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Original scientific paper

A novel *Wolbachia ftsZ* genotype in '*Candidatus* Phytoplasma solani' planthopper vector *Hyalesthes obsoletus* (Hemiptera: Fulgoromorpha: Cixiidae) associated with *Convolvulus arvensis*

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SUMMARY

Hyalesthes obsoletus (Hemiptera: Fulgoromorpha: Cixiidae) is a pan-European polyphagous planthopper known as a significant vector of the plant pathogenic bacterium '*Candidatus* Phytoplasma solani' (stolbur phytoplasma), which poses threats to various agricultural crops. A population of *H. obsoletus* associated with *Convolvulus arvensis* in Serbia was studied to investigate the presence and genetic diversity of *Wolbachia*, an endosymbiotic bacterium known for its promising biological control applications. Both insect-associated microorganisms, '*Ca*. P. solani' and *Wolbachia*, were found in the assessed *H. obsoletus* population. The analyzed vector population had a '*Ca*. P. solani' infection rate of 50%, while *Wolbachia* showed a high infection rate of 80%. *Wolbachia* presence displayed minimal variation across genders and was independent of individuals' phytoplasma-infection status. Genotyping of the identified '*Ca*. P. solani' strains revealed four previously described *stamp* genotypes (Rqg50/St1, Rqg31/St2, STOL/St4 and M5/St28). Notably, a single novel *Wolbachia ftsZ* genotype, designated WHo1, was found in the assessed *H. obsoletus* population, providing a valuable insight into the genetic diversity of *Wolbachia* endosymbionts within the Cixiidae family. Phylogenetic analysis demonstrated intricate relationships between WHo1 and other *Wolbachia* strains infecting hosts from diverse hemipteran suborders. Although *Wolbachia*-based strategies show promise for phytoplasma vector control, further research is needed to elucidate its potential interactions with '*Ca*. P. solani' and its effects on vector reproduction and fitness.

Keywords: *Hyalesthes obsoletus*, '*Candidatus* Phytoplasma solani', stolbur phytoplasma, *Wolbachia*, endosymbionts

INTRODUCTION

Hyalesthes obsoletus Signoret (Hemiptera: Fulgoromorpha: Cixiidae) is a pan-European polyphagous cixiid planthopper whose range extends eastward to the Middle East across the Mediterranean basin (Hoch & Remane, 1985). This insect is a prominent vector of the plant pathogenic bacterium '*Candidatus* Phytoplasma solani' (class Mollicutes), the stolbur phytoplasma, associated with plant diseases collectively referred to as "stolbur diseases" (Quaglino et al., 2013; Jović et al., 2019). *Hyalesthes obsoletus* occupies a broad ecological niche, primarily inhabiting xerothermic and ruderal environments, but its capacity for habitat exploitation allows it to extensively utilize agricultural landscapes whenever suitable host plants are available (Hoch & Remane, 1985; Holzinger et al., 2003; Nickel et al., 2003; Kosovac et al., 2018). Despite its polyphagous feeding strategy, *H. obsoletus* displays obligate host plant specialization for oviposition and nymphal development, being restricted in this context to a narrow range of hosts, specifically four plant species: *Urtica dioica* L., *Convolvulus arvensis* L., *Vitex agnus-castus* L., and *Crepis foetida* L. (Sforza et al., 1999; Sharon et al., 2005; Kaul et al., 2009; Kessler et al., 2011; Kosovac et al., 2018). Harboring '*Ca*. P. solani' as natural reservoirs, these plants serve as the starting points for diverse epidemiological cycles vectored by associated *H. obsoletus* populations that threaten crop yields across various agroecosystems (Langer & Maixner, 2004; Kosovac et al., 2016; 2019; 2023a; 2023b). Populations of *H. obsoletus* associated with *C. arvensis* have been linked to several '*Ca*. P. solani'-associated diseases of the tuf-b epidemiology in Serbia, including stolbur disease of pepper and eggplant, *Bois noir* disease of grapevine, potato stolbur disease and Rubbery Taproot Disease (RTD) of sugar beet (Aleksić et al., 1967; Mitrović et al., 2016; Kosovac et al., 2019; 2023a). Recent findings suggest that *H. obsoletus ex C. arvensis* populations may contribute to maize redness and tobacco stolbur, further expanding the range of the cultivated plants threatened by this vector and associated phytoplasma (Kosovac et al., 2023b). Molecular methods have significantly improved the research of stolbur disease epidemiology, enabling the tracing of '*Ca*. P. solani' strains through reservoir plants, vectors, and crops (Langer & Maixner, 2004; Aryan et al., 2014; Kosovac et al., 2023a; 2023b), and paving the way for more effective disease management strategies. Since traditional control measures, such as weed control and insecticide applications, have shown limited effectiveness (Mori et al., 2016; Riedle-Bauer & Brader, 2023), the challenge of stolbur disease control necessitates exploration of novel methods, including biological control that involves nematodes, fungi, and endosymbiotic bacteria (Gonella et al., 2011; Chuche et al., 2016; Iasur-Kruh et al., 2017; Moussa et al., 2021).

Wolbachia Hertig (α-Proteobacteria) are intracellular gram-negative bacteria with a host range that includes arthropods and nematodes (Werren, 1997). A specific classification scheme has been proposed for *Wolbachia,* classifying them within monophyletic supergroups (A-S) (Lefoulon et al., 2020). These well-known endosymbionts not only manipulate host reproduction (Hilgenboecker et al., 2008; Werren et al., 2008), but also significantly impact various host characteristics, such as fitness, metabolism, immunity, and the native microbiome (Stouthamer et al., 1999; Kambris et al., 2009; Hosokawa et al., 2010; Duan et al., 2020; Ju et al., 2020). *Wolbachia*'s unique manipulation of host biology has made it a powerful tool for control of mosquito-borne diseases, relying on mechanisms such as cytoplasmic incompatibility (CI) and pathogen blocking (PB) (Ross et al., 2019; Shropshire et al., 2020). The reported ability of *Wolbachia* to hinder viral replication in mosquitos (Sullivan, 2020) offers an impactful strategy potentially applicable for controlling insect-vectored plant pathogens.

Evidence of the *Wolbachia* CI effect has been documented in the planthopper virus vectors *Laodelphax striatellus* (Fallén) and *Sogatella furcifera* (Horváth). Despite being infected with the same *Wolbachia* strain, these vectors exhibited varying levels of CI, which appeared to correlate with different quantities of *Wolbachia* present in males (Noda et al., 2001). Research on the planthopper vector *Nilaparvata lugens* (Stål) revealed that the introduction of a specific *Wolbachia* strain isolated from *L. striatellus* can induce CI within its population (Gong et al., 2020). Moreover, this intervention has been shown to suppress the replication and transmission of rice ragged stunt virus (RRSV), highlighting the promising potential of *Wolbachia*-based strategies for managing plant diseases transmitted by hemipteran vectors (Gong et al., 2020).

Wolbachia infection has been documented in European cixiids, with the reported data for *Pentastiridius leporinus* (Linné), a vector of '*Ca*. P. solani' and '*Candidatus* Arsenophonus phytopathogenicus' to sugar beet

and potato (Bressan et al., 2008; 2009; Rinklef et al., 2024). Regarding the planthopper *H. obsoletus*, the presence of *Wolbachia* in its populations has been reported in Italy, and the detected *Wolbachia* strains were classified to supergroup B based on phylogenetic analysis of the 16S rRNA gene sequence (Gonella et al., 2011). Furthermore, *Wolbachia* was found in *H. obsoletus* populations associated with various plants across different regions, including *U. dioica*, *Salvia sclarea* L., and *Lavandula angustifolia* Mill. in France, *C. arvensis* in Germany, and *V. agnus-castus* in Israel (Chuche et al., 2016; Iasur-Kruh et al., 2017). These findings have allowed further research on the presence, diversity and potential applications of *Wolbachia* biocontrol strategies for managing this phytoplasma vector.

The presented study investigates the presence of the bacterial endosymbiont *Wolbachia* in *H. obsoletus* population associated with *C. arvensis* in Serbia, the primary vector of '*Ca*. P. solani' in this region. A two-step approach was employed: (1) analyzing '*Ca*. P. solani' infection and diversity in *H. obsoletus* using the stolbur phytoplasma-specific *stamp* gene (Fabre et al., 2011), and (2) examining *Wolbachia* presence, frequency, and genetic diversity employing the *ftsZ* gene, a well-established phylogenetic marker for *Wolbachia* (Schulenburg et al., 2000; Lo et al., 2002). Moreover, we have evaluated a potential correlation between '*Ca*. P. solani' infection and *Wolbachia* presence within the studied *H. obsoletus* population.

MATERIAL AND METHODS

Sampling insect material

Adult specimens of *H. obsoletus* were collected in July 2021 from the experimental fields of the Institute of Field and Vegetable Crops in Novi Sad, Serbia (GPS: 45°19'25.9"N 19°49'08.7"E). Entomological nets and mouth aspirators were used to sweep individual *C. arvensis* plants or patches within sugar beet plots and along their borders. The collected insects were transferred to 2 ml plastic tubes (Sarstedt, Germany) filled with 96% ethanol and transported to the laboratory. Morphological identification of the specimens was performed using a Leica S9E stereomicroscope. Following the taxonomic key of Hoch and Remane (1985), the specimens were identified as *H. obsoletus* based on the white pronotum and further examination of male genitalia. After confirming their identity, the insects were preserved in 96% ethanol at 4°C until further analysis.

DNA extraction

Total genomic DNA was extracted from individual *H. obsoletus* specimens using a non-destructive SDS-based protocol described by Rees et al. (2001) and Mahuku (2004), further modified by Kosovac et al. (2018). Initially, specimens were punctured and incubated overnight at 56°C in TES buffer (0.5% SDS, 20 mM Tris-HCl, 10 mM EDTA) supplemented with proteinase K (187.5 µg/ml). After homogenate separation and chloroform treatment (11,000 rpm, 10 min, repeated), the upper aqueous phase was precipitated with isopropanol and centrifuged (13,000 rpm, 15 min). The resulting DNA pellet was washed with 96% ethanol, air-dried, and resuspended in 50 µl TE buffer (50 mM of Tris, 1 mM of EDTA, pH 7.6). Extracted DNA was stored at -20°C.

Molecular detection and characterization of '*Ca*. P. solani' in *H. obsoletus*

Detection of '*Ca*. P. solani' was conducted using a nested PCR procedure targeting the *stamp* gene, which encodes the antigenic membrane protein of stolbur phytoplasma, in a total of 50 *H. obsoletus* specimens (25 males and 25 females). The PCR procedure employed primer pairs StampF/StampR0 and StampF1/StampR1 (Table 1). The final 25 µl PCR mixture contained 2 µl of isolated DNA, 1xDreamTaq PCR Master Mix (Thermo Scientific, Vilnius, Lithuania), and 0.4 µM of each primer. Thermal protocol followed the conditions described by Fabre et al. (2011). The '*Ca*. P. solani' positive control was the referent strain 429/19 (Ćurčić et al., 2021) from a collection of the Laboratory of Phytopathology of the Institute of Pesticides and Environmental Protection in Belgrade. PCR products (5 µl) were separated on a 1% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator. Nested *stamp* PCR amplicons from 10 '*Ca*. P. solani'-positive samples (five males and five females) were sequenced using the StampF1 primer by a commercial service (Macrogen Inc., South Korea). The obtained sequences were analyzed using the FinchTV v.1.4.0 software (http://www.geospiza.com) and aligned with ClustalW within the MEGA 11 software (Thompson et al., 1994; Tamura et al., 2021). The obtained sequences were compared with *stamp* genotypes available in NCBI GenBank database using BLAST (Basic Local Alignment Search Tool).

Table 1. Primers used for '***Ca***. P. solani' and *Wolbachia* detection and genotypisation, with their respective nucleotide sequences

Targeted gene	Primer name	Primer sequence 5'- 3'	Literature
stamp	StampF	GTAGGTTTTGGATGTTTTAAG	Fabre et al. (2011)
	StampR0	AAATAAAAGAACAAGTATAGACGA	
	StampF1	TTCTTTAAACACACCAAGAC	
	StampR1	AAGCCAGAATTTAATCTAGC	
ftsZ	ftsZunif	GG(CT)AA(AG)GGTGC(AG)GCAGAAGA	Lo et al. (2002)
	ftsZunir	GG(CT)AA(AG)GGTGC(AG)GCAGAAGA	

Molecular detection and characterization of *Wolbachia* in *H. obsoletus*

Following the detection of '*Ca*. P. solani' in *H. obsoletus* samples, a subset of 20 specimens (10 males and 10 females) was selected for *Wolbachia* analysis. This subset included an equal number of '*Ca*. P. solani'-positive and '*Ca*. P. solani'-negative individuals of both sexes. *Wolbachia* detection utilized the conventional PCR targeting the *ftsZ* gene (*Wolbachia* cell division gene) with primers ftsZunif and ftsZunir (Table 1). The final 25 µl PCR reaction mix contained 1 µl of isolated DNA, while the other components remained consistent with the protocol for the *stamp* gene. A thermal protocol according to Krstić (2017) was employed. Visualization of PCR products was performed as previously described. All *ftsZ* PCR amplicons were commercially sequenced (Macrogen Inc., South Korea) using the ftsZunir primer and the obtained sequences were compared with those available in the NCBI GenBank database using BLAST.

Reconstructing phylogenetic network of '*Ca*. P. solani'

To assess the genetic relatedness of '*Ca*. P. solani' genotypes infecting the studied *H. obsoletus* population, a phylogenetic median-joining network analysis was performed. This analysis compared the detected *stamp* genotypes with previously reported genotypes found in *H. obsoletus ex C. arvensis* in Serbia or plants infected by this vector (Mitrović et al., 2016; Kosovac et al., 2019; Kosovac et al., 2023a; 2023b). These included genotypes Rpm35/St3 (acc. no. KC703015), Vv24/St30 (KC703022), St81 (OP156885), St89 (OP156893), St94 (OP156898), St95 (OP156899), St97 (OR667032), St98 (OR667033), St99 (OR667034), and St102 (OR667037). The network analysis utilized a 474 bp fragment of the '*Ca*. P. solani' *stamp* gene sequence. The software NETWORK version 10.2 (Fluxus Engineering) (Bandelt et al., 1999) was employed with default settings. An ε parameter value of 0 was used, and maximum parsimony post-processing was implemented to create a network with the shortest possible trees.

Reconstructing phylogeny of *Wolbachia*

A *Wolbachia* genotype network was calculated to visualize the relationships between the *ftsZ* sequences from *H. obsoletus* and those of previously detected *Wolbachia* strains in Hemiptera insects (suborders Fulgoromorpha, Cicadomorpha, Heteroptera, and Sternorrhyncha). Median-joining analysis was performed based on a trimmed 435 bp *ftsZ* gene fragment using the previously defined values and settings. To infer the evolutionary relationships among the *Wolbachia* strains, a maximum likelihood phylogenetic tree was reconstructed using MEGA 11 (Tamura et al., 2021). The General Time Reversible (GTR) model was employed as a model of nucleotide substitution with 1000 replications. To root the tree, we included an *ftsZ* sequence from *Nasonia vitripennis* (Walker) (Hymenoptera), belonging to *Wolbachia* supergroup A, whereas all other strains employed in the median-joining and maximum likelihood analyses belong to supergroup B.

RESULTS

Genetic diversity of '*Ca*. P. solani'

Molecular detection of '*Ca*. P. solani' in the studied *H. obsoletus* population revealed a 50% infection rate (25/50 individuals). Sequencing of the '*Ca*. P. solani' *stamp* gene in 10 selected samples identified four previously described genotypes: Rqg50/St1 (acc. no. KC703019), Rqg31/St2 (KC703017), STOL/St4 (FN813261), and M5/St28 (KP337316). Rqg31/St2 was the most prevalent genotype, detected in 5/10 samples (Table 2). M5/St28 and STOL/St4 were each detected in two samples, while Rqg50/St1 was identified in only one isolate. All four '*Ca*. P. solani' genotypes were identified in females, while only Rqg50/St1 was absent in males, suggesting a minimal sex bias in genotype distribution (Table 2).

A median-joining network of the 14 *stamp* genotypes revealed the highest diversification from STOL/St4 genotype with six genotypes: St89, St95, St97, St98. St99, St102, clustering in a subnetwork derived from STOL (Figure 1). Forming a distinct cluster in the network, genotypes St81 and St94 were found to diverge from the centrally positioned Rqg31/St2, differing from it by only one nucleotide each. The Rqg50/St1 genotype, distant by 6 nucleotides from Rqg31/St2, clustered closely with Vv24/St30, while the genotype M5/St28 displayed genetic proximity to the Rpm35/St3 genotype (Figure 1).

Table 2. An overview of the '*Ca*. P. solani' and *Wolbachia* strains genotyped in the *H. obsoletus ex C. arvensis* subset. The table lists the '*Ca*. P. solani' original genotype name and its „St" variant (reviewed in Kosovac et al., 2023a); abbreviation HobsCa - *H. obsoletus ex C. arvensis*.

DNA isolate ID	HobsCa female/male (♀/♂)	'*Ca*. P. solani' presence (+/-)	'*Ca*. P. solani' *stamp* genotype	*Wolbachia* presence (+/-)	*Wolbachia ftsZ* genotype
113/22	♀	+	Rqg31/St2	+	WHo1
117/22	♀	+	Rqg31/St2	+	WHo1
118/22	♀	+	M5/St28	+	WHo1
230/21	♀	+	Rqg50/St1	+	WHo1
233/21	♀	+	STOL/St4	+	WHo1
246/21	♂	+	Rqg31/St2	+	WHo1
247/21	♂	+	STOL/St4	-	-
250/21	♂	+	Rqg31/St2	+	WHo1
251/21	♂	+	M5/St28	+	WHo1
92/22	♂	+	Rqg31/St2	+	WHo1
110/22	♀	-	/	+	WHo1
111/22	♀	-	/	+	WHo1
112/22	♀	-	/	+	WHo1
240/21	♀	-	/	+	WHo1
241/21	♀	-	/	+	WHo1
93/22	♂	-	/	-	-
95/22	♂	-	/	+	WHo1
96/22	♂	-	/	+	WHo1
242/21	♂	-	/	+	WHo1
244/21	♂	-	/	+	WHo1

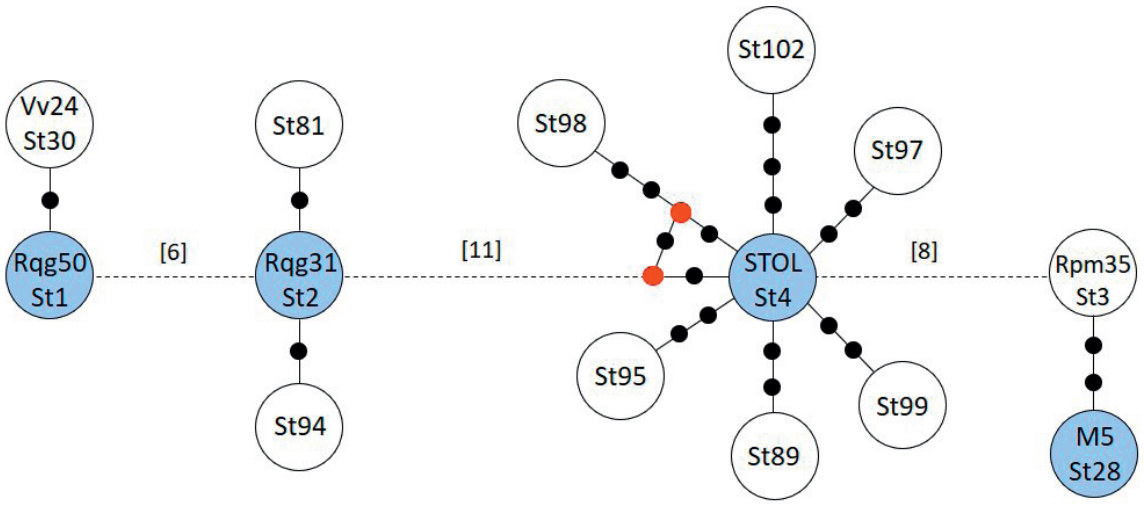

Figure 1. A median-joining network constructed using '*Ca*. P. solani' *stamp* genotypes found in the *H. obsoletus ex C. arvensis* population in this study (depicted with blue circles), and *stamp* genotypes previously found in populations of this vector associated with *C. arvensis* or transmitted by it on experimental plants (colourless circles). Within circles representing a specific *stamp* genotype, original genotype designations are provided alongside 'St' *stamp* sequence variant names (as reviewed in Kosovac et al., 2023a). The black dots on the connecting lines represent the number of nucleotide differences between linked genotypes. Dashed interconnecting lines represent disproportional distances between genotypes, with the number of mutations provided in square brackets above them. Red dots in the network are median vectors representing missing or unsampled intermediate genotypes.

Wolbachia infection patterns and *ftsZ* variability

Analysis of the *H. obsoletus* population revealed a high *Wolbachia*-infection rate, with 18/20 samples testing positive (Table 2). Notably, *Wolbachia* was detected in all ten female specimens (100% infection rate), while 8/10 males (80%) were positive. Sequence analysis of the *ftsZ* gene from these samples revealed the presence of a single *Wolbachia* genotype. Comparison with *ftsZ* sequences deposited in NCBI identified this genotype as a novel, so far unknown sequence variant, designated as WHo1 (acc. no. PP681126).

The analysis of *ftsZ* sequences using a median-joining network revealed a complex genetic relationship between *Wolbachia* and insect hosts (Figure 2A). The network encompassed *Wolbachia* strains from diverse hemipteran groups, including planthoppers (Fulgoromorpha), leafhoppers (Cicadomorpha), mirids and lace bugs (Heteroptera), and whiteflies and psyllids (Sternorrhyncha). The newly identified *Wolbachia* WHo1 *ftsZ* genotype from the planthopper *H. obsoletus* displayed intricate relationships with genotypes from all other insect groups. Within the network, a prominent double reticulation connected seven genotypes. WHo1 showed a genetic relatedness to *Wolbachia* strains found in the psyllid *Bactericera maculipenis* (abbreviated in Figure 2 as WBm) and the whitefly *Bemisia tabaci* (WBt). A *Wolbachia* strain from another planthopper, *Dictyophara europaea* (WDe), diverged from the whitefly *B. tabaci* (WBt) strain by three nucleotides. *Wolbachia* strains from the leafhopper *Philaenus spumarius* (WPhs1 and WPhs2) exhibited closer affinity to the mirid bug *Macrolophus pygmaeus* than to *Wolbachia* strains from the planthoppers (*H. obsoletus* and *D. europaea*). The network also revealed a second, distinct genetic cluster. This cluster comprised three *Wolbachia* strains from different suborders: a shared *ftsZ* genotype between the psyllid *Bactericera cockerelli* (WBc) and the lace bug *Pseudacysta perseae* (WPp), and a strain found in the leafhopper *Balclutha brevis* (WBb) (Figure 2A).

Maximum likelihood phylogenetic analysis corroborated the clustering pattern observed in the median-joining network (Figure 2A, 2B). All *ftsZ* sequences retrieved from the NCBI database for hemipteran hosts that belong to *Wolbachia* supergroup B, suggest the same affiliation for the newly described WHo1 genotype from *H. obsoletus*. Consistent with this, the maximum likelihood tree clearly separated the *N. vitripennis Wolbachia* strain (supergroup A) from the remaining sequences belonging to supergroup B

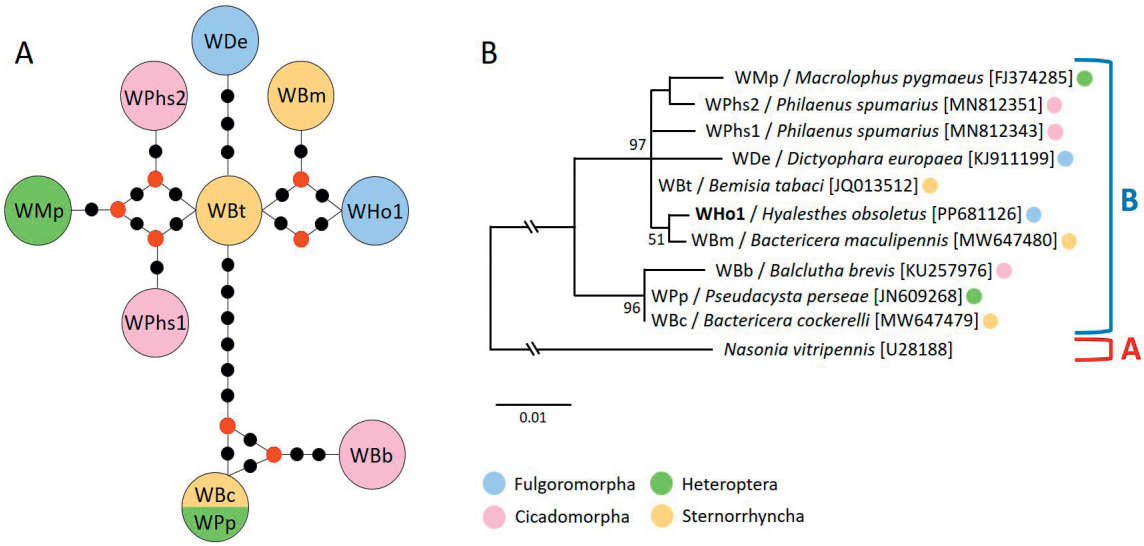

Figure 2. A – Median-joining network constructed using *Wolbachia ftsZ* genotypes found in *H. obsoletus ex C. arvensis* population in this study and genotypes from other hemipteran hosts. Each circle represents a unique *ftsZ* genotype, with given abbreviations provided for the purpose of this study and corresponding to the host. Black dots on connecting lines denote nucleotide differences between linked genotypes. Median vectors (red dots) indicate missing or unsampled intermediate genotypes. Colour coding (legend) corresponds to the specific suborder within the order Hemiptera. B – Maximum likelihood phylogenetic tree based on *Wolbachia ftsZ* genotypes including genotypes used in the median-joining network analysis (supergroup B) alongside a *Wolbachia* supergroup A strain isolated from *N. vitripennis*. Each branch is labelled with the *ftsZ* genotype abbreviation, insect host, and GenBank acc. number in square brackets. Circle colours next to the branches correspond to the specific Hemiptera suborder as defined in legend. Bootstrap support values above 50% are shown next to branches.

(Figure 2B). Within the B genetic group, the tree revealed two high-supported (96-97% bootstrap value) clades. Notably, the novel genotype WHo1 clustered closely with *Wolbachia* from *B. maculipennis* (WBm) within a secluded branch, overall mirroring the grouping observed in the median-joining network (Figure 2A). Likewise, genotypes WBb, WPp, and WBc formed a group on a separate branch, consistent with the network's second genetic cluster (Figure 2A).

One-sided co-occurrence of '*Ca*. P. solani' and *Wolbachia* in *H. obsoletus* population

Potential correlation between the occurrence of '*Ca*. P. solani' and *Wolbachia* in the *H. obsoletus* population was assessed through molecular detection and genotyping of both insect-associated microorganisms (Table 2). Nearly all samples positive for '*Ca*. P. solani' were found to be infected with *Wolbachia* (9/10), showing a 90% co-occurrence rate. However, the presence of *Wolbachia* in nearly all *H. obsoletus* individuals (18/20) was irrespective of sex and '*Ca*. P. solani' infection. Moreover, a single *Wolbachia* genotype, WHo1, was detected across all '*Ca*. P. solani'-infected samples, irrespective of the present *stamp* genotype. The two *H. obsoletus* individuals negative for *Wolbachia* were males, one infected with the '*Ca*. P. solani' STOL/St4 genotype and the other uninfected.

DISCUSSION

This study provides the first insight into *Wolbachia* presence and its genetic diversity within a population of the planthopper vector *H. obsoletus* associated with *C. arvensis* in Serbia. Additionally, the potential patterns of co-occurrence between *Wolbachia* and '*Ca*. P. solani' in assessed vector population were explored. Detection of four '*Ca*. P. solani' *stamp* genotypes (Rqg50/St1, Rqg31/St2, STOL/St4, and M5/St28) aligns with previous studies characterizing '*Ca*. P. solani' associated with *H. obsoletus ex C. arvensis* in Serbia (Mitrović et al., 2016; Kosovac et al., 2019; 2023a; 2023b). These genotypes belong to the tuf-b '*Ca*. P. solani' epidemiology, with STOL/St4 bridging both the tuf-b1 and newly described tuf-d cycles (Ćurčić et al., 2021; Kosovac et al., 2023a).

While the performed phytoplasma characterization relied solely on the *stamp* gene, it provided sufficient information to confirm the existing knowledge about '*Ca*. P. solani' epidemiology within this vector-host association.

The performed analysis of *Wolbachia* in the *H. obsoletus* population from Serbia, based on the *ftsZ* gene, provides some foundational data that pave the way for further investigation of this endosymbiont's role within this vector host. A novel *ftsZ* genotype was unveiled, WHo1, which lacks any matches with *Wolbachia* strains identified in other organisms. Identifying a novel *Wolbachia ftsZ* genotype in *H. obsoletus* expands the understanding of this endosymbiont diversity within the Cixiidae family. *Wolbachia* was previously characterized in populations of the planthopper *D. europaea* in Serbia, a vector of the *Flavescence dorée* phytoplasma (16SrV group) (Krstić et al., 2018). Our findings, demonstrating that the investigated *H. obsoletus* population harbours a single *Wolbachia* genotype (WHo1) alongside four '*Ca*. P. solani' genotypes, align with those of Krstić et al. (2018), revealing a higher level of diversity in the phytoplasma population (five genotypes) compared to the single *Wolbachia* strain observed within several assessed *D. europaea* populations. This consistency suggests a potential lack of direct correlation between the diversities of these co-occurring microorganisms within their insect hosts. However, Krstić et al. (2018) reported a higher *Wolbachia* infection rate in the vector's populations with low phytoplasma infection, suggesting a potential interaction between the two microorganisms. To gain a more reliable understanding of co-occurrence patterns between *Wolbachia* and '*Ca*. P. solani' in *H. obsoletus*, investigation across multiple vector's populations with varying phytoplasma infection rates would be valuable.

Further research, especially genotyping of the *Wolbachia* surface protein *wsp* gene and MLST analysis of the genes *gatB*, *coxA*, *hcpA*, and *fbpA*, should be conducted to thoroughly characterize the detected *Wolbachia* strains in *H. obsoletus* and precisely determine their sequence type (ST) and supergroup affiliation (Stouthamer et al., 1999; Baldo et al., 2006). Based on the performed phylogenetic analysis, it can be concluded that WHo1 genotype belongs to supergroup B, which is consistent with previously published data on this planthopper (Gonella et al., 2011). *Wolbachia*-infection levels detected in our study also follow a previously documented pattern with more females infected than males (Gonella et al., 2011). Full genotypisation of the *Wolbachia* strains infecting *H. obsoletus* is especially important since the presence of this endosymbiont was confirmed in its three out of four host plant associations (Chuche et al., 2016). It would be especially interesting to perform a comparative analysis of *Wolbachia* strains found in *H. obsoletus* and *P. leporinus* as both cixiids are vectors of '*Ca*. P. solani' (Bressan et al., 2008; 2009; Jović et al., 2019; Rinklef et al., 2024).

While *Wolbachia*-based strategies have been proven effective in targeting arbovirus transmission by female mosquitoes (reviewed in Gong et al., 2023), their applicability for controlling phytoplasma transmission still poses a challenge, given that both sexes are effective vectors (Alma et al., 2019). Although this endosymbiont holds promise for control of the vectors of plant pathogens (Gong et al., 2023), several key challenges remain. The most critical is identification of specific *Wolbachia* strains that induce CI, a necessary effect for population suppression, while the PB would be desirable to further enhance control. Unfortunately, many *Wolbachia* strains lack these effects. Once a suitable *Wolbachia* strain is identified and established within a vector's population, interventions like population suppression or replacement could be implemented in insect control programs.

The presented study, based on a limited sample size, cannot establish a correlation between *Wolbachia* and '*Ca*. P. solani' infection in *H. obsoletus*, nor any potential pathogen interference by *Wolbachia*. Future studies utilizing larger *H. obsoletus* sample size would elucidate the complex relations between *Wolbachia* and its insect host, as well as *Wolbachia*'s influence on vector-borne pathogen transmission dynamics (Zug & Hammerstein, 2015). Additionally, investigating the impact of the *Wolbachia* strain genotyped as WHo1 on the *ftsZ* gene on host fitness, as well as its potential for vector biocontrol within a multidisciplinary framework, holds potential for sustainable phytoplasma disease management. Therefore, further research on *Wolbachia* in *H. obsoletus* is a promising study field, as this vector plays a central role in disseminating '*Ca*. P. solani' across agroecosystems in Central and Southeastern Europe, especially considering the current limitations in stolbur disease control.

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Novi *ftsZ* genotip *Wolbachia* endosimbionta cikade *Hyalesthes obsoletus* (Hemiptera: Fulgoromorpha: Cixiidae) vektora '*Candidatus* Phytoplasma solani' asocirane sa *Convolvulus arvensis*

REZIME

Hyalesthes obsoletus (Hemiptera: Fulgoromorpha: Cixiidae) je polifagna cikada poznata kao značajan vektor fitopatogene bakterije '*Candidatus* Phytoplasma solani' (stolbur fitoplazma) koja nanosi štetu u proizvodnji brojnih poljoprivrednih kultura. U okviru odabrane populacije *H. obsoletus* asocirane sa biljkom *Convolvulus arvensis* u Srbiji, istraženi su prisustvo i genetička raznovrsnost endosimbiontske bakterije *Wolbachia*, poznate kao perspektivnog agensa u biološkoj kontroli. U ispitivanoj populaciji *H. obsoletus* pronađena su oba mikroorganizma, '*Ca*. P. solani' i *Wolbachia*. Stopa '*Ca*. P. solani' infekcije *H. obsoletus* populacije je bila 50%, dok je u slučaju *Wolbachia* iznosila 80%. Prisustvo *Wolbachia* nije bilo uslovljeno infekcijom fitoplazmom i pokazalo je minimalne varijacije između polova insekta. Genotipizacijom identifikovanih sojeva '*Ca*. P. solani' otkrivena su četiri prethodno opisana *stamp* genotipa (Rqg50/St1, Rqg31/St2, STOL/St4 i M5/St28). Novi *Wolbachia ftsZ* genotip, označen kao WHo1, je identifikovan u populaciji *H. obsoletus*, što pruža novi uvid u genetičku raznovrsnost *Wolbachia* endosimbionta insekata iz familije Cixiidae. Filogenetska analiza je pokazala složene odnose između WHo1 i *Wolbachia* sojeva iz domaćina različitih Hemiptera podredova. Iako strategije biološke kontrole zasnovane na *Wolbachia* imaju potencijal u kontroli vektora fitoplazmi, potrebna su dalja istraživanja kako bi se objasnile interakcije ovog endosimbionta sa '*Ca*. P. solani' kao i uticaj *Wolbachia* na reprodukciju i fitnes vektora.

Ključne reči: *Hyalesthes obsoletus*, '*Candidatus* Phytoplasma solani', stolbur fitoplazma, *Wolbachia,* endosimbionti