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Molecular biology

**MOLECULAR CHARACTERIZATION OF 'CANDIDATUS PHYTOPLASMA
SOLANI' FROM *Prunus avium* AND *Solanaceous* CROPS
IN DIFFERENT REGIONS OF AZERBAIJAN**

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'*Ca. P. solani*' has recently been detected in annual crops such as eggplant, pepper and tomatoes, also in declining cherry trees and common medlar in Azerbaijan. The aim of the present study was to carry out molecular characterization of stolbur phytoplasma isolates from infected *Solanaceous* crop, cherry and insect vectors in different regions of Azerbaijan by genotyping based non-ribosomal gene stamp. Surveys were conducted in Baku, Absheron and Guba regions. Genotyping by sequencing the stamp gene that encodes the antigenic membrane protein of Stolbur showed that stolbur isolates detected in Azerbaijan have eight genotypes; seven of Stamp genotypes grouped in the branch of *tuf*-type b stamp cluster III. The isolate AZ2012-PV1 had a Stamp genotype different from others and grouped in Stamp cluster II.

Keywords: '*Ca. P. solani*', RFLP, stamp gene amplification, sequence analysis, genotyping, *Prunus avium*, *Solanaceae*

Introduction

'*Candidatus Phytoplasma solani*' is widespread in all European and Mediterranean areas, and it can seriously affect quality and quantity of production (Quaglino et al., 2013). This phytoplasma induces stolbur disease, which affects a wide range of crops and wild plants including solanaceous crops, grapevine, lavender, strawberry, sugar beet, maize, stinging nettles and bindweed (Garnier, 2000). Symptoms of stolbur disease are leaf discoloration, stunting and abnormal floral development leading to sterility. It is transmitted by three *Fulguromorpha* planthoppers of the family *Cixiidae*, such as *Hyalesthes obsoletus* Signoret (Cargnus et al., 2012; Mori et al., 2013), *Pentastiridius leporinus* Linnaeus (Bressan et al., 2008) and *Reptalus panzeri* Loew (Jovic et al., 2007).

'*Ca. P. solani*' has been detected in annual crops such as eggplant, pepper and tomatoes, also in declining cherry trees and common medlar in Azerbaijan (Balakishiyeva et al., 2010; Balakishiyeva et al., 2012). The aim of the present study was to carry out molecular

characterization of stolbur phytoplasma isolates from infected *Solanaceous* crop, cherry and insect vectors in different regions of Azerbaijan by genotyping based non-ribosomal gene stamp. Surveys were conducted in Baku, Pirshagi, Mehdiabad and Guba regions. Genotyping by sequencing the stamp gene that encodes the antigenic membrane protein of Stolbur showed that stolbur isolates detected in Azerbaijan have eight genotypes; seven of Stamp genotypes grouped in the branch of *tuf*-type b stamp cluster III. The isolate AZ2012-PV1 had a Stamp genotype different from others and grouped in Stamp cluster II.

Material and methods

Plant samples and DNA extraction.

Different plant samples showing symptoms reminiscent of phytoplasma infection and symptomless plants, as negative control, were collected in September in Baku, Absheron (Pirshagi, Mehdiabad) and Guba regions (see list of samples in table 1).

Table 1

Plant samples from Baku, Mehdiabad and Guba regions

N°	Sample ID	Latin name (H)- healthy control	Place	DNA concentration ng/μl	PCR R16R2/F2n
1.	Ce1.AZ.12	<i>Prunus avium</i>	Mehdiabad	195	+
2.	Ce2.AZ.12	<i>Prunus avium</i>	Mehdiabad	173	-
3.	Ce3.AZ.12	<i>Prunus avium</i>	Mehdiabad	152	+
4.	Ce4.AZ.12	<i>Prunus avium</i>	Mehdiabad	116	-
5.	Ce5.AZ.12	<i>Prunus avium</i>	Baku	225	-
6.	Ce6.AZ.12	<i>Prunus avium</i>	Baku	332	-
7.	Ce7.AZ.12	<i>Prunus avium</i>	Baku	140	-
8.	Ce8.AZ.12	<i>Prunus avium</i>	Baku	145	+
9.	Ce9.AZ.12	<i>Prunus avium</i>	Guba	315	-
10.	Ce10.AZ.12	<i>Prunus avium</i>	Guba	170	-
11.	Ce11.AZ.12	<i>Prunus avium</i>	Guba	264	+
12.	Ce12.AZ.12	<i>Prunus avium</i>	Guba	120	-
13.	Ce13.AZ.12	<i>Prunus avium</i>	Guba	129	-
14.	Ce14.AZ.12	<i>Prunus avium</i>	Guba	156	-
15.	Ce15.AZ.12	<i>Prunus avium</i>	Guba	158	-
16.	Ce16.AZ.12	<i>Prunus avium</i>	Guba	225	-
17.	Ce17.AZ.12	<i>Prunus avium</i>	Guba	168	-
18.	Ce18.AZ.12	<i>Prunus avium</i>	Guba	235	-
19.	Ce19.AZ.12	<i>Prunus avium</i>	Guba	195	-
20.	Ce20.AZ.12	<i>Prunus avium</i>	Guba	107	-
21.	Ce21.AZ.12	<i>Prunus avium</i>	Guba	115	-
22.	Ce22.AZ.12	<i>Prunus avium</i>	Guba	144	-
23.	Ce23.AZ.12	<i>Prunus avium</i>	Guba	118	-
24.	Ce24.AZ.12	<i>Prunus avium</i>	Guba	566	-
25.	Ce25.AZ.12	<i>Prunus avium</i>	Guba	206	+
26.	Ce26.AZ.12	<i>Prunus avium</i> (H)	Guba	166	-
27.	Pv1.AZ.12	<i>Capsicum annuum</i>	Mehdiabad	191	+
28.	Pv2.AZ.12	<i>Capsicum annuum</i>	Mehdiabad	374	+
29.	Pv3.AZ.12	<i>Capsicum annuum</i>	Mehdiabad	410	+
30.	Pv4.AZ.12	<i>Capsicum annuum</i> (H)	Mehdiabad	493	-
31.	Au5.AZ.12	<i>Solanum melongena</i> (H)	Mehdiabad	345	-
32.	Au6.AZ.12	<i>Solanum melongena</i>	Mehdiabad	380	+

Phytoplasma detection. Detection of phytoplasma infection was performed on 2 μl of each DNA extract tested by 16SrDNA nested PCR with the universal primers for phytoplasmas R16mF2 / R16mR1 (external primers) followed by R16F2n / R16R2 (internal primers) (Gundersen & Lee, 1996). For each PCR reaction one positive control (reference phytoplasma isolate maintained in periwinkle experimental host at INRA greenhouse), healthy plant for each plant species and water as negative

controls were used to check the reliability of the reaction. The PCR products (7 μL) were analyzed by electrophoresis in the 1 X TBE buffer through 1% (w/v) agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator.

Restriction fragment length polymorphism (RFLP) analysis. The Nested PCR products were subjected to RFLP analysis with *Taq* I, *Alu*I and *Rsa*I restriction enzymes. 20 μl the enzymatic digestion mixture contained 0,5 μl

of each enzyme (5 U/μl), 2 μl of 10x buffer, 13,5 μl sterilized H₂O and 4 μl of PCR product. Digestion with *Taq* I was performed by incubation at 65° C for 2 hours. Digestions with *Alu*I and *Rsa*I restriction enzymes were realized by incubation at 37° C over night. Digested PCR products were then loaded on 2% agarose gel, stained with ethidium bromide and visualized on a transilluminator after electrophoresis.

Typing on non-ribosomal *stamp* gene.

All stolbur phytoplasma isolates obtained were subjected to genotyping on non-ribosomal *stamp* gene (Fabre *et al.*, 2011). The *stamp* gene was amplified by Nested PCR using the external primer pair Stamp-F (5'-GTAGGTTTTGGATGTTTAAAG-3') and Stamp-R0 (5'-AAATAAAAGAACAAGTATAGACGA-3') in first round and internal primer pair Stamp-F1 (5'-TTC TTTTAAA CACACCAAGAC-3') and Stamp-R1 (5'-AAGCCAGAATTTAATCTAGC-3') in Nested PCR. First round PCR condition were 4 min at 94°C as an initial denaturation step, followed by 35 cycles of 30s at 94°C, 30 s at 56°C and 1 min 30s at 72 °C, and an extension step of 7 min at 72°C. The Nested-PCR consisted of a denaturation step of 4 min at 94 °C and 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C followed by an extension step of 7 min at 72 °C. PCR products were analysed by electrophoresis in 1% agarose gel and stained with ethidium bromide.

Sequencing and phylogenetic analysis. Expected 637 bp amplicons were sequenced on both strands by BECKMAN-COULTER GENOMICS Company (Grenoble, France) on MegaBACE capillary sequencing instruments. Raw sequence chromatograms were assembled and edited using Staden package (Bonfield *et al.*, 1995). Multiple sequences alignments were performed using the Clustal W program (Thompson *et al.*, 1994) and phylogenetic analyses were conducted with MEGA version 5 (Tamura *et al.*, 2011) using maximum parsimony with randomized bootstrapping evaluation of branching validity.

Results and discussion

The large crop survey to evaluate the incidence of stolbur phytoplasma diseases were realized in September. Surveys were conducted in Baku, Absheron and Guba regions. During

surveys stunted cherry trees exhibiting yellowing were collected. For solanaceous crop, yellowing and stunted peppers and eggplants with virescence and phyllody symptoms were collected. Symptomless plants were also sampled as negative control.

Total DNAs extracted from fresh leaf midribs and DNA extracts examined for phytoplasma infection using 16S-rDNA amplification by Nested PCR with universal primer pairs (R16mF2 / R16mR1 followed by R16F2n / R16R2) according to the cycling conditions described in the original publication (Gundersen & Lee, 1996). Nested PCR result showed that while symptomless plants gave no amplification, expected 1250 bp amplicons were obtained from the infected cherry trees, peppers and eggplant. To identify the detected phytoplasmas obtained Nested PCR products were subjected to RFLP analysis with *Taq* I, *Alu*I and *Rsa*I restriction enzymes. Results acquired from RFLP analyses with enzymes *Taq* I, *Alu*I and *Rsa*I showed that the 16S-rDNA from the infected cherry trees, peppers and eggplant (Au6.AZ.2012) gave the same profile that the Stolbur phytoplasma reference strain (Molière) maintained at INRA Bordeaux in periwinkle *Catharanthus roseus*.

All of the stolbur phytoplasma isolates detected in Azerbaijan and the reference phytoplasma isolates maintained in periwinkle experimental host were subjected to genotyping on non-ribosomal *stamp* gene.

Genotyping by sequencing the *stamp* gene that encodes the antigenic membrane protein of Stolbur showed that stolbur isolates detected in Azerbaijan have eight genotypes; seven genotypes grouped in the branch of *tuf-type* b *stamp* cluster III (Figure). This branch groups Stolbur isolates of the east of the Mediterranean basin (Azerbaijan, Serbia and Lebanon). The isolate AZ2012-PV1 had a Stamp genotype different from others and grouped in Stamp cluster II. The isolate AZ2012-PV2 is a mixed infection of 2 different stolbur phytoplasma strains (AZ2012-PV2C and AZ2012-PV2T).

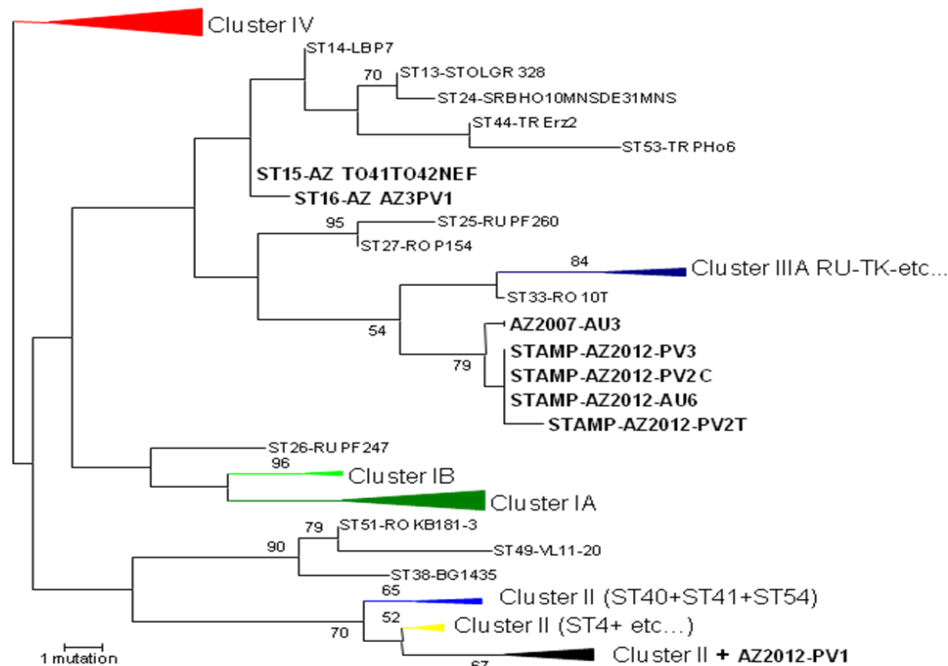


Fig. Maximum Parsimony analysis of STAMP sequences. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 52 most parsimonious trees (length = 140) is shown. The consistency index is (0.452830), the retention index is (0.871965), and the composite index is 0.510722 (0.394852) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (200 replicates) are shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 60 nucleotide sequences. The codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 613 positions in the final dataset. Evolutionary analyses were conducted in MEGA5

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AZƏRBAYCANIN MÜXTƏLİF BÖLGƏLƏRİNDƏ *Prunus avium* VƏ *Solanaceous* NÜMAYƏNDƏLƏRİNDƏ 'CANDIDATUS PHYTOPLASMA SOLANI' FİTOPLAZMASININ MOLEKULAR XARAKTERİSTİKASI

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'*Ca. P. solani*' fitoplazma növü bu yaxınlarda Azərbaycanda becərilən badımcın, bibər və pomidor kimi birillik tərəvəz bitkilərində, həmçinin qurumaqda olan albalı və əzgil ağaclarında aşkar edilmişdir. Bu tədqiqatın məqsədi Azərbaycanın şimal rayonlarında *Solanaceous* fəsiləsinə aid olan bitkilərdə, albalını və həşəratları yoluxdurmuş stolbur fitoplazmasının izolyatlarının qeyri-ribosomal *stamp* geninə görə genotipləşdirilməsi əsasında molekulyar xarakteristikasının aparılmasıdır. Monitorinqlər Bakıda, Abşeron və Quba rayonlarında aparılmışdır. Stolbur fitoplazmasının antigen membran zülalını kodlaşdıran *stamp* geninin sekvens olunması ilə genotipləşdirilməsi nəticəsində Azərbaycanda stolbur izolyatlarının səkkiz genotipi aşkar edilmişdir; yeddi *Stamp* genotipi III *stamp* klasterin *tuf-tip b* qolunda qruplaşmışdır. AZ2012-PV1 izolyatı isə digər *stamp* genotiplərindən fərqlənir və II *Stamp* klasterində yerləşmişdir.

Aşar sözlər: '*Ca. P. solani*', *RFLP*, *stamp* geninin amplifikasiyası, sekvens analiz, genotipləşdirmə, *Prunus avium*, *Solanaceae*

МОЛЕКУЛЯРНАЯ ХАРАКТЕРИСТИКА 'CANDIDATUS PHYTOPLASMA SOLANI' ИЗ *Prunus avium* И ПРЕДСТАВИТЕЛЕЙ *Solanaceous* В РАЗЛИЧНЫХ РЕГИОНАХ АЗЕРБАЙДЖАНА

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В Азербайджане недавно были обнаружены '*Ca. P. solani*' в таких однолетних культурах, как баклажаны, перец и томат, а также в засыхающих деревьях вишни и мушмулы германской. Целью данной работы явилось дать молекулярную характеристику изолятов фитоплазмы столбура из зараженных пасленовых культур, вишни и насекомых в северных регионах Азербайджана генотипированием, основанном на нерибосомальных *stamp* генах. Мониторинги были проведены в Баку, Абшеронском и Губинском районах. Генотипирование с помощью секвенирования гена *stamp*, который кодирует антигенный мембранный белок столбура, показало, что изоляты столбура, обнаруженные в Азербайджане, имеют восемь генотипов; семь *stamp* генотипов, сгруппированы в *tuf-type b* III *stamp* кластере. Изолят AZ2012-PV1 отличается от других *Stamp* генотипов и находится в II *Stamp* кластере.

Ключевые слова: '*Ca. P. solani*', *RFLP*, амплификация *stamp* гена, секвенс-анализ, генотипирование, *Prunus avium*, *Solanaceae*