysis of the temporal distribution of subtype frequencies in apple trees revealed that exclusively subtype AT-1 in combination with four rpX subtypes was present in South Tyrol in the period from 2002 to 2004. From 2006 onwards subtype AT-2/rpX-A became dominant with an average frequency of 90%. The data obtained suggest that there may be a co-adaptation of particular '*Ca*. P. mali' subtypes with different insect vector species.

Keywords *Cacopsylla melanoneura* · *Cacopsylla picta* · *Malus domestica* · Molecular genetic typing · Rhodanese-like protein gene · Ribosomal protein L22 gene

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Introduction

Apple proliferation is an apple tree disease of increasing importance in Central and Southern Europe. The disease manifests in a variety of symptoms including witches' brooms, enlarged stipules, growth suppression, foliar reddening as well as undersized fruit of poor quality and yield losses (Seemüller and Schneider 2007). The pathogen '*Candidatus* Phytoplasma mali' is transmitted to apple trees through grafting (Kartte and Seemüller 1988), by the insect vectors *Cacopsylla picta* and *C. melanoneura* (Frisinghelli et al. 2000; Tedeschi and Alma 2004) and/or through natural root bridges (Baric et al. 2008a; Ciccotti et al. 2008).

Phytoplasmas are bacteria of the class Mollicutes without cell walls that provoke diseases in several

hundreds of plant species (Lee et al. 2000). They are obligate intracellular parasites restricted to the phloem sieve elements of their host plants. In addition, these plant pathogens can survive and multiply in different tissues of insect vectors (Christensen et al. 2005). Since phytoplasmas cannot be cultivated in vitro, molecular analysis methods are compulsory for classification and diagnosis, and have notably contributed to a better understanding of the epidemiology of phytoplasma diseases (Lee et al. 2000; Seemüller and Schneider 2004).

Differentiation of '*Ca.* P. mali' subtypes has been based on PCR-RFLP analysis (Jarausch et al. 2000; Martini et al. 2008; Casati et al. 2010), SSCP analysis (Schneider and Seemüller 2009) and/or DNA sequence analysis (Schneider and Seemüller 2009; Casati et al. 2010; Seemüller et al. 2010; Danet et al. 2011) of different genomic regions. The application of these methods has helped to reveal the genetic diversity of '*Ca.* P. mali' in several geographic areas (Jarausch et al. 2000; Cainelli et al. 2004; Martini et al. 2008; Schneider and Seemüller 2009; Casati et al. 2010; Danet et al. 2011) and to gain insight into the pathological relevance of different strains (Seemüller and Schneider 2007; Seemüller et al. 2010). These analysis procedures, however, involve several time-consuming and labour-intensive steps, making high-throughput screening difficult. Fast and reliable determination of different '*Ca.* P. mali' subtypes would be an important tool to analyse the epidemiology and spread of this pathogen. For this reason, a fast, sensitive and easy-to-apply method for simultaneous typing of '*Ca.* P. mali' at two genetic loci was devised. This novel approach combines two methods suitable for analysis with an automated sequencing system: terminal restriction fragment length polymorphism (T-RFLP; Liu et al. 1997) and the analysis of the variable number of tandem repeats (VNTR; Nakamura et al. 1987). The locus analysed by T-RFLP is a putative rhodanese-like protein gene (Bonnet et al. 1990; Kube et al. 2008; GenBank accession number: CU469464), termed PR3 by Jarausch et al. (2000), which shows two point mutations that allow differentiation of three different subtypes of '*Ca.* P. mali': AT-1, AT-2 and AP (Jarausch et al. 2000). The VNTR analysis is based on the ribosomal protein L22 gene (*rpl22*) displaying a variable copy number of a 12-nucleotide repeat sequence and permitting discrimination of four different subtypes, rpX-A, rpX-B, rpX-C and rpX-D (Martini et al. 2008).

The newly devised typing approach was used to analyse a collection of DNA isolates from apple trees and two psyllid species tested positive for '*Ca.* P. mali'. The samples were obtained between 2002 and 2010 in South Tyrol (Northern Italy), the largest interconnected apple growing area in Europe, covering more than 18,000 ha of intensive orchards. In this area, apple proliferation has sporadically been noticed since the 1960s but initially remained restricted to vigorous apple trees on seedling rootstocks (reviewed in Baric et al. 2010a). In 1998 the disease first appeared and started to spread in high-density orchards, reaching a maximum in 2006 when an average of 1% of the apple trees planted in South Tyrol (approximately half a million trees) was affected. Using the molecular typing approach, we sought to trace back the spread of apple proliferation in this geographic region.

Materials and methods

Sample material

The present study includes a collection of DNA isolates obtained from a total of 310 '*Ca.* P. mali'-infected apple trees sampled between 2002 and 2010. Samples were collected exclusively from commercial orchards in the apple growing region of the North Italian province of South Tyrol (Table 1). In 2002 and 2003 samples were taken by collaborators of the South Tyrolean Extension Service from orchards affected by symptoms in order to confirm the presence of '*Ca.* P. mali'. An exception in this period was the orchard from Ritten/Renon (RI), which was part of a monitoring study due to a higher incidence of apple proliferation-diseased trees observed in this particular geographic area. Between 2006 and 2010 orchards with ≥9 trees analysed were sampled because of an acute disease outbreak. In the Eisack/Isarco river valley, an area characterised by very low infection rates with '*Ca.* P. mali' and the absence of *C. picta*, symptomatic trees were actively searched and sampled in 2009 and 2010 (sampling sites AB, EL, KR, NA and VA). Total genomic DNA was extracted from dissected root phloem using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The presence of '*Ca.* P. mali' was confirmed by a highly specific and sensitive real-time PCR assay (Baric and Dalla Via 2004; Baric et al. 2006).

Table 1 Samples of apple trees from South Tyrol infected with '*Candidatus* Phytoplasma mali' included in the molecular typing study

Sampling site	Abbreviation	Cultivar	No. of orchards	No. of trees analysed	Age of trees (years)	Year of sampling
Brixen/Bressanone	BR	'Golden Delicious'	1	1[a]	n.d.	2002
Glurns/Glorenza	GL	'Golden Delicious'	2	6[a]	n.d.	2002
Latsch/Laces	LT	'Golden Delicious'	2	2[a]	n.d.	2002
Bozen/Bolzano	BO	'Golden Delicious', 'Red Delicious'	3	3[a]	n.d.	2003
Laimburg	LB	'Cripps Pink'	1	1[a]	n.d.	2003
Prad/Prato	PR	'Golden Delicious'	1	1[a]	n.d.	2003
Vilpian/Vilpiano	VI	'Braeburn', 'Jonagold'	2	2[a]	n.d.	2003
Ritten/Renon	RI	'Golden Delicious'	1	15[b]	6–8	2002–2004
Lana 1	LA1	'Red Delicious'	1	34[c]	1–2	2006–2007
Neumarkt/Egna	NE	'Red Delicious'	1	53[d]	24	2006
Tscherms/Cermes	TS	'Golden Delicious'	1	9[a]	16	2006
Allitz/Alliz	AL	'Golden Delicious'	1	22[d]	6	2007
Laimburg	LB	'Gala'	1	10[d]	7	2007
Lana 2	LA2	'Golden Delicious'	1	13[a]	12	2007
Tramin/Termeno	TR	'Golden Delicious'	1	13[d]	22	2007
Völlan/Foiana	VO	'Red Delicious'	1	23[d]	4	2007
Oberplars/Plars di sopra	OB	'Golden Delicious'	1	36[e]	5–7	2007–2009
Kuens/Caines	KU	'Golden Delicious', 'Red Delicious'	2	9[a]	14 23	2008
Albeins/Albes	AB	'Golden Delicious'	1	1[a]	12	2009
Lana 3	LA3	'Golden Delicious'	1	39[a]	12	2009
Natz/Naz	NA	'Golden Delicious'	2	2[a]	20, >12	2009, 2010
Elvas	EL	'Golden Delicious'	1	2[a]	17	2010
Kranebitten/Costa d'Elvas	KR	'Braeburn'	1	1[a]	10	2010
Marling/Marlengo	MA	'Golden Delicious'	1	10[a]	11	2010
Vahrn/Varna	VA	'Golden Delicious'	2	2[a]	15, 21	2010
Total			33	310		

n.d. no date

[a] samples analysed using exclusively the new T-RFLP/VNTR typing approach

[b] 7 samples analysed with both conventional PCR-RFLP and new T-RFLP/VNTR typing approach; 8 samples analysed with the new typing approach only

[c] 25 samples analysed with both conventional PCR-RFLP and new T-RFLP/VNTR typing approach; 9 samples analysed with the new typing approach only

[d] all samples analysed with both conventional PCR-RFLP and new T-RFLP/VNTR typing approach

[e] 18 samples analysed with both conventional PCR-RFLP and new T-RFLP/VNTR typing approach; 18 samples analysed with the new typing approach only

In addition, a smaller set of DNA isolates from *C. melanoneura* and *C. picta* from South Tyrol, tested positive for '*Ca.* P. mali', were subjected to molecular genetic typing at two loci. The 15 samples of *C. melanoneura* were obtained in 2002, 2003 and 2006 (Wolf et al. 2003; Baric et al. 2010b), while the 19 samples of *C. picta* were collected in 2006 (Baric et al. 2010b). In 2002, specimens of *C. melanoneura* were collected between February 18 and March 18 in orchards located in Dorf Tirol/Tirolo, Ritten/Renon, Glurns/Glorenza, Kranebitten/Costa d'Elvas, Natz/Naz and Lana; in 2003, they were collected on March

20 and April 02 in Fragsburg and Schenna/Scena; and in 2006, on May 22 in Naturns/Naturno. Specimens of *C. picta* were collected between April 14 and May 03, 2006 in orchards located in Tisens/Tesimo, Nals/Nalles, Lana, Tscherms/Cermes and Naturns/Naturno (Baric et al. 2010b).

PCR-RFLP analysis

Two distinct genomic regions were analysed to differentiate subtypes of '*Ca*. P. mali'. A 776 bp fragment containing parts of a putative rhodanese-like protein gene was amplified using primers AP10 and AP13 (Jarausch et al. 2000). The 20 µl reaction volume contained 1 µM of each primer, 200 µM dNTPs, 1 × GeneAmp PCR Buffer II (10 mM Tris–HCl, pH 8.3, 50 mM KCl; Applied Biosystems Foster City, CA, USA), 2 mM $MgCl_2$ (Applied Biosystems), 1 Unit AmpliTaq Gold Polymerase (Applied Biosystems) and approximately 20 ng template DNA. PCR reactions consisted of 35 amplification cycles performed in a GeneAmp PCR System 2700 (Applied Biosystems), preceded by a 10 min denaturation step at 94°C and followed by an elongation step for 5 min at 72°C. Cycle conditions were as follows: 30 s at 94°C, 30 s at 63°C and 60 s at 72°C.

An approximately 1 kb fragment containing parts of ribosomal protein genes *rpl*22 and *rps*3 was amplified in a nested PCR (Martini et al. 2008). Primers rpAP15f2 and rp(I)R1A were used for the first round of PCR, followed by the amplification of 50-fold diluted PCR products with primers rpAP15f and rpAP15r. The reaction setup was the same as described above. Both PCR reactions were carried out for 35 cycles after an initial denaturation for 10 min at 94°C, followed by a final elongation for 8 min at 72°C. Cycles consisted of 30 s at 94°C, 30 s at 54°C for primers rpAP15f2/rp(I)R1A and 55°C for primers rpAP15f/rpAP15r, respectively, and 90 s at 72°C. PCR products were separated and visualised on 1.5% SeaKem LE agarose gels (Lonza Group Ltd, Basel, Switzerland) stained with ethidium bromide.

PCR products containing the putative rhodanese-like protein gene were aliquoted in two 5-µl portions and digested with 5 U of the restriction enzymes *BspH*I (an *Rca*I isoschizomere) and *Hinc*II, respectively, according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). 8 µl of the nested PCR product containing parts of the ribosomal protein gene were digested with the restriction enzyme *Alu*I for 2 h at 37°C (New England Biolabs). Restriction enzyme digests (8 µl) were analysed by agarose gel electrophoresis and ethidium bromide staining using a mixture of 1.5% SeaKem LE agarose and 1.5% MetaPhor agarose (both Lonza Group Ltd).

T-RFLP analysis

The T-RFLP method allows detection of different sizes of fluorescently labelled terminal fragments generated by restriction digest of PCR-amplified variants of a particular genomic region using an automated DNA sequencer (Liu et al. 1997). This sensitive and high-throughput method was adapted to distinguish the three subtypes AT-1, AT-2 and AP (Jarausch et al. 2000) based on the analysis of the rhodanese-like protein gene (PR3). The sequence of the corresponding gene fragment was obtained from GenBank (accession number L22217) and Primer Express Software (Applied Biosystems) was used to design specific primers which flanked the two point mutations defining the three subtypes (Fig. 1a). Both the forward primer APnrpTYP-F (5′- GTA CAC AAA AAT TAA AAA TC -3′) and the reverse primer APnrpTYP-R (5′- ATC TAT ATC TTT ATC TAG TTT -3′) were fluorescently labelled at the 5′-end with D4-PA WellRED Dye (Sigma-Proligo, Boulder, CO, USA). The 283 bp target sequence was PCR-amplified in a 10 µl reaction mixture containing 200 nM of each primer, 200 µM of each dNTP, 1 × GeneAmp PCR Buffer II, 2 mM $MgCl_2$ and 0.5 U AmpliTaq Gold polymerase (all Applied Biosystems) as well as approximately 20 ng genomic DNA. The PCR reaction was carried out under the following conditions: 10 min initial denaturation at 94°C, followed by 35 cycles consisting of 30 s at 94°C, 30 s at 45°C and 60 s at 72°C, and a final extension of 7 min at 72°C. Immediately after amplification, 6.3 U of each of the restriction enzymes *BspH*I and *Hinc*II and 1 × NEB4 buffer (New England Biolabs) were added to the PCR product volume and incubated at 37°C for 2 h.

Analysis of the Variable Number of Tandem Repeats (VNTR)

Martini et al. (2008) described a polymorphism distinguishing different '*Ca.* P. mali' subtypes based on the presence of a variable number of tandem

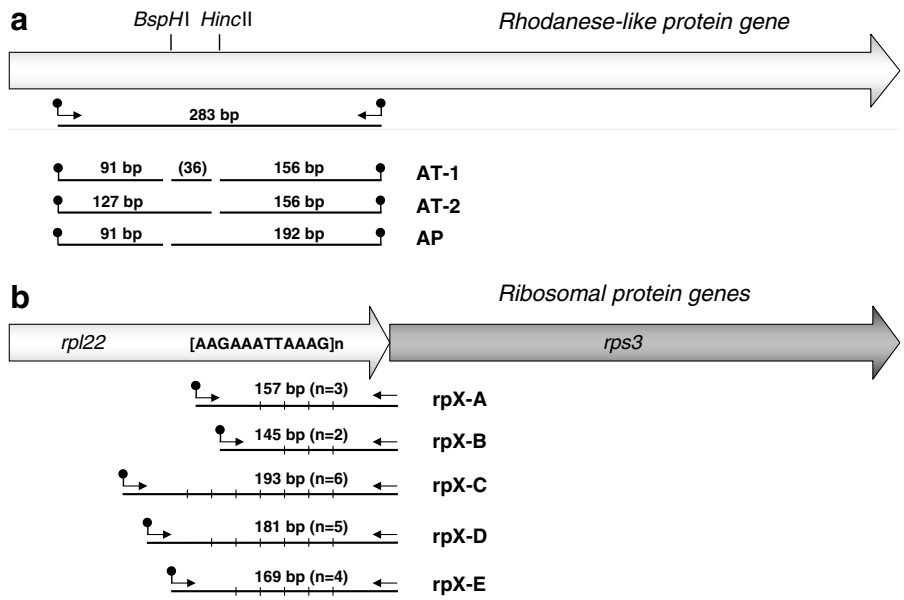

Fig. 1 Principle of the T-RFLP and VNTR analyses developed for the typing of '*Ca.* P. mali'. **a** Distinction of the three subtypes AT-1, AT-2 and AP is based on two point mutations at the restriction sites of *BspH*1 and *Hinc*II within the rhodanese-like protein gene (PR3) (Jarausch et al. 2000). The PCR product (*solid line*; 283 bp) is amplified using fluorescently labelled forward and reverse primers (*arrows* marked with *filled circles*). The resulting terminal restriction fragments characterising the '*Ca.* P. mali' subtypes AT1, AT2 and AP are shown as *solid lines*, the position of the fluorophore is indicated by *filled circles*. **b** VNTR analysis is based on the variable copy number of a 12 bp motif within the ribosomal protein gene *rpl22* (Martini et al. 2008). The forward primer is fluorescently labelled (*arrows* marked with *filled circles*) whereas the unlabelled reverse primer (*arrow*) is spanning the boundary between the *rpl22* gene and the adjacent *rps3* gene. The fragment lengths of the five known subtypes and the corresponding number of the repeat units are indicated

repeats (VNTR) of a 12 bp sequence within the ribosomal protein gene *rpl22* (in the following referred to as rpX). As a faster and more sensitive alternative to the nested PCR-RFLP approach, we designed new primers flanking the region with the repeating unit and delivering shorter PCR fragments (<200 bp) which can be separated and visualised on an automated DNA sequencer (Fig. 1b). Primers were designed based on a 1,291 bp sequence obtained from GenBank (accession number EF193368) and the forward primer APrpsTYP-F (5′-GTT TCC GCG AGC TAA AGG T-3′) was fluorescently labelled at the 5′-end with D3-PA WellRED Dye, while the reverse primer APrpsTYP-R (5′- GGA TTA GAT TTT TGT CCC ATG AA-3′) was unlabelled. Reaction mixtures (10 μl) contained 200 nM of each primer, 200 μM of each dNTP, 1 × GeneAmp PCR Buffer II, 2 mM $MgCl_2$ and 0.5 U AmpliTaq Gold polymerase (Applied Biosystems) and approximately 20 ng genomic DNA. PCR conditions were as follows: initial denaturation for 10 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at 57°C and 30 s at 72°C, and a final extension for 7 min at 72°C.

Simultaneous separation and visualisation of fluorescently labelled T-RFLP and VNTR fragments

Since T-RFLP and VNTR fragments were labelled with different fluorophores, it was possible to analyse both genomic regions of each sample in the same lane of an automated DNA sequencer CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) (Fig. 2). 2.5 μl of the restriction digest of the APnrpTYP-F/APnrpTYP-R PCR product and 1 μl of the APrpsTYP-F/APrpsTYP-R PCR product were mixed with 40 μl Sample Loading Solution and 0.4 μl of the internal Size Standard 400 (both Beckman Coulter). Capillary electrophoresis was run on the CEQ 8000 Genetic Analysis System (Beckman Coulter) for 35 min at 7.5 kV. Sizing of the fluorescently labelled fragments was performed relative to the internal

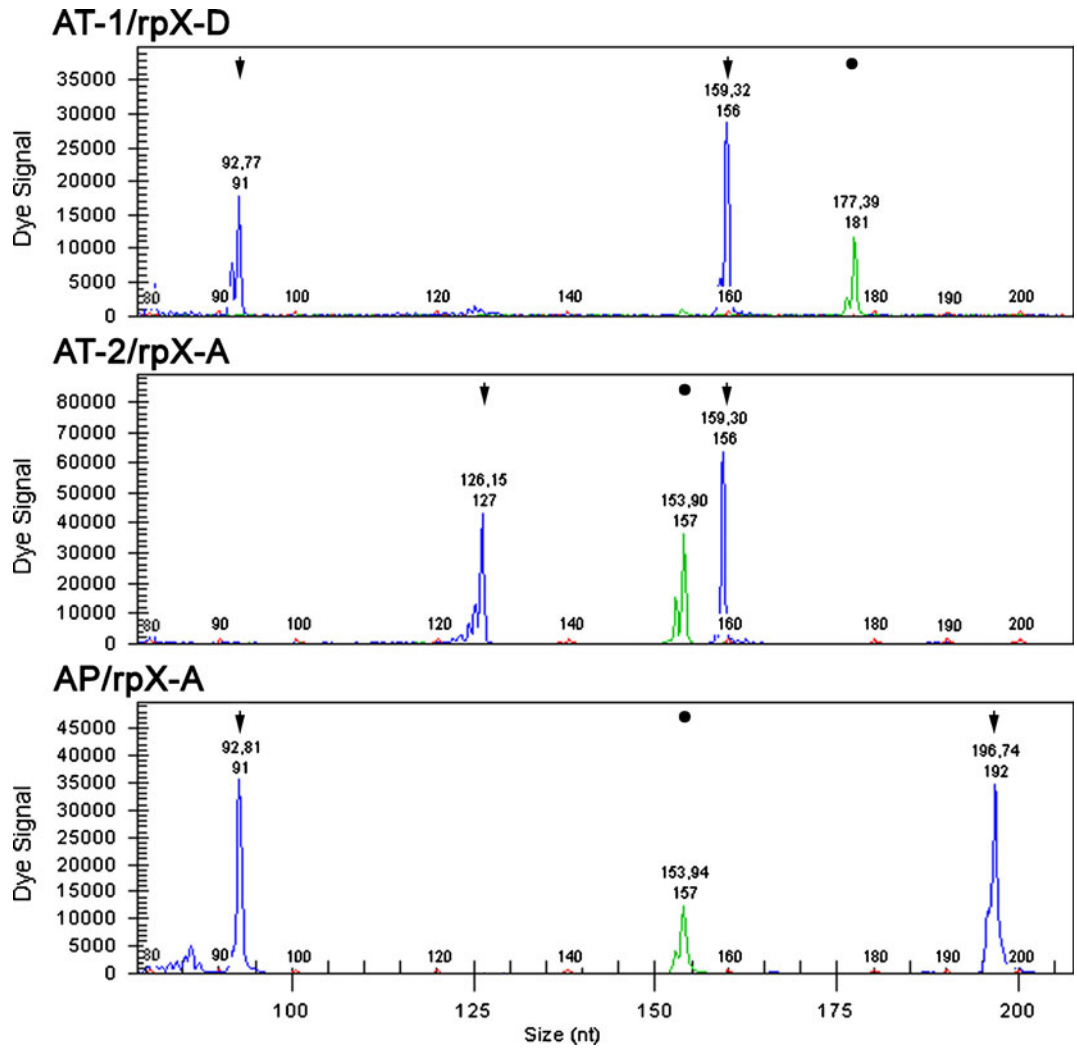

Fig. 2 Molecular profiles obtained by combining T-RFLP analysis of the putative rhodanese-like protein gene (*blue*; marked with *arrows* at the top of each electropherogram) and VNTR analysis of the ribosomal protein L22 gene (*green*; marked with *black dots* at the top of each electropherogram) for three reference strains, AT-1/rpX-D, AT-2/rpX-A and AP/rpX-A. Numbers on the top of the peaks refer to the estimated fragment length (above) and the expected fragment length (below) in bp. The small peaks on the bottom labelled with integer values represent the internal size standard

molecular standard using the CEQ 8000 Fragment Analysis Software of the same manufacturer.

Results

Performance evaluation of T-RFLP and VNTR analyses

To test the newly developed typing approach, reference DNA samples of the '*Ca*. P. mali' subtypes AT-1/rpX-C, AT-1/rpX-D, AT-2/rpX-A and AP/rpX-A were analysed, which were kindly provided by M. Martini (University of Udine, Italy). A profile with three different peaks was obtained for each sample: two peaks labelled with D4 (blue) from the T-RFLP analysis and one peak labelled with D3 (green) relative to the VNTR analysis (Fig. 2). The estimated fragment lengths differed slightly from the expected values but were stable among samples. T-RFLP analysis resulted in the fragment combinations 93+159 bp for subtype AT-1, 126+159 bp for subtype AT-2 and 93+197 bp for subtype AP (values rounded off to whole numbers). Estimated fragment lengths of subtypes rpX-A, rpX-C

and rpX-D were 154 bp, 189 bp and 177 bp, instead of the expected 157 bp, 193 bp and 181 bp. The estimated fragment lengths of subtypes rpX-B and rpX-E found in samples from South Tyrol were 142 bp and 166 bp (instead of the expected 145 bp and 169 bp) (see also Fig. 1).

In a second step, the sensitivity and specificity of the newly developed approach was determined by analysing 171 DNA isolates from '*Ca*. P. mali'-positive apple trees with the conventional PCR-RFLP procedure and the combined T-RFLP and VNTR approach in parallel. The ribosomal protein gene could be successfully typed in all samples using both, the PCR-RFLP and the VNTR analysis. For the rhodanese-like protein gene, instead, this was possible for 162 of the 171 samples using PCR-RFLP and for all samples applying the T-RFLP approach (Table 2). Results obtained by the two procedures were concordant in all cases except one in which the T-RFLP analysis revealed the presence of a mixed infection (AP/AT-1), whereas PCR-RFLP was able to identify only one subtype (AP). With respect to the ribosomal protein subtypes, both the conventional and the new approach detected a mixed infection (rpX-A/rpX-D) in the same sample (Table 2). Specificity of the new primer sets for the T-RFLP and VNTR analysis was not tested on non-infected samples as the typing procedure was intended to be used for DNA isolates in which the presence of '*Ca*. P. mali' has previously been confirmed by an adequate diagnostic method.

Table 2 Comparison of '*Ca.* P. mali' typing results obtained by conventional PCR-RFLP and the newly developed T-RFLP and VNTR analyses

Analysed genomic region	Subtype	n samples	
		PCR-RFLP	T-RFLP/VNTR analysis
Rhodanese-like protein gene	AT-1	13	16
	AT-2	148	154
	AP	1	–
	AT-1/AP (mixed)	–	1
	Total	162	171
Ribosomal protein gene	rpX-A	156	156
	rpX-B	–	–
	rpX-C	3	3
	rpX-D	9	9
	rpX-E	2	2
	rpX-A/rpX-D (mixed)	1	1
	Total	171	171

Comparison of the conventional typing process using PCR-RFLP with the newly developed combined T-RFLP/VNTR approach resulted in a considerable reduction of handling steps. Moreover, data analysis was remarkably facilitated due to the simultaneous electrophoresis of both T-RFLP and VNTR fragments and the acquisition of numeric data that can easily be processed with a computer (Fig. 2).

Identification of a new subtype based on the variable number of tandem repeats within the ribosomal protein gene *rpl22*

In 13 samples of apple trees (and also 11 of *C. melanoneura*), a previously unknown profile was determined by analysing the variable number of tandem repeats within the ribosomal protein gene *rpl22*. The fragment size estimated by VNTR analysis was 166 bp, corresponding to a calculated fragment size of 169 bp when four copies of the 12 bp repeat motif were present (Fig. 1). The existence of the new subtype was confirmed by conventional PCR-RFLP. In addition, the amplified DNA fragment of the new rpX subtype was cloned into the pGEM-T Easy plasmid vector (Promega, Madison, WI, USA) and sequenced according to the procedure described in Baric et al. (2008b). Finally, sequence analysis confirmed the presence of four copies of the 12 bp repeat in the new subtype denominated rpX-E following the nomenclature of Martini et al. (2008). The nucleotide sequence of the newly discovered rpX subtype of '*Ca*. P. mali' was deposited in GenBank under the accession number HQ702275.

Frequency of '*Ca.* P. mali' subtypes in samples from South Tyrol

After the successful evaluation of the new typing procedure on a subset of 171 '*Ca*. P. mali'-positive samples, an additional set of DNA isolates from 139 infected apple trees was analysed using only T-RFLP and VNTR analysis, resulting in a total of 310 '*Ca*. P. mali'-infected apple trees for which the molecular genetic profile was determined at two loci (Table 1). The combined T-RFLP/VNTR approach was also

used for typing 34 psyllid samples previously tested positive for '*Ca*. P. mali'.

Analysis of the two variable sites in the rhodanese-like protein gene of '*Ca.* P. mali' revealed that the most prevalent subtype in South Tyrol was AT-2 with 251 infected plants, followed by AT-1 found in 57 infected apple trees. Subtype AP was found in one plant and in another plant a mixed infection with subtypes AP and AT-1 was detected. VNTR analysis of the ribosomal protein gene confirmed the presence of the four previously described rpX subtypes as well as the newly discovered subtype rpX-E in South Tyrol. rpX-A was by far the most frequent subtype, present in 262 out of 310 phytoplasma-positive apple trees, followed by rpX-D (26), rpX-E (13), rpX-B (5), rpX-C (3) and rpX-A + rpX-D (1).

Combining the molecular genetic profiles obtained at two loci distinguished the following subtypes in infected apple trees: AT-2/rpX-A (81.0%), AT-1/rpX-D (8.4%), AT-1/rpX-E (4.2%), AT-1/rpX-A (3.2%), AT-1/rpX-B (1.6%), AT-1/rpX-C (1.0%), AP/rpX-A (0.3%) and one mixed infection with AP + AT-1/rpX-A + rpX-D (0.3%). The comparison of the frequencies of subtype combinations showed that subtypes AT-2 and AP were exclusively associated with rpX-A. Subtype AT-1, although less frequent than AT-2, was found in combination with all rpX-subtypes (Table 3).

In *C. melanoneura* samples the most frequent subtype was AT-1/rpX-E, followed by AT-1/rpX-D and AT-1/rpX-C. All *C. picta* samples typed at the two genetic loci displayed subtype AT-2/rpX-A (Table 4).

Table 4 Occurrence of '*Ca.* P. mali' subtypes in infected psyllids sampled in South Tyrol between 2002 and 2006

Subtype	*C. melanoneura*			*C. picta*
	2002[a]	2003	2006[b]	2006[b]
AT-1/rpX-C		1/5		
AT-1/rpX-D	1/9	2/5		
AT-1/rpX-E	8/9	1/5	1/1	
AT-1/rpX-DE		1/5		
AT-2/rpX-A				19/19

[a] Wolf et al. (2003); samples were kindly provided by C. Poggi Pollini (University of Bologna, Italy)

[b] Baric et al. (2010b)

Temporal distribution of '*Ca.* P. mali' subtypes in apple tree samples

This study included apple tree samples collected between 2002 and 2010. Analysis of the temporal distribution of subtype frequencies in the 31 samples collected in the period from 2002 to 2004 revealed that only subtype AT-1 in combination with four rpX subtypes was distributed in South Tyrol (Fig. 3a). From 2006 onwards, subtype AT-2/rpX-A became dominant with an average frequency of 90%. Subtype AP/rpX-A was found in one sample in 2010 and in another sample in 2007 in the context of a mixed infection (subtype AT-1 + AP/rpX-A + rpX-D) (Fig. 3b, Table 3).

Table 3 Frequency of combined '*Ca.* P. mali' subtypes in infected apple trees sampled in South Tyrol between 2002 and 2010

Subtype	Year of sampling								Period	
	2002	2003	2004	2006	2007	2008	2009	2010	2002–2004	2006–2010
AT-1/rpX-A	0.143	0.100	0.143		0.009	0.067	0.034	0.125	0.129	0.021
AT-1/rpX-B	0.286						0.017		0.129	0.004
AT-1/rpX-C				0.037						0.011
AT-1/rpX-D	0.428	0.600	0.714	0.037	0.009		0.034	0.187	0.548	0.032
AT-1/rpX-E	0.143	0.300	0.143	0.037	0.009	0.200			0.194	0.025
AT-2/rpX-A				0.889	0.963	0.733	0.915	0.625		0.899
AP/rpX-A								0.063		0.004
AT-1 + AP/ rpX-A + rpX-D					0.009					0.004
N samples (N sites)	14 (4)	10 (5)	7 (1)	81 (3)	108 (7)	15 (2)	59 (4)	16 (5)	31	279

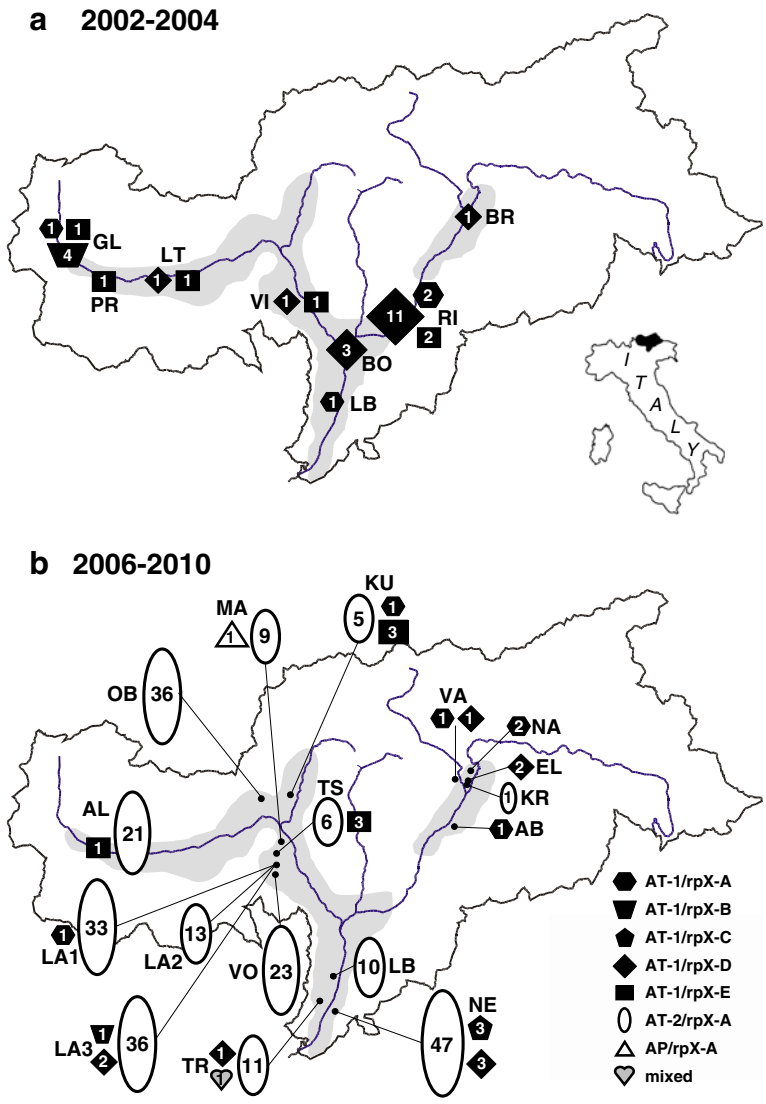

Fig. 3 Map of South Tyrol showing the position of sampling sites where '*Ca*. P. mali'-infected apple trees were sampled and the occurrence of different subtypes in the periods 2002–2004 (**a**) and 2006–2010 (**b**). Each subtype is represented by a different symbol. The numerals in the symbols indicate the number of trees at each locality carrying a particular combined subtype. The apple growing area of South Tyrol is indicated in grey

Discussion

Molecular typing procedures have become important tools for microbial identification and epidemiological studies (Li et al. 2009). These approaches are of particular relevance for uncultivable organisms, such as phytoplasmas, for which phenotypic differentiation is impossible. We have presented a rapid and easy-to-use method with enhanced sensitivity for the simultaneous typing of '*Ca*. P. mali' at two genetic loci. This approach is suitable for the application in high-throughput environments and can be applied to infected plant and psyllid samples. The separation and visualisation of PCR and T-RFLP fragments labelled with different fluorophores is based on the use of an automated sequencer. By the choice of additional fluorophores or the design of PCR/T-RFLP systems with distinct fragment lengths, the new typing assay could be easily extended to include additional genetic loci and hence to increase the resolution of the typing procedure.

The molecular typing of a collection of DNA isolates from '*Ca*. P. mali'-infected apple trees and a smaller set

of psyllid samples at two genetic loci has allowed some insights into the intraspecific diversity of the pathogen and its spread in South Tyrol. Our study covered almost a complete decade which, based on the prevalence of different subtypes of '*Ca*. P. mali', can be divided into two periods. The first period, from 2002 to 2004, was characterised by the occurrence of the five subtypes AT-1/rpX-A, AT-1/rpX-B, AT-1/rpX-C, AT-1/rpX-D and AT-1/rpX-E, and the complete absence of subtype AT-2/rpX-A. In the second period, starting from 2006, the frequency of AT-1-associated subtypes has drastically decreased and a single subtype, AT-2/rpX-A, became predominant in more than 90% of the investigated apple trees sampled from zones with acute disease outbreak.

The temporal distribution pattern of '*Ca*. P. mali' subtypes in South Tyrol coincides fairly well with the occurrence and population density of the two insect species *C. melanoneura* and *C. picta*, which have been proven to be the vectors of '*Ca*. P. mali' (Frisinghelli et al. 2000; Tedeschi and Alma 2004). Until 2004, *C. picta* was not known in the investigation area (Wolf and Zelger 2006), while *C. melanoneura* reached high densities on apple trees, provoking substantial feeding damages in some orchards in the 1990s (reviewed in Baric et al. 2010a). Soon after its appearance, *C. picta* spread throughout the apple growing region of South Tyrol, except the valley of the Eisack/Isarco river, reaching a maximum population density of 1.8 individuals per branch in 2006 (Walch 2006). The same year saw a massive outbreak of apple proliferation, with more than half a million apple trees showing unambiguous apple proliferation symptoms. Targeted vector control and mandatory uprooting of diseased trees ultimately contributed to the reduction of the population size of both *Cacopsylla* species as well as the incidence of apple proliferation-infected plants in South Tyrol (reviewed in Baric et al. 2010a).

The molecular typing data obtained in this study indicate that apple proliferation could have spread in two waves in South Tyrol. Since AT-1 displayed higher molecular genetic diversity as it was coupled with all five rpX subtypes, it appears to have been in this area for a longer time. Besides the exclusive presence of AT-1-associated subtypes, the first period of the disease's spread was characterised by high population densities of *C. melanoneura*. These were also found infected with '*Ca*. P. mali' (Wolf et al. 2003) and the small number of samples that could be further analysed showed similar pathogen subtype combinations as infected apple trees in the same period (see Tables 3 and 4). Therefore, *C. melanoneura* seems to have been the main vector of apple proliferation in the first years of its spread in this region. The sudden occurrence and predominance of subtype AT-2/rpX-A in various orchards of South Tyrol suggests a founder effect that coincides with the appearance and spread of *C. picta*. This species could have immigrated to South Tyrol from the neighbouring Province of Trentino where it had been present for many years (Frisinghelli et al. 2000). Moreover, *C. picta* specimens collected in the Trentino area and carrying '*Ca*. P. mali' were infected with subtype AT-2 in 96% of the cases, and this subtype was also found in more than 80% of the infected apple trees (Cainelli et al. 2004).

While *C. picta* was shown to be an efficient vector of '*Ca*. P. mali' in several independent transmission trials (Frisinghelli et al. 2000; Jarausch et al. 2004a; Seemüller et al. 2004; Carraro et al. 2008), experiments performed with infected specimens of *C. melanoneura* succeeded only in North-Western Italy (Tedeschi and Alma 2004; Tedeschi et al. 2003). Comprehensive transmission trials with the latter species in Germany completely failed to transmit the pathogen to healthy test plants (Mayer et al. 2009). In Trentino, however, only one infection was achieved in a total of 278 experiments performed over a six-year period, resulting in a transmission rate of 0.36% (Mattedi et al. 2008). Mayer et al. (2009) thus speculated about the existence of distinct populations of *C. melanoneura* which may vary in their capacity to acquire and transmit the pathogen. Alternatively, Casati et al. (2010) suggested that particular subtypes of '*Ca*. P. mali' could be adapted to the transmission by different vector species. This assumption is supported by the strict co-occurrence of AT-1-associated subtypes of '*Ca*. P. mali' and *C. melanoneura* in several regions of North-Western Italy, where *C. melanoneura* is considered to be the most important vector of apple proliferation (Casati et al. 2010). Moreover, despite the availability of only a small number of infected samples of this species, the same pattern was found in the first period of our study. On the other hand, European regions with *C. picta* as the principal disease vector are characterised by the dominance of '*Ca*. P. mali' subtypes AT-2 (Cainelli et

al. 2004; Jarausch et al. 2000) or AP (Jarausch et al. 2000, 2004b; Martini et al. 2008). In South Tyrol, subtype AT-2/rpX-A was detected in all investigated specimens of *C. picta* as well as in the major part of the orchard trees sampled between 2006 and 2010 due to acute symptom outbreaks. The only exception was the Eisack/Isarco river valley where infection rates with '*Ca*. P. mali' were generally low and *C. picta* has up to now never been found (Walch 2006; M. Zöschg, personal communication). In this area, seven of the eight apple trees sampled in 2009 and 2010 displayed AT-1-associated subtypes (see sampling sites AB, EL, KR, NA and VA in Fig. 3b).

A comparison of the distribution of sampling sites in the two periods determined in this study shows that the areas affected by apple proliferation do not exactly overlap (Fig. 3). During the first years of its spread in South Tyrol, apple proliferation remained mainly restricted to orchards located at higher altitudes. From 2005 onwards, in particularly the region around Meran/Merano was affected (reviewed in Baric et al. 2010a). For this reason, 8 of 20 orchards from the second period were located in this area (LA1-3, KU, MA, OB, TS and VO). It is known that apple trees can be infected with '*Ca*. P. mali' without showing any symptoms for years (Carraro et al. 2004). Therefore, the year of sampling is not an indication of the time point of pathogen transmission and it cannot be excluded that subtype AT-2/rpX-A was already present in this area before. However, this study clearly shows that the younger massive symptom outbreak of apple proliferation in certain zones of South Tyrol can be related to this particular subtype of '*Ca*. P. mali'.

So far, none of the studies addressing the vectoring capacity of *C. melanoneura* or *C. picta* has specified the molecular subtype of '*Ca*. P. mali' employed in the transmission experiments (Frisinghelli et al. 2000; Jarausch et al. 2004a; Seemüller et al. 2004; Tedeschi and Alma 2004; Carraro et al. 2008; Mattedi et al. 2008; Mayer et al. 2009). The present work indicates that the availability of such information could be of utmost relevance for assessing and better understanding the co-adaptation of particular '*Ca*. P. mali' strains with certain insect species as well as different potential plant reservoirs. Thus, future studies, involving a higher number of insect specimens, should test the hypothesis that subtype AT-2/rpX-A may be more efficiently acquired and transmitted by *C. picta* than by *C. melanoneura*, while the latter species may be better adapted to the acquisition and transmission of AT-1-associated subtypes. In this context, it will be further necessary to elucidate the molecular mechanisms that may be responsible for the pathogen-insect specificity. Besides ecological implications, knowledge about the vectoring ability of different insect species and the molecular background of host-phytoplasma interaction would contribute to a better disease control of apple proliferation.

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