

# ‘*Candidatus phytoplasma solani*’ genotypes associated with potato stolbur in Serbia and the role of *Hyalesthes obsoletus* and *Reptalus panzeri* (hemiptera, cixiidae) as natural vectors

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**Abstract** A progressive spread of stolbur-associated symptoms observed in potato fields in Serbia over the past few years initiated the study on disease epidemiology and transmission pathways performed during 2013 and 2014. Inspection of potato fields on 12 localities in northern Serbia revealed high incidence (60 % of symptomatic plants) and wide dispersal (100 % of inspected localities) of ‘*Candidatus Phytoplasma solani*’. A qualitative analysis of Auchenorrhyncha fauna in affected potato fields identified 16 species, however only *Hyalesthes obsoletus*, *Reptalus panzeri* and *R. quinquecostatus* tested positive for ‘*Ca. P. solani*’. Multilocus typing of strains associated with field collected potato plants and insects had been performed to identify the propagation scenario underlying the threatening epidemics. Combined analyses of the *tuf*, *stamp* and *vmp1* genes detected ten genotypes, seven of which were shared by the potato plants and insects, confirming their interaction, with no clear species-specific association of certain ‘*Ca. P. solani*’ genotypes

with plausible insect vectors. Semi- field experiments with naturally ‘*Ca. P. solani*’-infected *H. obsoletus* and *R. panzeri* confirmed the ability of both species to successfully transmit the pathogen to potato plants and induce symptoms characteristic of stolbur disease. The third putative vector *R. quinquecostatus* shared genotypes of ‘*Ca. P. solani*’ with potato plants and other two cixiids, and though not tested in this study should not be ruled out as a potential vector. Our study revealed rather complex epidemiology of potato stolbur in Serbia involving several possible routes of horizontal transmission and provided experimental evidence for two natural planthopper vectors.

**Keywords** *Hyalesthes obsoletus* · Multilocus typing · Potato stolbur · *Reptalus panzeri* · *Reptalus quinquecostatus* · Vector

## Introduction

‘*Candidatus Phytoplasma solani*’ belonging to the 16SrXII-A ribosomal subgroup (Quaglino et al. 2013), formerly known as stolbur phytoplasma, is a widely distributed European-native pathogen causing diseases on many cultivated plants, including tomato, potato, tobacco, pepper, celery, carrot, parsley, grape, maize etc. (reviewed in EFSA Panel on Plant Health 2014). Over a past decade, ‘*Ca. P. solani*’ was registered in association with several economically important diseases in Serbia, including maize redness, Bois Noir

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and potato stolbur (Jović et al. 2007; Jović et al. 2011; Cvrković et al. 2014). Furthermore, it was registered infecting celery, kale, garden bean and various weeds (Ivanović et al. 2011; Trkulja et al. 2011; Mitrović et al. 2015a; Cvrković et al. 2014).

In the European Union, ‘*Ca. P. solani*’ has a quarantine status and is one of the most significant phytoplasma infecting potato (EPPO/CABI 1996). Potato stolbur caused by the ‘*Ca. P. solani*’ is an economically important disease having a significant impact on potato production. Severe outbreaks of potato stolbur have been reported in Czech Republic, Hungary, Romania, and Russia, causing 30 to 80 % of yield losses and reduction in tuber and seed quality (Ember et al. 2011). ‘*Ca. P. solani*’ is transmitted by grafting and vegetative propagation of infected host plants, and by the vectors *Hyalosthes obsoletus*, *Reptalus panzeri* and possibly others (EFSA Panel on Plant Health 2014). Propagation of this phytoplasma across Europe is evident, being reported in Germany, Czech Republic, Switzerland, Turkey, Bulgaria, Romania, Russia, Serbia, Belgium, Greece (Girsova et al. 2008; Ember et al. 2011; Jović et al. 2011; Tahzima et al. 2013; Holeva et al. 2014; EFSA Panel on Plant Health 2014).

A progressive spread with tendency of growing epidemics of potato stolbur disease has been observed in Serbia after its first molecular confirmation (Jović et al. 2011), imposing the urgency to establish an effective disease management program. However, building a platform for the successful control strategy is challenging considering the epidemiology of potato stolbur being far from resolved. Epidemiological cycle of ‘*Ca. P. solani*’ and transmission pathways are in close correlation with the host plant/insect vector associations. Several native plants as reservoirs and many hemipteran species found to be natural carriers of ‘*Ca. P. solani*’ contribute to rapid and wide expansion in the field. ‘*Ca. P. solani*’ has been reported in several wild plants in Serbia such as *Sorghum halepense*, *Cirsium arvense*, *Convolvulus arvensis*, *Daucus carota*, *Picris hieracioides*, *Erigeron annuus*, *Cynodon dactylon* (Jović et al. 2009; Cvrković 2010; Mitrović et al. 2012), that are common weeds in different crops and could act as pathogen reservoirs.

Inspection of potato fields in Serbia for potential hemipteran vectors revealed a dominance of *Psammotettix alienus*, *Hyalosthes obsoletus*, *Reptalus quinquecostatus*, *R. panzeri* and *Euscelis incisus* (Mitrović et al. 2015b), all previously reported to be

natural carriers of ‘*Ca. P. solani*’ (Trivellone et al. 2005; Riedle-Bauer et al. 2006; Jović et al. 2009; Cvrković 2010; Cvrković et al. 2014). Furthermore, *R. panzeri* is a proven vector of this phytoplasma in maize fields and vineyards (Jović et al. 2007, 2009; Cvrković et al. 2014) and *H. obsoletus* in vineyards and sugar beet fields (Maixner 1994; Sforza et al. 1998; Bressan et al. 2008). Composition of the Auchenorrhyncha fauna in the potato fields in Serbia and the presence of ‘*Ca. P. solani*’ in the wild plants indicate a rather complex epidemiology of potato stolbur disease, with plausible several spread pathways.

In regard to the unknown epidemiology of the potato stolbur disease, major goals addressed in this study were to: i) determine the ‘*Ca. P. solani*’ presence in the potato growing regions of northern Serbia; ii) characterize the ‘*Ca. P. solani*’ strains associated with potato; iii) identify potential vectors among Auchenorrhyncha and characterize the ‘*Ca. P. solani*’ strains harbored by the insects; and iv) test the transmission ability of *H. obsoletus* and *R. panzeri* to vector the potato stolbur disease.

## Material and methods

### Field sampling

In July of 2013 and 2014, a total of 12 potato fields in northern Serbia were inspected for the ‘*Ca. P. solani*’ - associated symptoms (Table 1). Leaf midribs with petioles or aerial tubers from the symptomatic plants were sliced, distributed in 1 g aliquots and kept at  $-20^{\circ}\text{C}$  till analysed molecularly. Sampled plants were submitted to PCR/RFLP analysis for identification and characterization of ‘*Ca. P. solani*’. At the time of plant sampling from the inspected potato fields, planthoppers and leafhoppers were collected as well with the entomological sweep net and a mouth aspirator. Insects were collected from the bordering rows and along the diagonal in the potato fields (Table 2). Captured specimens were identified using taxonomic keys described by Holzinger et al. (2003) and Biedermann and Niedringhaus (2004) and stored individually in plastic vials with 96 % ethanol at  $4^{\circ}\text{C}$  until examined for phytoplasma presence. In terms of resolving the epidemiological cycle(s) of the potato stolbur disease, the assemblages of weeds in the potato fields were also registered including the crops in the surroundings.

**Table 1** The list of inspected potato fields in Serbia, ‘*Ca. P. solani*’ detection in the plant and insect material, weeds assemblages in potato fields and the surrounding crops

Locality	GPS	Weeds surrounding potato field	Crops in the vicinity of potato field	No of infected potato plants/No of analyzed plants	‘ <i>Ca. P. solani</i> ’-positive Auchenoorrhyncha captured in potato fields
Titel	N45°12'38.6" E20°16'14.3"	<i>Amaranthus retroflexus</i> , <i>Ambrosia artemisiifolia</i> , <i>Chenopodium album</i> , <i>Convolvulus arvensis</i> , <i>Dactylis glomerata</i> , <i>Sorghum halepense</i>	Maize	3/7	–
Bački Petrovac	N45°20'46.6" E19°37'47.2"	<i>A. artemisiifolia</i> , <i>Cirsium arvense</i> , <i>C. arvensis</i> , <i>C. album</i>	Soya	5/8	<i>Hyalesthes obsoletus</i> , <i>Reptalus panzeri</i> , <i>R. quinquecostatus</i>
Novi Sad	N45°14'30.8" E19°42'53.5"	<i>C. arvensis</i> , <i>C. album</i> , <i>D. glomerata</i> , <i>Setaria viridis</i>	Tomato	6/9	–
Futog	N45°14'28.6" E19°39'38.8"	<i>A. retroflexus</i> , <i>C. arvense</i> , <i>A. artemisiifolia</i> , <i>C. arvensis</i> , <i>C. album</i> , <i>S. viridis</i>	Soya, cabbage	2/7	<i>H. obsoletus</i>
Begeč	N45°14'28.5" E19°36'39.4"	<i>A. artemisiifolia</i> , <i>C. arvensis</i> , <i>S. halepense</i> , <i>C. arvense</i>	Soya, maize	5/8	<i>H. obsoletus</i> , <i>R. panzeri</i>
Gložan	N45°16'10.1" E19°34'54.3"	<i>C. arvensis</i> , <i>C. album</i> , <i>D. glomerata</i> , <i>C. arvense</i> , <i>S. halepense</i>	Maize, grapevine	4/7	–
Deronje	N45°88'04.3" E19°13'48.2"	<i>A. retroflexus</i> , <i>A. artemisiifolia</i> , <i>C. album</i> , <i>C. arvensis</i>	Maize, soya	5/8	<i>H. obsoletus</i>
Despotovo	N45°27'32.8" E19°19'32.3"	<i>C. arvensis</i> , <i>A. artemisiifolia</i> , <i>S. halepense</i>	Soya, grapevine, maize	5/7	<i>H. obsoletus</i> , <i>R. panzeri</i>
Bela crkva	N44°63'34.3" E21°26'42.6"	<i>A. retroflexus</i> , <i>C. arvensis</i> , <i>S. halepense</i> , <i>C. arvense</i>	Sunflower, pepper, wheat	6/8	<i>H. obsoletus</i> , <i>R. quinquecostatus</i>
Kušići	N44°52'30.0" E21°27'47.1"	<i>A. retroflexus</i> , <i>C. arvensis</i> , <i>A. artemisiifolia</i> , <i>Agropyrum repens</i> , <i>C. album</i> , <i>C. arvense</i> , <i>D. glomerata</i> , <i>Cichorium intybus</i> , <i>Portulaca oleraceae</i> , <i>S. halepense</i>	Cabbage, maize, wheat	4/9	<i>H. obsoletus</i> , <i>R. panzeri</i> , <i>R. quinquecostatus</i>
Stara Pazova	N44°58'54.3" E20°19'12.9"	<i>A. retroflexus</i> , <i>C. arvensis</i> , <i>C. album</i> , <i>C. arvense</i>	Pepper, tomato, bean, carrot	2/5	–
Srednjevo	N44°40'40.9" E21°30'54.3"	<i>A. retroflexus</i> , <i>C. arvensis</i> , <i>S. halepense</i>	Maize	8/9	<i>H. obsoletus</i> , <i>R. panzeri</i>

**Table 2** The list of hemipteran species collected in the potato fields in Serbia with ‘*Ca. P. solani*’ incidence

Family	Subfamily	Species/No of localities where present	No of stolbur positive specimens/No of collected specimens
Cixiidae	Cixiinae	<i>Hyalesthes obsoletus</i> /10	38/118
		<i>Reptalus panzeri</i> /8	16/57
		<i>R. quinquecostatus</i> /7	10/38
Delphacidae	Asiracinae	<i>Asiraca clavicornis</i> /1	0/4
	Delphacinae	<i>Dicranotropis hamata</i> /5	0/17
Tettigometridae		<i>Tettigometra atra</i> /1	0/2
Issidae	Issinae	<i>Issus coleoptratus</i> /1	0/1
Cercopidae	Cercopinae	<i>Lepyronia coleoptrata</i> /1	0/2
Membracidae	Smiliinae	<i>Stictocephala bisonia</i> /2	0/7
Cicadellidae	Agalliinae	<i>Anaceratagallia ribauti</i> /3	0/19
	Cicadellinae	<i>Cicadella viridis</i> /1	0/3
	Typhlocybinae	<i>Typhlocybina</i> sp./3	0/8
	Deltocephalinae	<i>Doratura impudica</i> /2	0/9
		<i>Euscelis incisus</i> /7	0/59
		<i>Psammotettix alienus</i> /10	0/60
		<i>Errastunus ocellaris</i> /3	0/11

#### PCR detection and characterization of ‘*Ca. P. solani*’

Total DNA was extracted from potato plants using the CTAB method (Angelini et al. 2001) and from the insect material applying a modified CTAB method according to Gatineau et al. (2001). Initial phytoplasma identification in the field-collected plant and insect material was based on a nested PCR analysis with ‘*Ca. P. solani*’-specific stol11 primers F2/R1 and F3/R2 (Clair et al. 2003), following a protocol by Radonjić et al. (2009). DNA amplification was performed in 20 µl volumes, PCR products were separated on 1 % agarose gel, stained with ethidium bromide and visualized with a UV transilluminator.

Molecular characterization of ‘*Ca. P. solani*’ associated with potato and insects in Serbia was based on a multilocus typing of: i) *tuf* gene encoding translation elongation factor Tu, ii) *stamp* gene encoding the antigenic membrane protein, and iii) *vmp1* gene encoding a putative membrane protein. The *tuf* gene was amplified in a nested PCR analysis using the primer pairs Tuf1f/r and TufAYf/r, following a protocol described by Langer and Maixner (2004). PCR products were subjected to restriction analysis using *Hpa*II endonuclease (Fermentas, Lithuania) according to the manufacturer’s instructions. The restriction products were separated by QIAxcel advanced system (Qiagen) for automated

capillary electrophoresis, using a Screening Gel Cartridge (Qiagen). Following parameters were applied for fragments separation: sample injection voltage 5 kV, sample injection time 8 s, separation voltage 6 kV and separation time 320 s. The QX alignment marker for 15 bp/5 kb (Qiagen) and the QX DNA size marker FX174/HaeIII (Qiagen) were used to align the resulting restriction fragments and for fragment size comparisons, respectively. Reference strains used for comparison of *tuf* gene restriction profiles were the *tuf*-a type from naturally infected *H. obsoletus* from the Middle-Rhine and the *tuf*-b from populations of the same species originating from the Mosel region of Germany (kindly provided by Maixner M., Bernkastel-Kues).

The *stamp* gene was amplified by nested PCR with StampF/R0 primers followed by StampF1/R1, with PCR conditions according to Fabre et al. (2011). Sequencing of the *stamp* gene was performed by Macrogen Inc. (Seoul, Korea), and sequences were deposited in the GenBank database under the accession numbers KP877583–KP877604. The *stamp* sequences were edited using FinchTV v.1.4.0. (<http://www.geospiza.com>), and compared to the ‘*Ca. P. solani*’ reference strains using ClustalW integrated into a MEGA5 software (Tamura et al. 2011).

A fragment of the *vmp1* gene was amplified in a nested PCR with the primer pair StolH10F1/R1

(Cimerman et al. 2009), followed by the inner primer pair TYPH10F/R (Fialová et al. 2009). The TYPH10F/R amplicons were digested with *RsaI* restriction enzyme. The restriction fragments were separated by capillary electrophoresis as described above. The reference strains for *vmp1* digestion pattern comparison were taken from Cvrković et al. (2014). For the *vmp1* profiles identified as V2-TA and V7-A, additional *TaqI* and *AhlI* digestions were performed to distinguish them from the reference V2 and V7 profiles, respectively.

### Field-cage transmission trials

Transmission trials of ‘*Ca. P. solani*’ to potato were performed in the experimental field of the Institute for Plant Protection and Environment in Zemun, following the method described by Jović et al. (2007). Experiments were conducted with naturally ‘*Ca. P. solani*’-infected adults of *H. obsoletus* and *R. panzeri* that were previously found harboring the pathogen and occurring in significant numbers in the potato fields under study. Four net cages (2 × 2 × 2.5 m), two for each cixiid species, were set up in the semi-field conditions on May 8th, 2014. In each cage, a total of 24 plants of potato were seeded (variety Lizeta/6 plants in 4 rows).

**Experiment with *Hyalesthes obsoletus*** On July 7th, 2014, 150 adults of *H. obsoletus* were collected from *Convolvulus arvensis* in the bordering rows of the potato field on the locality Bački Petrovac. On this site we have previously captured specimens carrying the ‘*Ca. P. solani*’ (Table 1). *Hyalesthes obsoletus* adults were released in one field cage with healthy potato seedlings, while the other cage was used as a control. The plants from both cages were sampled on August 24th, 2014 and submitted to molecular analysis.

**Experiment with *Reptalus panzeri*** In total, 120 adults of *R. panzeri* had been collected on July 8th, 2014 from *Sorghum halepense* and potato plants on the locality Srednjevo with previously detected presence of naturally ‘*Ca. P. solani*’-infected populations of this cixiid. Captured *R. panzeri* specimens were released in one cage with potato seedlings, while the other cage was used as a control. On August 25th, 2014 plants from the test and control cages were sampled and analyzed for the phytoplasma presence.

DNA was isolated from leaves and/or aerial tubers and PCR conducted using the ‘*Ca. P. solani*’ - specific

stol11 nested PCR protocol to identify the phytoplasma. For further characterization, *tuf*, *stamp* and *vmp1* genes were amplified and analyzed, as described above.

## Results

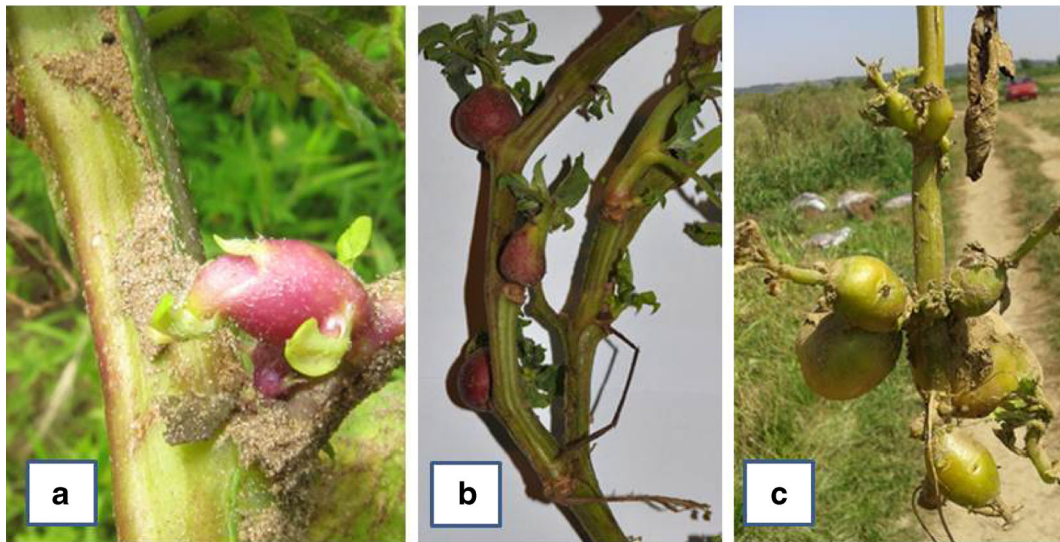
### Detection and characterization of ‘*Ca. P. solani*’ in plant material

At the time of sampling, the symptoms observed in the potato fields on 12 localities in northern Serbia varied from leaf yellowing to fully developed symptoms in form of aerial tubers with purplish discoloration on stems or on the ground surface, rubbery stems and tubers, shortened internodes or swollen stems (Fig. 1). The presence of ‘*Ca. P. solani*’ was identified in 55 out of 92 sampled potato plants (60 %) expressing symptoms from leaf upward rolling and yellowing to aerial tubers development and spongy stems, on all inspected sites (Table 1). The remaining 37 plants that were tested negative expressed only symptoms of leaf yellowing with no aerial tubers formation.

The *tuf* gene was successfully amplified in all ‘*Ca. P. solani*’ positive samples and digested with *HpaII* endonuclease. Comparison of the restriction profiles with the reference strains determined that all 55 potato plants were infected with a *tuf-b* type.

Characterization of the *stamp* gene identified six ‘*Ca. P. solani*’ genotypes associated with the infected potato plants in Serbia. BLAST analysis of the *stamp* sequences determined the 100 % identity with one of the reference strains either Rqg31 (KC703017), Rqg50 (KC703019), STOL (FN813261), Rpm35 (KC703015), M5 (KP337316) or BG4560 (FN813252) (Table 3). *RsaI* digestion of *vmp1* amplicons distinguished four profiles corresponding to the reference strains V2-TA, V7-A, V4, V14. Additional restriction analyses with *Taq* and *AhlI* confirmed the trueness of the genotypes previously identified as V2-TA and V7-A, discriminating them from the referent V2 and V7, respectively.

In terms of comprehensive diversity of ‘*Ca. P. solani*’, the following nine *tuf/stamp/vmp1* genotypes were characterized in association with the potato plants in the field: *tuf-b*/STOL/V2-TA (22 %), *tuf-b*/M5/V14 (14 %), *tuf-b*/Rqg31/V14 (13 %), *tuf-b*/Rqg31/V4 (11 %), *tuf-b*/Rpm35/V14 (11 %), *tuf-b*/Rqg50/V4 (11 %), *tuf-b*/Rqg31/V2-TA (9 %), *tuf-b*/Rqg50/V14 (7 %) and *tuf-b*/BG4560/V7-A (2 %) (Table 3; Fig. 2).



**Fig. 1** Symptoms associated with the potato stolbur in Serbia. A – enlarged view of the aerial tuber on the potato stem with purplish discoloration produced in semi-field transmission experiment with *Hyalesthes obsoletus*; B – shortened internodes, swollen and rubbery

stems, with aerial tubers and purplish discoloration (locality Bački Petrovac); C – impact of the potato stolbur in the field - plants rapidly devastated and hyper production of non-consumable aerial tubers on the stems and the ground level (locality Kušići)

Inspection of potato fields for the weed assemblages revealed *Convolvulus arvensis* as prevalent and present on all localities. The wild compartment of plants in the potato fields also included *Chenopodium album*, *Sorghum halepense*, *Cirsium arvense*, *Dactylis glomerata*, *Amaranthus retroflexus*, *Cichorium intybus*, *Ambrosia artemisiifolia*, *Agropyrum repens*, *Portulaca oleraceae*, *Setaria viridis* (Table 1), most being common weeds in other crops besides potato fields in Serbia. The crops grown next to the inspected potato fields in northern Serbia were maize, wheat, bean, carrot, soya, cabbage, sunflower, pepper, tomato and grapevine (Table 1).

#### Diversity of Auchenorrhyncha fauna and association with ‘*Ca. P. solani*’

A qualitative analysis of the hemipteran fauna in the potato fields on 12 localities in Serbia determined diversity of 16 species (Table 2). Seven species belong to the family Cicadellidae, three were Cixiidae, two Delphacidae, while the families Aphrophoridae, Tettigometridae, Issidae and Cercopidae were present with one species. *Psammotettix alienus* and *H. obsoletus* were the species with the widest dispersal, being collected from 10 inspected potato fields, followed by *R. panzeri* on eight, and *R. quinquecostatus* and *Euscelis incisus* on seven localities. Other hemipteran

species have been detected in one to five of the inspected potato fields (Table 2). Overall, the most dominant species in terms of abundance was *H. obsoletus* with 118 specimens collected, while the number of other captured planthoppers and leafhoppers ranged from 1 to 60 specimens (Table 2). In spite of a significant number of Auchenorrhyncha species captured in the potato fields, ‘*Ca. P. solani*’ was detected only in *H. obsoletus*, *R. panzeri* and *R. quinquecostatus* (Tables 1 and 3).

Molecular analyses determined the presence of ‘*Ca. P. solani*’ in 38 out of 118 captured *H. obsoletus* specimens (32 %) from eight localities (80 %) (Table 1). RFLP analysis of the *tuf* gene determined a *tuf-b* type in all insects harboring the phytoplasma. The *stamp* genotyping identified the presence of strains sharing a 100 % identity with reference strains Rqg31, Rqg50 or Rpm35 (Table 3). Digestion of the *vmp1* profiles determined association with V2-TA, V14 and V4 genotypes. Overall, six *tuf/stamp/vmp1* genotypes were detected to be harbored by the field collected *H. obsoletus*: *tuf-b/Rqg31/V2-TA* (21 %), *tuf-b/Rqg50/V4* (18 %), *tuf-b/Rqg31/V4* (18 %), *tuf-b/Rqg31/V14* (16 %), *tuf-b/Rpm35/V14* (16 %), and *tuf-b/Vv24/V4* (11 %) (Table 3; Fig. 2).

In total, 16 out of 57 captured specimens of *R. panzeri* (28 %) were determined to carry ‘*Ca. P. solani*’, on five locations where sampled (62.5 %)

**Table 3** ‘*Ca. P. solani*’ genotypes associated with field collected hemipterans and affected potato plants from twelve inspected locations in northern Serbia

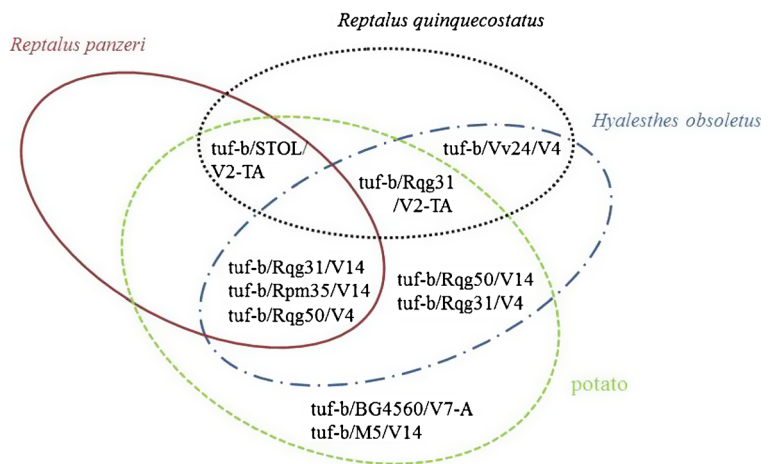
Stolbur phytoplasma comprehensive genotypes			Host	No of isolated genotypes
<i>tuf</i> <sup>a</sup>	<i>Stamp</i> <sup>b</sup> (isolate/Acc. No.)	<i>vmpI</i> <sup>c</sup>		
tuf-b	STOL (PS1/KP877583)	V2-TA	Potato	12
tuf-b	STOL (PS1Rp/KP877584)	V2-TA	<i>R. panzeri</i>	8
tuf-b	STOL (PS1Rq/KP877585)	V2-TA	<i>R. quinquecostatus</i>	5
tuf-b	Rpm35 (PS7/KP877596)	V14	Potato	6
tuf-b	Rpm35 (PS7Ho/KP877597)	V14	<i>H. obsoletus</i>	6
tuf-b	Rpm35 (PS7Rp/KP877598)	V14	<i>R. panzeri</i>	2
tuf-b	M5 (PS3/KP877587)	V14	Potato	8
tuf-b	BG4560 (PS2/KP877586)	V7-A	Potato	1
tuf-b	Rqg31 (PS4/KP877588)	V4	Potato	6
tuf-b	Rqg31 (PS4Ho/KP877589)	V4	<i>H. obsoletus</i>	7
tuf-b	Rqg31 (PS5/KP877590)	V14	potato	7
tuf-b	Rqg31 (PS5Ho/KP877591)	V14	<i>H. obsoletus</i>	6
tuf-b	Rqg31 (PS5Rp/KP877592)	V14	<i>R. panzeri</i>	4
tuf-b	Rqg31 (PS6/KP877593)	V2-TA	Potato	5
tuf-b	Rqg31 (PS6Ho/KP877594)	V2-TA	<i>H. obsoletus</i>	8
tuf-b	Rqg31 (PS6Rq/KP877595)	V2-TA	<i>R. quinquecostatus</i>	2
tuf-b	Rqg50 (PS8/KP877599)	V4	Potato	6
tuf-b	Rqg50 (PS8Ho/KP877600)	V4	<i>H. obsoletus</i>	7
tuf-b	Rqg50 (PS8Rp/KP877601)	V4	<i>R. panzeri</i>	2
tuf-b	Rqg50 (PS9/KP877602)	V14	Potato	4
tuf-b	Vv24 (PS10Ho/KP877603)	V4	<i>H. obsoletus</i>	4
tuf-b	Vv24 (PS10Rq/KP877604)	V4	<i>R. quinquecostatus</i>	3

<sup>a</sup> *Tuf HpaII* RFLP profiles according to Langer and Maixner (2004)

<sup>b</sup> *Stamp* genotypes represented by the reference strains (Fabre et al. 2011; Cvrković et al. 2014; Atanasova et al. 2015)

<sup>c</sup> *VmpI* RFLP profiles according to Murolo et al. (2010, 2013). Profiles V2-TA and V7-A were determined by digestion using *RsaI* and *TaqI* + *AluI* enzymes (aberration -TA) or *RsaI* and *AluI* (aberration -A) according to Cvrković et al. (2014)

**Fig. 2** Propagation pathways of ‘*Ca. P. solani*’ strains associated with potato (green circle), *R. panzeri* (red circle), *R. quinquecostatus* (black circle) and *H. obsoletus* (blue circle) based on the multilocus typing. *Tuf/stamp/vmpI* genotypes shared by potato plants and cixiid species were designated in the circles intersections



(Table 3). Digestion of the *tuf* gene amplicons determined only the *tuf-b* type. Amplified strains were identical with one of the reference *stamp* genotypes Rqg31, Rqg50, Rpm35 or STOL. The *RsaI* restriction profiles of the *vmp1* gene were corresponding to the reference genotypes V2-TA, V4 or V14. Combined characterization of three genes revealed four genotypes carried by the *R. panzeri* i.e. *tuf-b*/STOL/V2-TA (50 %), *tuf-b*/Rqg31/V14 (25 %), *tuf-b*/Rqg50/V4 (12.5 %) and *tuf-b*/Rpm35/V14 (12.5 %) (Table 3; Fig. 2).

'*Ca. P. solani*' was identified in ten specimens out of 38 captured of *R. quinquecostatus* (26 %) from three localities (25 %) (Table 1). Multilocus typing detected three genotypes *tuf-b*/STOL/V2-TA (50 %), *tuf-b*/Rqg31/V2-TA (20 %) and *tuf-b*/Vv24/V4 (30 %) (Table 3; Fig. 2).

#### Field-cage transmission trials

**Experiment with *Hyalesthes obsoletus*** At the time of sampling in the cage with plants exposed to *H. obsoletus*, the symptoms of potato stolbur disease were developed in form of leaves yellowing, rubbery stems, shorten internodes and aerial tubers on stems with purplish discoloration (Fig. 1A). Plants from the control cages remained symptomless. PCR analysis confirmed the presence of '*Ca. P. solani*' in 23 out of 24 potato plants exposed to *H. obsoletus* (96 %), while the control plants all tested negative. Restriction analysis of the *tuf* gene identified the *tuf-b* type in all infected potato plants. Comparison of the *stamp* gene sequences determined the 100 % identity with one of the referent genotypes Rqg31, Rqg50 or Rpm35 (Table 4). Amplified *vmp1* genes were digested with *RsaI* producing the profiles corresponding to the referent isolates V2-TA, V14 and V4. Additional digestion with *Taq* and *AfuI* distinguished the V2-TA profile from the referent V2. Combining the three genes, six genotypes were determined in the transmission tests with *H. obsoletus* i.e. *tuf-b*/Rqg31/V4 (26.1 %), *tuf-b*/Rqg31/V2-TA (17.3 %), *tuf-b*/Rqg50/V4 (17.3 %), *tuf-b*/Rqg31/V14 (13.1 %), *tuf-b*/Rqg50/V14 (13.1 %), and *tuf-b*/Rpm35/V14 (13.1 %).

**Experiment with *Reptalus panzeri*** In the cage with potato plants exposed to *R. panzeri*, fully developed symptoms associated with '*Ca. P. solani*' were registered on 11 plants (45.8 %), including aerial tubers with purplish discoloration and spongy stems. Other 13 exposed

plants in the cage remained asymptomatic. Molecular analyses detected phytoplasma in all plants expressing typical symptoms, whereas all other test plants were phytoplasma free. Control plants showed no symptoms development and tested negative for the phytoplasma presence. Characterization of the '*Ca. P. solani*' strains transmitted to potato by *R. panzeri* based on the sequences of the *tuf*, *stamp* and *vmp1* genes, revealed a single genotype *tuf-b*/STOL/V2-TA associated with all infected plants (Table 4).

#### Discussion

Pathways of transmission and propagation are determined by the interaction between the host plants of phytoplasma and its insect vectors. '*Ca. Phytoplasma solani*' has a wide range of diverse host plants, but many of them represent dead-end hosts for this phytoplasma because the vectors do not develop on them (reviewed in EFSA Panel on Plant Health 2014). However, '*Ca. P. solani*' continually persists in the natural compartment hosted by plants such as *Convolvulus arvensis*, *Calystegia sepium*, *Urtica dioica*, *Sorghum halepense*, *Cirsium arvense* which are reservoirs of phytoplasma, as well as the hosts of its vectors (Langer and Maixner 2004; Bressan et al. 2008; Jović et al. 2009).

Epidemiological studies of '*Ca. P. solani*' associated with Bois Noir disease and maize redness in Serbia provided molecular and experimental evidence of *H. obsoletus*, *R. panzeri* and *R. quinquecostatus* involvement in transmission and propagation of this phytoplasma (Jović et al. 2007, 2009; Cvrković et al. 2014). *Hyalesthes obsoletus* and *R. panzeri* were confirmed vectors, while *R. quinquecostatus*, although failed to transmit phytoplasma in the experiments, has never been discarded as a potential vector considering a substantial rate of field collected specimens carrying '*Ca. P. solani*' phytoplasma (Cvrković 2010; Cvrković et al. 2014).

Widespread occurrence of potato stolbur disease in Serbia corresponds to the presence of known cixiid vectors in the potato fields, as well as the weeds known as natural reservoirs of '*Ca. P. solani*' and host plants of the vectors. Overall, 16 hemipteran species were captured in potato fields on twelve localities, but only *H. obsoletus*, *R. panzeri* and *R. quinquecostatus* were tested positive for '*Ca. P. solani*'. *Hyalesthes obsoletus* was the most common species present on ten localities,



**Table 4** Molecular characterization of ‘*Ca. P. solani*’ strains associated with field collected *H. obsoletus* and *R. panzeri* and transmitted to potato plants in field-cage experiments

Vector species	Collection site/weeds in the surrounding	No of released insects	No of exposed plants	No of symptomatic plants*	No of ‘ <i>Ca. P. solani</i> ’-infected plants	<i>tuf/stamp/vmp1</i> ‘ <i>Ca. P. solani</i> ’ genotype (No of plants)
<i>Hyalosthes obsoletus</i>	Bački Petrovac/ <i>Convolvulus arvensis</i>	150	24	23	23	tuf-b/Rqg31/V4 (6)
						tuf-b/Rqg31/V14 (3)
						tuf-b/Rqg31/V2-TA (4)
						tuf-b/Rqg50/V4 (4)
<i>Reptalus panzeri</i>	Srednjevo/ <i>Sorghum halepense</i>	120	24	11	11	tuf-b/Rqg50/V14 (3)
						tuf-b/Rpm35/V14 (3)
						tuf-b/STOL/V2-TA (11)

\*symptoms were in form of leaves yellowing, rubbery stems, shorten internodes and aerial tubers with purplish discoloration in both field- cage experiments. There were no differences between the symptoms developed on potato plants infected by *H. obsoletus* or *R. panzeri*

followed by *R. panzeri* detected on eight and *R. quinquecostatus* on seven out of 12 inspected localities.

The most dominant weed present in all inspected potato fields was *Convolvulus arvensis*, one of the major ‘*Ca. P. solani*’ reservoirs responsible for persistence of phytoplasma. In literature, the *tuf-b* strain type of ‘*Ca. P. solani*’ was described to be primarily associated with bindweed (reviewed in Cvrković et al. 2014). Among other native plants present in the potato fields, *Cirsium arvense* and *Sorghum halepense* were previously reported harboring ‘*Ca. P. solani*’ in Serbia (Jović et al. 2009; Cvrković 2010), thus represent potential natural reservoirs as well. Moreover, the same weeds are suitable host plants for cixiid vectors. For example, *R. panzeri* nymphs were reported feeding on roots of *S. halepense*, *C. arvensis* and *C. arvense* in maize fields in Serbia (Jović et al. 2009), while *C. arvensis* is a known host for nymphs and adults of *H. obsoletus* (Holzinger et al. 2003). Additional factor which could contribute to the growing epidemics of potato stolbur disease in terms of alternative pathways of horizontal propagation is the fact that potato fields in northern Serbia are surrounded by the crops which had been previously reported to host the ‘*Ca. P. solani*’, e.g. maize (Jović et al. 2007), grapevine (Cvrković et al. 2014), and garden bean (Mitrović et al. 2015a).

Although the adults of *R. panzeri*, *R. quinquecostatus* and *H. obsoletus* were infected with ‘*Ca. P. solani*’ in the inspected potato fields in a significant rate, it was unclear when these insects acquired the phytoplasma. Cixiids are characteristics for laying eggs in the soil surrounding the host plants and the nymphs feeding on the roots. Therefore, acquisition of ‘*Ca. P. solani*’ may be achieved by overwintering nymphs feeding on infected roots of some of the natural reservoirs, and then transmission to potato plants by the cixiid adults during their activity from mid-June onwards.

Polyphagous vectors could contribute to genetic differentiation and local adaptation of phytoplasma and consequently to different transmission scenarios (reviewed in Cvrković 2010). Characterization of ‘*Ca. P. solani*’ strains associated with potato plants and insects provided molecular evidence on plausible pathways underlying the epidemics in the field. Combined analyses of the *tuf*, *stamp* and *vmp1* genes detected ten ‘*Ca. P. solani*’ genotypes, with no clear species-specific association with insect vectors and independent epidemiological cycles of potato stolbur. Moreover,

genotypes detected in potato fields matched the isolates from the vineyards and maize, as well as insects sampled in these crops in Serbia. It indicates several routes of horizontal transmission through exchange of ‘*Ca. P. solani*’ strains between the crops via polyphagous vectors and their host plants within the weed compartment.

Genotype tuf-b/STOL/V2-TA was reported as the most dominant in the field collected potato plants, *R. panzeri* and *R. quinquecostatus*. The results obtained in the semi-field experiment confirmed that *R. panzeri* can successfully transmit tuf-b/STOL/V2-TA strain of ‘*Ca. P. solani*’ to potato plants. Prevalence of this genotype in the field collected plants and two cixiid species indicates several transmission pathways plausible in situ. This assumption is substantiated by the previous reports of tuf-b/STOL/V2-TA genotype dominance in grapevine in Serbia, *R. quinquecostatus* from vineyards, *R. panzeri* collected from maize fields, as well as successful transmission of this genotype to grapevine by *R. panzeri* collected from the vineyards in Serbia (Cvrković et al. 2014). Clearly the plants responsible for the maintenance of this genotype in high frequency in the natural environment are common weeds in different crops and also host plants shared by the vectors.

Genotype tuf-b/Rqg31/V4 was reported for the first time in Serbia, harbored by field collected potato plants and *H. obsoletus*, and successfully transferred by this cixiid to potato plants in the transmission trial. They also shared other genotype tuf-b/Rqg31/V2-TA, including field collected *R. quinquecostatus* as well. This genotype has been previously registered in grapevine and *R. quinquecostatus* populations captured in Serbian vineyards (Cvrković et al. 2014), grapevine in Austrian vineyards (Aryan et al. 2014) and *H. obsoletus* associated with *Convolvulus arvensis* in Montenegro (Kosovac et al. 2015).

Two genotypes tuf-b/Rqg31/V14 and tuf-b/Rpm35/V14 were detected in the field collected potato plants, *R. panzeri*, *H. obsoletus* and experimental plants exposed to *H. obsoletus*. First one has been unknown to date in Serbia, but had been registered in *H. obsoletus* associated with *Convolvulus arvensis* from Montenegro (Kosovac et al. 2015). The second genotype was detected in *R. panzeri* populations from maize fields in Serbia (Cvrković et al. 2014) and grapevine, *C. arvensis* and *H. obsoletus* populations associated with *C. arvensis* and *Vitex agnus-castus* in Montenegro (Kosovac et al. 2015).

Genotype tuf-b/Rqg50/V4 was isolated from the field sampled potato, *H. obsoletus*, *R. panzeri* and potato from the transmission test with *H. obsoletus*. This genotype has been found previously in *R. quinquecostatus* and *R. panzeri* collected from the Serbian vineyards (Cvrković et al. 2014), grapevine and *H. obsoletus* from *C. arvensis* in Montenegro (Kosovac et al. 2015) and grapevine and *R. panzeri* from Austrian vineyards (Aryan et al. 2014).

Genotypes tuf-b/BG4560/V7-A and tuf-b M5/V14 were isolated only from the field collected potato plants. The first genotype has been reported from *R. quinquecostatus* from Serbian vineyards (Cvrković et al. 2014), while the second genotype from *H. obsoletus* specimens associated with *C. arvensis* in Macedonian vineyards (Atanasova et al. 2015).

A tuf-b/Rqg50/V14 genotype was solely restricted to the field collected plants and potato exposed to *H. obsoletus* in transmission trial. It has already been reported in association with grapevine (Cvrković et al. 2014), garden bean (Mitrović et al. 2015a) and carrot (data not shown) in Serbia, *H. obsoletus* in Macedonian vineyards (Atanasova et al. 2015) and grapevine in Italy (Murolo and Romanazzi 2015).

Several scenarios might be underlying the growing epidemics of potato stolbur involving the three cixiids interacting. With potato plants and insects carrying the same genotypes, *H. obsoletus* and *R. panzeri* should be considered to play an important role as natural vectors in the epidemiology of the potato stolbur in Serbia. Semi field experiments confirmed the ability of the two tested cixiid species to transmit ‘*Ca. P. solani*’ to potato, however with a twice higher vector performance expressed by *H. obsoletus*. Genotypes isolated from the infected plants in the semi-field experiments corresponded to the genotypes identified in field collected plants and insects, confirming the involvement of both cixiids in the propagation of ‘*Ca. P. solani*’ in potato fields in Serbia.

*Reptalus quinquecostatus* is reported as a natural carrier of ‘*Ca. P. solani*’ (Trivellone et al. 2005; Cvrković et al. 2014), but never confirmed as the vector. However, this cixiid should not be discarded as a potential link in the propagation of stolbur disease within the potato fields. The genotype tuf-b/BG4560/V7-A, reported only from the potato plants, has been detected so far only in *R. quinquecostatus* from Serbian vineyards (Cvrković et al. 2014). In the specimens collected in potato fields, *R. quinquecostatus* predominantly carried tuf-b/STOL/V2-TA which was the most

dominant genotype isolated from the potato plants in the field as well. The same genotype this cixiid also shares with *R. panzeri* indicating the possibility of joint host plants within the weed assemblages, from which they acquire or infect with ‘*Ca. P. solani*’. Furthermore, *R. quinquecostatus* shares the genotypes tuf-b/Vv24/V4 and tuf-b/Rqg31/V2-TA with *H. obsoletus*.

High incidence and wide dispersal of potato stolbur disease in northern Serbia clearly indicate a progressive epidemic. It undoubtedly confirms the need for urgent actions to be undertaken in terms of prevention of further propagation and yield losses. However, establishment and spread of ‘*Ca. Phytoplasma solani*’ cannot be controlled easily, being influenced by the prevalence of natural reservoirs in the weed compartment and by the presence and density of vector(s) populations.

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