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RESEARCH PAPERS

Survey and multigene characterization of stolbur phytoplasmas on various plant species in Serbia

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Summary. Stolbur phytoplasmas were detected in 116 out of more than 200 samples from nine plant species collected in 2009–2010 in Serbia. Phytoplasmas were detected and identified by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) amplified 16S rDNA. While all strains were identical on the level of 16S rDNA sequences, one strain represented a unique variant of stolbur phytoplasma with a SNP producing a new *Tru1*I restriction site. RFLP analyses of *tuf* gene amplicons, using *Hpa*II restriction enzyme, showed profiles identical to tuf type II profile in all stolbur phytoplasma strains from Serbia. Seminested PCR for amplification of the *rp* gene yielded aspecific amplicons with nearly half of the samples examined. However, in the 23 samples which yielded amplicons of expected size, RFLP analysis with *Alu*I restriction enzyme showed profiles with some variations. RFLP analyses of the amplified complete *secY* gene, using *Tru1*I and *Alu*I restriction enzymes, showed profiles indistinguishable from each other. In phylogenetic analyses of *secY* gene, strains belonging to tuf type I formed a lineage separate from the strains belonging to tuf type II. To our knowledge this is the first report of stolbur phytoplasma in valerian (*Valeriana officinalis*) adding a new plant species to the already wide natural host range of stolbur phytoplasma.

Key words: ribosomal protein, *secY* gene, *tuf* gene, valerian.

Introduction

Phytoplasmas are wall-less prokaryotes which are restricted to phloem of infected plants and their vectors, which include sap-sucking hemipteran insects such as leafhoppers, planthoppers and psyllids. Stolbur phytoplasma is considered as plant pathogen of European and Mediterranean origin, which infects a wide range of cultivated and wild plants. Recently, however, stolbur was also reported in China (Garnier, 2000; Duduk *et al*., 2010). This organism belongs to the 16SrXII-A ribosomal group, '*Candidatus* Phytoplasma solani' (Quaglino *et al*., 2013). Stolbur was reported for the first time in Serbia on pepper in 1949 (Martinović and Bjegović, 1950), and since then the disease was reported in many other cultivated crops and ornamental plants (grapevine, corn, peach, tobacco, chrysanthemum, wheat, coneflower, St. John's wort) as well as in wild plant species (*Convolvulus arvensis*, *Sonchus oleraceus, S. halepense*) (Duduk *et al*., 2004; 2006; Jović *et al*., 2009; Pavlović *et al*., 2011; 2012). Two planthoppers, *Hyalestes obsoletus* and *Reptalus panzeri* are reported as vectors of the phytoplasma in Serbia (Aleksić *et al*., 1967; Jović *et al*., 2009; Mori et al., 2013).

Although stolbur phytoplasmas are known to have low variability in 16S rDNA, some genetic diversity was reported on 16S rDNA as well as on some non-ribosomal DNA such as *tuf*, *secY* and *vmp1* genes (Langer and Maixner, 2004; Pacifico *et al*., 2007; Šeruga Musić *et al*., 2008; Cimerman *et al*., 2009; Quaglino *et al*., 2009; Fabre *et al*., 2011). It has been reported that three variants of *tuf* gene (tuf type I, II and III) of stolbur phytoplasmas are asso-

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ciated with different weed reservoir plants (Langer and Maixner, 2004).

In the present study, a 2 year survey was carried out for identification and multigene characterization of 116 strains of stolbur phytoplasma on various crops and from different regions of Serbia. The aim was to investigate presence of 16S rDNA variability, identify tuf type and estimate variability of *secY* and *rp* genes in these strains.

Materials and methods

Sample collection and nucleic acid extraction

During 2009 and 2010 more than 200 plant samples, from nine plant species grown on open fields and showing symptoms resembling phytoplasma infection (Table 1), were collected from 15 locations in different regions of Serbia. Total nucleic acids were extracted from 0.5 g of fresh leaf midrib tissue from each sample, following the protocol described by Doyle and Doyle (1990), dissolved in TE buffer and stored at -20ºC. Nucleic acids were diluted in sterile distilled water 1:100 before performing PCR assays. Additional strains (SB1 and 21OS) (Šeruga Musić *et al*., 2011) of stolbur phytoplasma from grapevine with "bois noir" disease were also included in the analyses as tuf type I reference strains.

Two strains, 284/09 and 142/09, were micropropagated in Murashige and Skoog (MS) medium after grafting on tobacco seedling and directly, respectively.

16S ribosomal DNA

For phytoplasma identification in collected samples, direct PCR assays with the universal phytoplasma primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and nested PCR on obtained amplicons with primer pair R16F2n/R2 (Lee *et al*., 1995) were carried out. Each 25 μL PCR mix contained 1 μL of DNA template, 1× PCR Master Mix (Fermentas, Vilnius, Lithuania) and 0.4 μM of each primer. Samples lacking DNA were employed as negative controls. As a template for nested PCR, 1 μL of direct PCR amplicon diluted 30× in sterile water was used. Thirty-five PCR cycles were performed, for both amplifications, under the following conditions: 1 min (2 min for the first cycle) for 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. Six microlitres of PCR products were separated in 1% agarose gel, stained with ethidium bromide and visualized with UV transilluminator.

Identification of the detected phytoplasmas was performed with RFLP analysis using *Tru1*I (Fermentas, Vilnius, Lithuania) restriction enzyme on P1/P7 or R16F2n/R2 amplicons. Restriction products were separated in 8% polyacrylamide gel, stained and visualized as described above.

Non-ribosomal genes

For amplification of the *tuf* gene, the fTufAy/rTufAy primer pair was used in direct or in nested PCR assays followed by fTuf1/rTuf1 primers (Schneider *et al*., 1997). Primer pairs used for amplification of the *rp* gene were rpF1C/rp(I)R1A in direct or in seminested PCR followed by rpL2F3/rp(I)R1A (Martini *et al*., 2007), while for amplification of the *secY* gene direct PCR with AYsecYF1/AYsecYR1 primer pair (Lee *et al*., 2006) was performed. For all reactions PCR mix was as described above.

Amplification of the *tuf* gene consisted of 35 PCR cycles under the following conditions: 30 sec for denaturation step at 95°C, 30 sec for annealing at 45°C and 1 min for primer extension at 72°C. Conditions for the amplification of the *secY* and *rp* genes were the same as for amplification of 16S rDNA, except that the annealing temperature was 55°C for the *secY* gene, while for amplification of *rp* gene 50°C and 55°C annealing temperatures were used in separate reactions. PCR products were visualized as described above.

RFLP analysis of the amplified *tuf* gene was performed using *Hpa*II restriction enzyme, while the analyses of the amplified *secY* and *rp* genes were performed using *Tru1*I and *Alu*I restriction enzymes. *Hpa*II restriction products obtained were separated in 2% agarose or 8% polyacrylamide gel, while *Tru1*I and *Alu*I products were separated, respectively, in 8% and 6% polyacrylamide gel. All gels were stained and visualized as described above. Analysis of the *tuf* gene was performed on all samples in which stolbur phytoplasma (16SrXII-A) was detected, while analyses of *secY* and *rp* genes were performed on 43 representative samples belonging to all plant species (Table 1) and on both tuf type I reference strains.

Sequencing and sequence analyses

The direct P1/P7 and AYsecYF1/R1 -amplified products, from nine and seven representative sam-

Table 1. Stolbur phytoplasma strains and phytoplasma DNA fragments amplified using polymerase chain reaction assays.

Plant host	Locality	Genes ampliffied		Acc. numbers of sequenced strains	
		tuf	*secY*[a]	Strain	16S rDNA, *secY*[b]
Tobacco	Ečka	19/19	5/5	142/09	JQ730739 (1,707), JQ730747 (1,256)
				284/09	JQ730740 (1,706), FO393427
	Bavanište	10/11	0/0		
	Inđija	5/5	2/2		
	Debeljača	2/2	0/0		
Carrot	Begeč	8/8	3/3		
Pepper	Begeč	8/8	2/3		
Peach	Slankamen	0/1	0/1		
Corn	Ečka	0/1	0/1		
	Stara Pazova	1/1	0/1		
	Perlez	2/4	1/1	121/09	JQ730750 (1,735), JX645764 (1,256)
	Idvor	1/1	0/0		
Grapevine	Radmilovac	4/4	0/0		
	Bela Crkva	15/18	4/7	138/10	JQ730746 (1,709), JX645768 (1,256)
	Krčedin	2/3	1/2		
	Aleksandrovac	3/5	3/4	125/10	JQ730745 (1,712), JQ730748 (1,256)
Periwinkle	Belgrade	10/10	5/5	204/10	JQ730744 (1,687), JX645766 (1,264)
				198/10	JQ730743 (1,645), JX645767 (1,256)
	Ečka	1/1	1/1		
Parsley	Pančevo	10/10	3/3	231/09	JQ730741 (1,707), FO393428
Valerian	Pančevo	2/4	2/4	224/09	JQ730742 (1,690), JX645765 (1,222)

[a] Number of positive/number of tested samples.
[b] Obtained sequences length in bp is given in brackets.

ples, respectively, were purified using the mi-PCR purification kit (Metabion International AG, Martinsried, Germany). The products were sequenced by commercial service (Macrogen Inc., Soul) in both directions with the two forward primers P1 and R16F2n and the reverse primer P7 for 16S rDNA and with AYsecYF1 and AYsecYR1 for *secY* gene amplicons. Amplicon of *secY* gene of tuf type I control strain SB1 was also sequenced as described above. Obtained sequences were assembled using Pregap4 from the Staden program package (Staden *et al*., 2000), deposited in the NCBI under the accession numbers given in Table 1, aligned using Clustal W (Thompson *et al*., 1997) from the Molecular Evolutionary Genetics Analysis program-MEGA5 (Tamura *et al*., 2011) and searched for SNPs in Bioedit program (Hall, 1999). Two sequences of *secY* gene belonging to strains 284/09 and 231/09 were also included in the analyses (Siewert *et al*., 2012). Additional full *secY* stolbur sequences were retrieved from the NCBI all belonging to tuf type II: these were PO (FM163376), Rus93 (GU004346) and PTV (GU004355). However, since there was no full *secY* gene sequence available for a strain belonging to tuf type I, strains 1925

(AM992094) and GR13 (FN813284) from grapevine were used to represent tuf type I, and strain GGY from bindweed (AM992093) to represent tuf type II (Martini *et al*., 2008; Fialová *et al*., 2009).

The obtained sequences of the complete *secY* gene were aligned with the retrieved ones using Clustal W. The evolutionary history was inferred using the Maximum Parsimony (MP) and Maximum Likelihood (ML) methods (MEGA5). The MP tree was obtained using the Close-Neighbour-Interchange algorithm with search level 3, in which the initial trees were obtained with the random addition of sequences (ten replicates). For ML, a sequence evolution model was first chosen using the "find best model" option in MEGA5. Initial tree(s) for the heuristic search were obtained automatically. For both analyses the "Gaps/Missing Data Treatment" option was set to "use all sites". To estimate the statistical significance of the inferred clades, 1,000 bootstrapping was performed to estimate the stability and support for the inferred clades.

Results

Out of more than 200 samples tested, 115 samples showed *Tru1*I RFLP profiles of R16F2n/R2 amplicons indistinguishable from each other and referable to those of stolbur phytoplasma, 16SrXII-A (Lee *et al*., 1998). One sample (284/09) showed profile slightly different from, but clearly similar to, those of stolbur phytoplasma (Figure 1). One month after grafting of the 284/09 infected plant material on a tobacco seedling and symptom expression, nucleic acids were extracted and the presence of the same unique strain was confirmed in the grafted seedling.

In the rest of the symptomatic samples that were tested, phytoplasma different from stolbur or no phytoplasma were detected, so these were excluded from further analyses.

The nine sequences obtained from P1/P7 amplicons ranged from 1,645 to 1,735 bp in length containing 16S rDNA and spacer region. The search for SNPs revealed no differences at any nucleotide position, showing complete identity (100%) among eight of them, while one sample (284/09) showed one nucleotide difference [position 183 (A/G)], introducing an additional *Tru1*I restriction site. Comparison of the eight identical phytoplasma 16S rDNA sequences using BLAST with those retrieved from GenBank showed 100% homology of 16S rDNA sequence with a stolbur strain from grapevine (bois noir stolbur phytoplasma strain 425_05, EU010010). Blast search revealed that the nucleotide position difference between the strain 284/09 and all other stolbur sam-

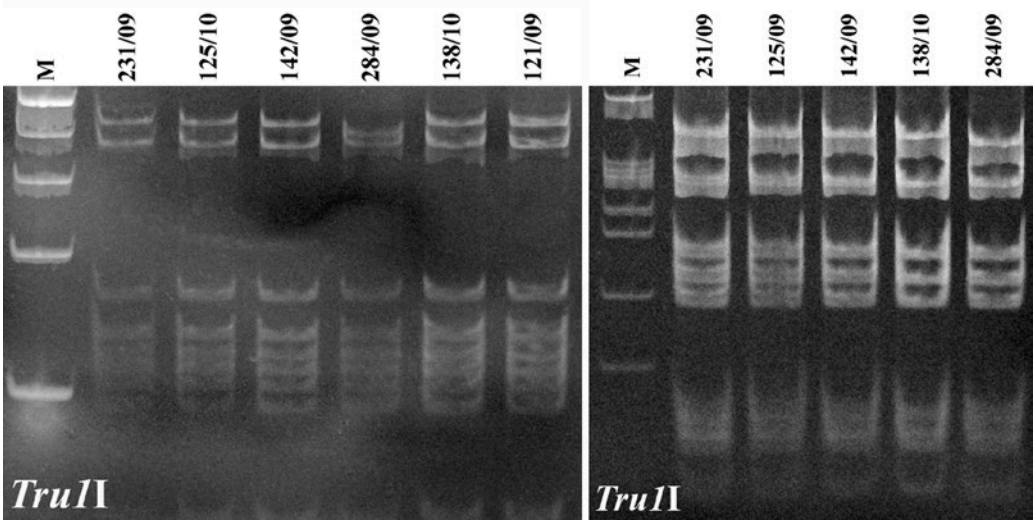

Figure 1. Polyacrylamide gel (8%) showing *Tru1*I-RFLP patterns of P1/P7 (left) and R16F2n/R2 (right) amplicons for different stolbur strains (see Table 1). M at left, molecular marker GeneRuler 100 bp DNA Ladder (Fermentas) [fragment sizes (bp) from top to bottom are 500, 400, 300, 200 and 100]; M at right, molecular marker ϕX174 DNA/BsuRI (*Hae*III digested) (Fermentas) [fragment sizes (bp) from top to bottom are 603, 310, 281, 271, 234, 194, 118 and 72].

ples tested (nt A on the position 183) is unique for the 284/09 strain; while all other sequences deposited in the GenBank had nt G on that position.

Nested PCR with fTuf1/rTuf1 followed by the fTufAy/rTufAy primer pair yielded amplicons of expected size (*c.* 950 bp) from 103 samples, while no amplification was obtained from 13 samples. RFLP analysis of fTufAy/rTufAy amplicons, using *Hpa*II restriction enzyme, showed profiles indistinguishable from each other and identical to the tuf type II profile described by Langer and Maixner (2004) (Figure 2).

Analysis of *rp* and *secY* genes was performed on 43 representative stolbur samples and both reference strains. Seminested PCR on the *rp* gene yielded aspecific amplicons (amplicon not, or not only, of expected size, which is *c.* 1,200 bp), with nearly half of samples employed. However, 23 samples yielded amplicons of expected size and were subjected to RFLP analyses with *Tru1*I and *Alu*I restriction enzymes. While the RFLP analysis with *Tru1*I restriction enzyme revealed no variability, *Alu*I restriction enzyme showed profiles with some variations highlighted with arrows in Figure 3.

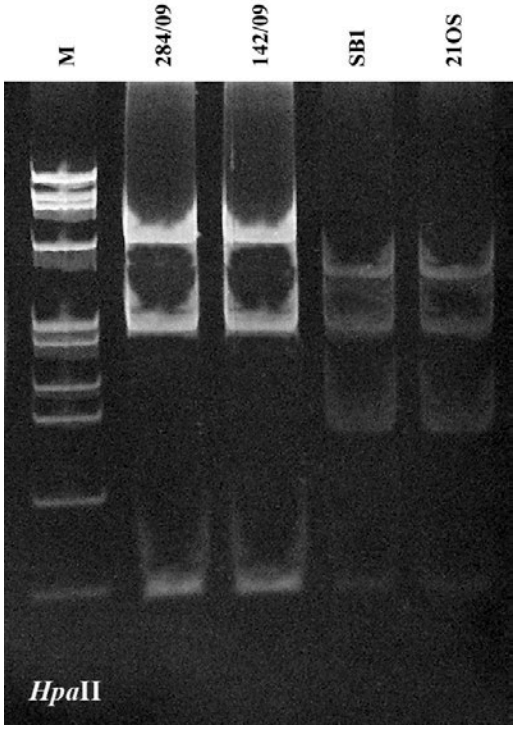

Figure 2. Polyacrylamide gel (8%) showing *Hpa*II-RFLP patterns of f/rTufAy amplicons for different stolbur strains (Table 1). M, molecular marker ϕX174 DNA/BsuRI (*Hae*III digested) [fragment sizes (bp) from top to bottom are 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118 and 72].

Out of the 43 samples tested, direct PCR with the AYsecYF1/R1 primer pair yielded amplicons of expected size (*c.* 1,300 bp) from 32 samples and both reference strains (Table 1). RFLP analyses of amplified *secY* gene, using *Tru1*I and *Alu*I restriction enzymes, showed profiles indistinguishable from each other (Figure 4).

All stolbur complete *secY* gene sequences obtained, ranged from 1,222 to 1,264 bp in length, were deposited in NCBI GenBank under the accession numbers given in Table 1. The complete *secY* gene sequence obtained from tuf type I reference strain SB1 (1,262 bp) was deposited in NCBI GenBank under accession number JQ730749. From Serbian stolbur samples, two genotypes were obtained that differ in one base pair [position 459 (G/A)], which is synonymous when translated. On the other hand, *secY* sequence of the tuf type I control strain SB1 differed from strains belonging to tuf type II in six or seven nucleotides (positions 103, 368, 376, 459, 805, 806 and 1102), resulting, in both cases, in five differences in predicted amino acid sequence.

Phylogenetic analyses of the *secY* gene using Maximum Parsimony algorithm resulted in the most parsimonious phylogenetic trees (data not shown) that had similar topology to that of Maximum Likelihood. One of 280 equally parsimonious trees is shown in Figure 5. Strains GR13, 1925 and SB1, all belonging to tuf type I, formed a lineage separate from the other stolbur strains belonging to tuf type II.

Discussion

Presence of stolbur phytoplasma in Serbia is known from 1949 (Martinović and Bjegović, 1950). Results from the present study confirm that stolbur phytoplasma is widespread in Serbia and present in a wide range of plant species. It is also confirmed that there is low genetic diversity of 16S rDNA of stolbur phytoplasmas in Serbia, even in samples collected from various plant species and different geographical regions of the country. While all tested strains but one have stolbur RFLP profiles indistinguishable from each other, and from published profiles with 100% homology of the 16S rDNA sequence with a stolbur strain from the GenBank, the strain 284/09 represents an unique variant of stolbur phytoplasma with a SNP which produces *Tru1*I restriction site. Al-

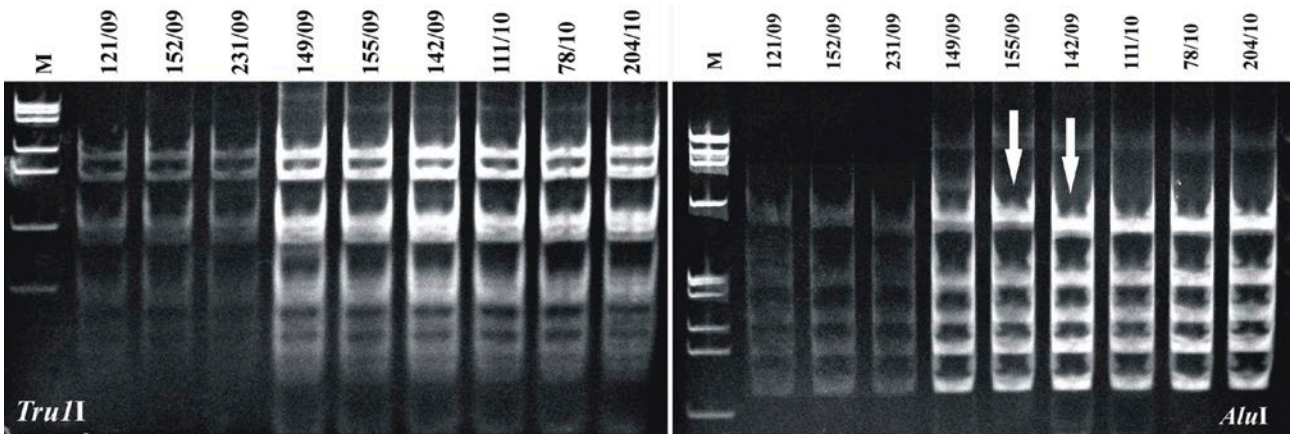

Figure 3. Polyacrylamide gel showing *Tru1*I (left) and *Alu*I (right)-RFLP patterns of rpF1C/rp(I)R1A PCR amplicons for different stolbur strains (Table 1). Hosts and localities were: 152/09, carrot, Begeč; 149/09, parsley, Pančevo; 155/09, carrot, Begeč; 111/10, tobacco, Inđija; 78/10, periwinkle, Ečka. M, molecular marker FX174DNA/BsuRI (*Hae*III digested) [fragment sizes (bp) from top to bottom are left: 603, 310, 281, 271, 234, 194, 118 and 72; right: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194 and 118].

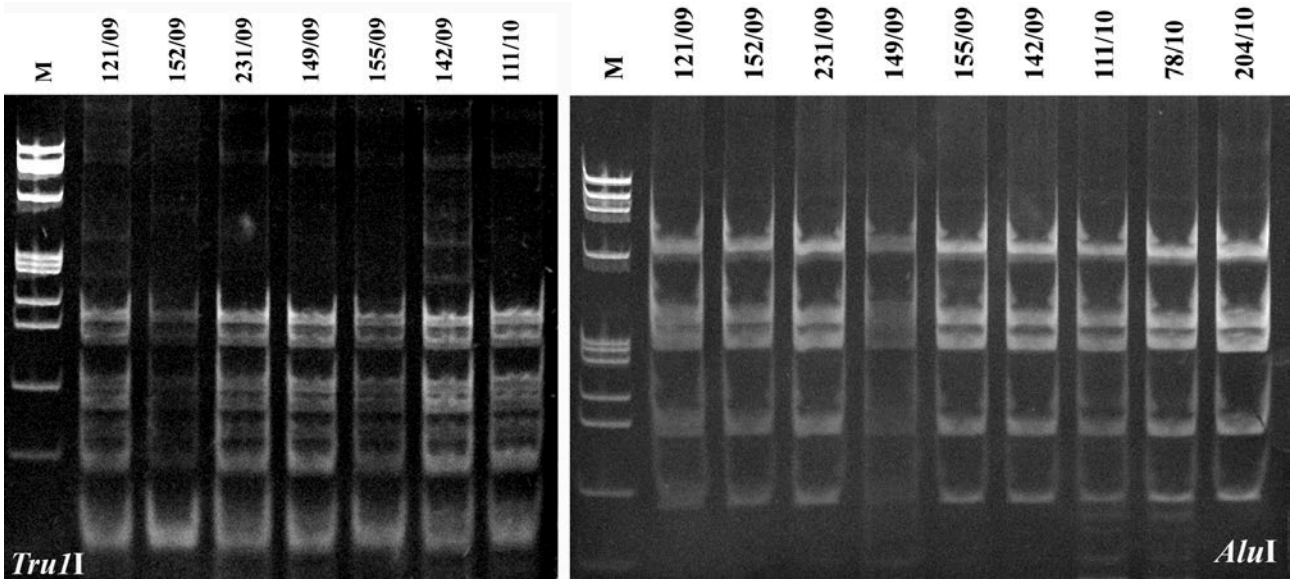

Figure 4. Polyacrylamide gel showing *Tru1*I (left) and *Alu*I (right)-RFLP patterns of *secY* amplicons. Stolbur strains and molecular marker M are as in Figure 2.

though several SNPs in 16S rDNA of stolbur strains from various plant species have been revealed so far, using different techniques (Šeruga Musić *et al*., 2008; Quaglino *et al*., 2009), the SNP on the position 183 and on *Tru1*I restriction site is not present in any sequence deposited in GenBank. To verify persistence of this polymorphism, grafting of the 284/09 plant material was performed and the presence of the same unique strain was confirmed in the grafted seedling. In order to maintain the strains 284/09 and 142/09, they were successfully micropropagated and maintained in infected tobacco shoots by micropropagation as part of the phytoplasma collection located at the University of Bologna (Bertaccini, 2010).

Since RFLP analyses of the *tuf* gene was reported to be in correlation with the reservoir plant host (Langer

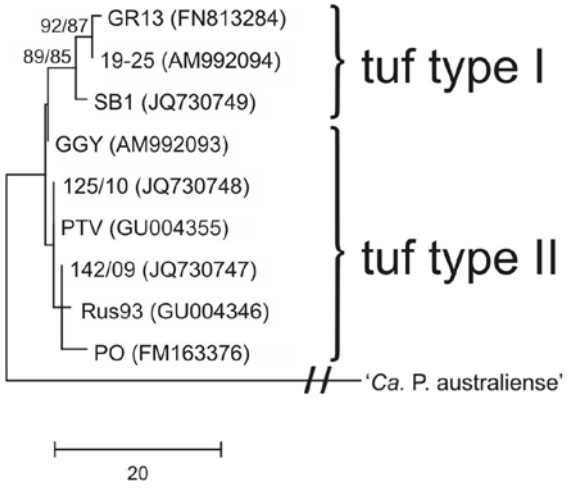

Figure 5. Phylogenetic tree constructed by parsimony analyses for nine stolbur phytoplasma strains based on *secY* gene sequences. '*Ca.* P. australiense' (NC_010544) was used as an outgroup taxon to root the tree. GenBank accession numbers are given in parentheses, and Tuf type affiliation is shown on the right side of the tree. Bootstrap support values (measures of support for the inferred subclades) referred to maximum parsimony / maximum likelihood are shown on the nodes (only values above 50% are shown). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar represents 20 nucleotide substitutions.

and Maixner, 2004) and partial *secY* gene sequence was used for studying molecular variability of stolbur phytoplasmas (Fialová *et al*., 2009); and no information about molecular variability of ribosomal protein genes for stolbur phytoplasmas is available, therefore these more variable single copy genes were used for finer strain differentiation of stolbur strains from serbia.

Results of *tuf* gene analysis in Serbian stolbur strains confirmed the presence of only tuf type II, which is, by Langer and Maixner, 2004, in Germany associated with *C. arvensis* as the plant reservoir. This is in agreement with well-known presence of stolbur phytoplasma in *C. arvensis* and its transmission by the polyphagus insect *H. obsoletus* from the weed to other various plant hosts (Aleksić *et al*., 1967). Thus, we suggest that *C. arvensis* plays an important role in stolbur phytoplasma epidemiology in Serbia.

The reason for specific amplification of ribosomal protein from only 23 samples out of 43 tested, even when different PCR conditions were used, is likely to be that the primers used were initially designed for phytoplasmas related to '*Candidatus* Phytoplasma asteris' (Martini *et al*., 2007).

For amplification of the *secY* gene only direct PCR was used, which could explain why this resulted in amplification of only 32 out of 43 samples. RFLP analyses of the *secY* gene on representative strains showed no polymorphism. However, search for SNPs among the complete *secY* gene sequences obtained revealed that two genotypes could be distinguished among Serbian strains, with the same predicted amino acid sequence. The tuf type I reference strain SB1 differed from Serbian tuf type II strains in five predicted amino acids. Two out of five observed predicted amino acid differences are located in part of the *secY* gene which is not available when the partial *secY* gene sequence is analyzed (Fialová *et al*., 2009). In phylogenetic analyses, strains belonging to tuf type I formed a lineage separate from those belonging to tuf type II, based on the complete *secY* gene sequence.

The SNP in the 16S rDNA of strain 284/09 is shown to be unrelated with variations in other marker genes tested. To our knowledge this is the first report of stolbur phytoplasma in valerian (*Valeriana officinalis*) adding a new plant species to wide natural host range of stolbur.

Acknowledgments

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