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Genetically divergent '*Candidatus* Phytoplasma solani' isolates in Croatian vineyard pathosystems suggest complex epidemiological networks

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Abstract

'Candidatus Phytoplasma solani' (CPs), a phytoplasma endemic to the Euro-Mediterranean basin is a causative agent of several plant diseases, including the grapevine yellows disease "bois noir" (BN). As different CPs strains have been shown to have different ecological reservoirs and pathways for spread, the genetic characterization of CPs strains is a prerequisite, and better control of BN relies on the identification of reservoir plants. The variability of the phytoplasma genotypes involved in the BN pathosystem in Croatian vineyards was assessed by a multilocus sequence typing (MLST) approach. The genotyping was performed on selected grapevine, wild plants, and insects collected within the eleven years of national survey conducted in all Croatian viticultural regions. The extensive *tuf, secY, stamp*, and *vmp1* genes-based MLST analyses revealed two new genotypes for *stamp* and *vmp1* genes, designated as ST59 and V28, respectively, and overall identified 28 different CPs MLST genotypes. The prevalent MLST genotype in grapevine CPsSqt21 (S6/ST6/V18/tuf-b2) was widespread in nine counties across Uplands, Slavonia, and Danube wine regions and was affiliated to the known vector *Hyalesthes obsoletus* and to *Urtica dioica*. The other two most frequent genotypes were the *U. dioica*-associated CPsSqt28 (S39/ST46/V3/tuf-a) and the *C. arvensis*-associated CPsSqt2 (S1/ST9/V4/tuf-b1). CPs of different vmp1 genotypes was also detected in *Cixius wagneri* specimens originating from different parts of Croatia. In addition, CPs was detected in several *Dichtyophara europaea* insects and in two new potential plant reservoirs *Ailanthus altissima* and *Robinia pseudoacacia*. The substantial number of found MLST genotypes indicates the presence of several independent epidemiological cycles and is certainly a consequence of a unique geographical position of Croatia, bridging the different eco-climatic areas of central and south-eastern Europe.

Keywords Grapevine, Bois noir, Sequence variants, MLST, Molecular epidemiology

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Background

Phytoplasmas encompass a very diverse group of wall-less plant pathogenic bacteria that infect numerous plant species worldwide and cause severe economic impacts to the agricultural sector (Hogenhout et al. 2008; Namba 2019). As intracellular parasites restricted to the plant phloem they depend on phloem-feeding insects for transmission (Weintraub and Beanland 2006). This "host switching" between two different kingdoms, plant and insect hosts, is a unique biological trait among mollicutes that can be linked to complex molecular mechanisms and genotypic variations (Oshima et al. 2011, Oshima et al. 2019).

Bois noir (BN) disease belongs to the grapevine yellows (GY) complex, that corresponds to phytoplasma-associated diseases which cause similar symptoms but have different aetiologies (Belli et al. 2010; ConsTable 2010). The cause of the BN disease has been attributed to '*Candidatus* Phytoplasma solani' (CPs), belonging to the 16SrXII-A ribosomal subgroup (Daire et al. 1993; Maixner 1994; Quaglino et al. 2013). Although widespread in Euro-Mediterranean region, BN disease is not epidemically transmitted within vineyards and is therefore regarded as less threatening in comparison to the quarantine classified, epidemic GY *Flavescence dorée* (FD) disease (Maixner 2011; Jeger et al. 2016).

However, increase of BN incidence has been reported in recent decades from various grapevine-growing regions marked by severe yield losses and reduced fruit quality in Europe (Aryan et al. 2014; Cvrković et al. 2014; Johannesen and Riedle-Bauer 2014; Atanasova et al. 2015; Ember et al. 2018). The increase in disease incidence and symptom severity is particularly accentuated in the vineyards with most susceptible cultivars such as Chardonnay, Riesling, Cabernet Sauvignon, Barbera, Sauvignon Blanc, and Sémillon (EFSA Panel on Plant Health (PLH), 2014; Ember et al. 2018).

While different polyphagous cixiid planthoppers (Hemiptera: Cixiidae) are reported to transmit CPs in many herbaceous and woody plants, *Hyalesthes obsoletus* Signoret is considered to be the principal vector of CPs to grapevine (Maixner et al. 1994). A role in CPs transmission to grapevine has been proven also for *Reptalus panzeri,* although that role is mainly restricted to Banat region of Serbia, and recently for *Dictyophara europea* (Cvrković et al. 2014, 2022). *H. obsoletus* can feed on various herbaceous plants, but it is most commonly associated with *Convolvulus arvensis* (field bindweed), *Urtica dioica* (stinging nettle), *Vitex agnus-castus* (monk's pepper/chaste tree), and *Crepis foetida* (stinking hawk's-beard) on which it completes its life cycle from eggs and nymphs to adults (Langer and Maixner et al. 2004; Sharon et al. 2005; Bressan et al. 2007; Johannesen et al. 2012; Kosovac et al. 2016, 2019). On the other hand, many plants (such as grapevine) act as occasional feeding source for *H. obsoletus*, thus representing dead-end hosts in the BN epidemiological cycle (Johannesen et al. 2008).

CPs spreads through highly complex disease cycle comprising several, possibly intermixed epidemiological networks (Langer and Maixner 2004; Jović et al. 2009; Kosovac et al. 2016, 2019; Mori et al. 2016; Quaglino et al. 2019). To enable identification of host plant-vector pairs in BN epidemiological pathways, various molecular methods have been implemented. CPs strains involved in BN disease can be tied to different epidemiological cycles through multilocus sequence typing (MLST). *Tuf* gene, encoding the translation elongation factor Tu (Schneider et al. 1997; Langer and Maixner 2004), *secY*, encoding a translocation protein (Fialová et al. 2009), and the two variable surface protein genes-*vmp1* (Cimerman et al. 2009; Fialová et al. 2009; Pacifico et al. 2009) and *stamp* (Fabre et al. 2011) are frequently used to evaluate CPs genetic diversity.

On the basis of the gene *tuf,* CPs strains are considered to be related to different natural epidemic cycles in the field. Two main genotypes: tuf-type a and b2 (known also as ab) are associated with stinging nettle whilst tuf-type b1 is found in bindweed and a broad range of other herbaceous host plants (Langer and Maixner 2004; Johannesen et al. 2008, 2012; Pacifico et al. 2009; Murolo et al. 2010; Aryan et al. 2014).

Grape growing and winemaking are an important part of Croatian heritage and cultural identity (Batović and Kukoč, 1987). The exceptional geographic position with three viticulture climate zones (Winkler et al. 1974) and tempestuous trading history enabled a great number of grape cultivars to be grown on Croatian territory during past centuries. Different climate and terrain conditions in four Croatian grapevine-growing regions (Fig. 1) (Law on wine 2019) affect the variety and quality of grape varieties resulting in production of unique regional wines. Moreover, for a small viticultural area, Croatia has a relatively high number of native cultivars constituting an important gene pool in Europe. Investigating the impact of CPs infection on international and indigenous grape varieties is therefore of the utmost importance, not just for the future of Croatian viticulture but also for the preservation of high genetic variability that lies behind some valuable traits (Maletić et al. 2015).

BN disease is widespread in Croatia with *H. obsoletus* considered to be the major vector of CPs to grapevine (Budinšćak et al. 2005; Mikec et al. 2006; Plavec et al. 2015; Plavec et al. 2018). Sporadic outbreaks occur in different grapevine-growing regions, in places with noticeable repercussions. Although several small-scale case studies were conducted over the years (Škorić et al.

Fig. 1 Map of the four Croatian grapevine growing regions according to the Law on wine (2019): Croatian Uplands (CUP) + Slavonia and Croatian Danube (SLD) (Continental Croatia), Croatian Istria and Kvarner (ISK) + Dalmatia (DAL) (Coastal Croatia), and administrative districts (counties) of Croatia. Abbreviations represent the counties sampled for CPs characterisation as presented in the Table 1: VS/Vukovar-Srijem, OB/Osijek-Baranja, BP/Brod-Posavina, PS/Požega-Slavonia, VP/Virovitica-Podravina, KK/Koprivnica-Križevci, ME/Međimurje, VŽ/ Varaždin, KZ/Krapina-Zagorje, SM/ Sisak-Moslavina, ZG/Zagreb, KA/Karlovac, IST/Istria, and ZD/Zadar. Abbreviations for counties where CPs positive samples are not found were omitted from the map. Map is generated by using the template map from https://d-maps.com/carte.php?num_car=5359&lang=en) (accessed on 24 May 2022)

1998; Šeruga et al. 2000; Mikec et al. 2006; Šeruga Musić et al. 2011; Plavec et al. 2015), we present the first comprehensive multigene typing of CPs isolates collected countrywide.

The main objectives of this study were: i) to assess the variability of CPs strains from naturally infected grapevines, wild plants, and insects by MLST, ii) to study the prevalence and distribution of CPs strains in Croatian grapevine-growing regions, and iii) to determine the presence of CPs strains in Auchenorrhyncha species in CPs affected vineyards.

Results

CPs detection in plants and insects

Throughout the national survey conducted from 2009 to 2020, typical symptoms of GY disease were observed in all Croatian grapevine growing regions. Continental Croatia (Croatian Uplands, Slavonia, and Croatian Danube) and a part of Coastal Croatia (Istria and Kvarner) were regularly affected. In the other part of Coastal Croatia (Dalmatia) symptoms of GY were rarely observed. During the survey period BN disease in vineyards was recorded in 14 out of 21 counties (Fig. 1).

The presence of CPs was confirmed in 510 samples out of 3336 grapevine samples analysed by triplex-real time PCR, while the significant part (675 samples) were positive for *Flavescence dorée* phytoplasmas (FDp) presence and partially subjected to MLST analysis as shown by Plavec et al. (2019). Among the other 89 collected and analysed plants, CPs was detected in *C. arvensis, Ailanthus altissima*, *Robinia pseudoacacia*, and *Polygonum aviculare*. Hemipteran insect species were sporadically collected and analysed from 2004 to 2020 and CPs was identified in 47 out of 267 specimens analysed, including *H. obsoletus, C. wagneri*, and *D. europaea. R. cuspidatus* was observed in several locations of the country but positive specimens were detected only in one of the previous studies (Additional file 1: Table S1; Mikec et al. 2006). A number of CPs positive isolates were chosen for genotyping based on their location in an attempt to include as many affected grapevine-growing counties (Fig. 1). In total, 78 plant and 20 insect samples were further characterised by MLST (Table 1).

CPs strain characterization by MLST sequence analysis

All four molecular markers were successfully amplified by nested PCR from most of the samples designated for the characterization. The phylogenetic analysis of *tuf* sequences showed the presence of three CPs tuf sequence types (tuf-a, tuf-b1, and tuf-b2) (data not shown). The most prevalent genotype was tuf-b1 (in 34/78 plant samples and 9/20 insect samples). Tuf-a genotype was identified in 17/78 plant samples while tuf-b2 was found in 27/78 plant and 11/20 insect samples. Tuf-a was detected only in grapevine samples, tuf-b2 in grapevine, *H. obsoletus*, and *D. europea* whilst tuf-b1 was detected in seven different hosts-grapevine plants *C. arvensis, A. altissima*, *R. pseudoacacia* and insects *H. obsoletus* and *C. wagneri* (Table 1).

The sequence analysis performed on *secY* gene amplicons enabled the identification of five genotypes: S1, S4, S6, S7, and S39 (Fig. 2). The genotype S6 was prevalent and detected in 26/78 plant and 8/20 insect samples. The genotype S4 was also frequently detected (17/78 plant and 7/20 insect samples) and was found in all plant hosts analysed and in all types of insect samples. Genotypes S1 and S6 were detected both in the grapevine and *H. obsoletus*. Finally, genotypes S7 and S39 were present only in grapevine samples and always in combination with tuf-a type (Table 1).

The *vmp1* typing revealed 8 *vmp1* genotypes within our CPs strain populations (Fig. 3). Among the identified genotypes, V3, V4, V2-TA, V14, V17, V18, and V23 were formerly designated and detected in other studies (Murolo et al. 2010, Murolo et al. 2014; Cvrković et al. 2014; Delić et al. 2015; Kosovac et al. 2016). The comparison with *vmp1* representative strains enabled the identification of one new, previously unreported genotype, designated as V28 according to the SEE-ERANET nomenclature (Foissac et al. 2013). This new genotype was detected only in *A. altissima* and *C. wagneri* (Table 1). The genotype V18 was prevalent in grapevine (20/78) and insect samples (8/20) followed by genotypes V3 (18/78 for plant and 3/20 for insect samples) and V4 (15/78 for plant and 1/20 for insect samples). The V4 had the most diverse set of hosts and was found in the grapevine, *C. arvensis, R. pseudoacacia*, and *H. obsoletus* (Table 1).

The comparison with the *stamp* gene dataset allowed the identification of thirteen genotypes. Twelve genotypes, namely ST4, ST6, ST9, ST13, ST19, ST22, ST23, ST29, ST38, ST46, ST48, and ST52 correspond to previously published reference strains (Fabre et al. 2011; Cvrković et al. 2014; Kosovac et al. 2016). The *stamp* sequence isolated from a single sample of *H. obsoletus* (4K) was different from other *stamp* sequences in the dataset. According to the SEE-ERANET nomenclature (Foissac et al. 2013), this new genotype was designated as ST59 (Fig. 4). The most abundant CPs *stamp* genotype was ST6, detected in 20/78 plant and 7/20 insect samples. It was found exclusively in grapevine and *H. obsoletus*. Genetic variant ST9 was found in several hosts (grapevine, *R. pseudoacacia, C. arvensis*, and *H. obsoletus*) and also frequently detected (17/78 plant and 2/20 insect samples). ST4 genotype was found only in *A. altissima, H. obsoletus*, and *C. wagneri,* whilst ST38 was seen in grapevine and *D. europea*. All other *stamp* variants were found exclusively in grapevine (Table 1).

In total, 28 MLST genotypes were detected. The prevalent MLST genotype was S6/ST6/V18/tuf-b2 hereafter referred as CPsSqt21, infecting almost one third of the analysed samples (27/98; 27.55%) and found both in *H. obsoletus* vector and grapevine samples. Two other most common MLST genotypes were S39/ST46/V3/tuf-a hereafter referred as CPsSqt28 (12/98; 12.24%) and S1/ST9/V4/tuf-b1 hereafter referred as CPsSqt9 (9/98;

Table 1 Analysed phytoplasma-infected plant and insect samples with information on '*Ca.* Phytoplasma solani' MLST genotyping

Host	Sample (Collection site/Year)	'*Ca.* P. solani ' MLST genotype				[a]County	[b]Grapevine growing region
		***secY* sequence**	***stamp* sequence**	***vmp1* sequence**	***tuf* sequence**		
Plant samples							
Vitis vinifera	342 (Motovun/2011)	S1	ST22	V14	tuf-b1	IST	
	438 (Momjan/2016); 19–9 (Šeget/2009);	S4	ST22	V14	tuf-b1		
	PO1 (Poreč/2015); 510 (Pula/2016); 719 (Grožnjan/2016);	S39	ST46	V3	tuf-a		**ISK**
	18–9 (Šeget, 2009)	S39	ST19	V3	tuf-a		
Vitis vinifera	10–10 (Vivodina/2010)	S1	ST9	V4	tuf-b1	KA	**CUP**
	11–10 (Vrškovac/2010)	S6	ST6	V18	tuf-b2		
	308 (Škaljevica/2013)	S4	ST29	V2-TA	tuf-b1		
Vitis vinifera	GBr2, GBr4 (Brckovština/2011); 344 (Sv. Vid/2012)	S6	ST6	V18	tuf-b2	KK	
Convolvulus arvensis	Br8 (Brckovština/2011)	S4	ST9	V4	tuf-b1		
Vitis vinifera	653 (Sv.I. Žabno/2015	S39	ST46	V3	tuf-a		
Vitis vinifera	37–10 (Sv. Križ Začretje/2010)	S1	ST22	V2-TA	tuf-b1	KZ	
	38–10 (Sv. Križ Začretje/2010); 533 (Radoboj/2016)	S6	ST6	V18	tuf-b2		
	1073 (Zlatar/2017)	S39	ST46	V3	tuf-a		
Vitis vinifera	2–9 (Železna Gora/2009)	S4	ST22	V4	tuf-b1	ME	
	4–9 (Vukanovec/2009)	S4	ST29	V2-TA	tuf-b1		
	7–9 (Vukanovec/2009)	S6	ST23	V3	tuf-a		
	GVu1, Gvu2 (Vukanovec/2011)	S6	ST6	V18	tuf-b2		
Vitis vinifera	13–10 (Dugi Vrh/2010); 40–10, 41–10 (Vinica/2010); 290 (Falinić Breg/2013)	S6	ST6	V18	tuf-b2	VŽ	
	22–10 (Cerovec/2010)	S1	ST52	V2-TA	tuf-b2		
	59–10 (Cestica/2010)	S1	ST9	V4	tuf-b1		
	60–10 (Cestica/2010)	S39	ST46	V3	tuf-a		
Vitis vinifera	29–9 (Jastrebarsko/2009); 7–10 (Plešivica/2010);	S39	ST46	V3	tuf-a	ZG	
	31–9, 33–9 (Šibice/2009); 8–10 (Plešivica/2010); 305 (Sveta Jana/2012); 785 (Zagreb/2016)	S6	ST6	V18	tuf-b2		
	5–10 (Plešivica/2010)	S4	ST9	V4	tuf-b1		
	9–10 (Dol/2010)	S4	ST29	V2-TA	tuf-b1		
	JS2 (Jaska/2015)	S4	ST29	V14	tuf-b1		
	JS4 (Jaska/2015)	S1	ST22	V14	tuf-b1		
Vitis vinifera	393 (Popovača/2015)	S1	ST9	V4	tuf-b1	SM	**CUP**
Vitis vinifera	42–9, 43/9 (Kamenac/2009); 611 (Erdut/2016)	S1	ST9	V4	tuf-b1	OB	**SLD**
	385 (Feričanci/2015)	S7	ST19	V3	tuf-a		
	ZJ1 (Zmajevac/2015)	S6	ST52	V2-TA	tuf-b2		
	DA1 (Dalj/2016)	S1	ST9	V14	tuf-b1		
	SU2 (Suza/2016)	S1	ST13	V14	tuf-b1		
	ZJ17 (Zmajevac/2015)	S4	ST9	V4	tuf-b1		
Vitis vinifera	380 (Pleternica/2015)	S6	ST6	V18	tuf-b2	PS	
Vitis vinifera	264 (Vukosavljevica/2014)	S1	ST22	V14	tuf-b1	VP	
	764 (Orahovica/2016)	S6	ST6	V18	tuf-b2		

Table 1 (continued)

Host	Sample (Collection site/Year)	*'Ca.* P. solani' MLST genotype				[a]County	[b]Grapevine growing region
		secY sequence	*stamp* sequence	*vmp1* sequence	*tuf* sequence		
Vitis vinifera	37–9 (Ilok/2009)	S6	ST52	V23	tuf-b2	VS	
	38–9 (Ilok/ 2009); 247 (Opatovac/2014)	S1	ST9	V4	tuf-b1		
	39–9 (Ilok/2009)	S7	ST19	V3	tuf-a		
	40–9 (Ilok/2009); IL11 (Ilok/2016)	S6	ST6	V18	tuf-b2		
	24–10 (Dalj/2010)	S4	ST29	V14	tuf-b1		
	25–10 (Dalj/2010)	S6	ST52	V23	tuf-b2		
	31–10 (Ilok/2010)	S1	ST13	V2-TA	tuf-b1		
	33–10 (Ilok/2010)	S4	ST48	V14	tuf-b1		
	IL4 (Ilok/2015)	S4	ST29	V23	tuf-b1		
	LO5 (Lovas/2016)	S1	ST9	V14	tuf-b1		
	IL7 (Ilok/2016)	S4	ST29	V2-TA	tuf-b1		
Ailanthus altissima	IL9Aa (Ilok/2016)	S4	ST4	**V28**	tuf-b1		
Convolvulus arvensis	IL10Ca (Ilok/2016)	S4	ST9	V4	tuf-b1		
Robinia pseudoacacia	IL10Rp (Ilok/2016)	S4	ST9	V4	tuf-b1		
Vitis vinifera	434 (Brodski Stupnik/2012)	S7	ST19	V3	tuf-a	BP	
	BS2 (Brodski Stupnik/2016)	S4	ST38	V14	tuf-b2		
Vitis vinifera	458 (Škabrnja Prkos/2015)	S6	ST23	V3	tuf-a	ZD	**DAL**
	1690 (Nadin/2019)	S1	ST9	V17	tuf-b1		
Insect samples							
Hyalesthes obsoletus	11 K (Novigrad Istarski/2005)	S1	ST4	V17	tuf-b1	IST	**ISK**
Cixius wagneri	CW-K (Kaštelir/2015)	S4	ST4	**V28**	tuf-b1		
Hyalesthes obsoletus	4 K (Vivodina/2004)	S1	**ST59**	V3	tuf-b1	KA	**CUP**
Hyalestes obsoletus	Ho1-1 (Marija Bistrica/2017)	S4	ST22	V14	tuf-b1	KZ	
	Ho2-4 (Marija Bistrica/2017	S6	ST6	V18	tuf-b2		
Dictyophara europaea	De7B (Mrzlo Polje/2019)	S4	ST 38	V3	tuf-b2		
	De7F (Mrzlo Polje/2019)	ND	ST 38	V3	tuf-b2		
Hyalesthes obsoletus	H17, H18, H21, H24 (Vukanovec/2011)	S6	ST6	V18	tuf-b2	ME	
Dictyophara europaea	N3c/N3f (Sveti Urban/2020)	S6	ST6	V18	tuf-b2		
Hyalesthes obsoletus	16 K (Varaždin Breg/2005)	S1	ST22	V14	tuf-b1	VŽ	
Dictyophara europaea	De3 (Cestica/2017)	S4	ST38	V3	tuf-b2		
Hyalestes obsoletus	HoN2 (Ivanec/2020)	S6	ST52	V18	tuf-b2		
Hyalesthes obsoletus	1 K (Jastrebarsko/2004)	S4	ST9	V4	tuf-b1	ZG	
	135 K (Jastrebarsko/2005)	S1	ST9	V14	tuf-b1		
Cixius wagneri	CW7 (Ilok/2015)	S4	ST4	ND	tuf-b1	VS	**SLD**
	CW-ZJ2 (Zmajevac/2015)	S4	ST4	**V28**	tuf-b1	OB	

[a] Abbreviations in the table correspond to administrative districts (counties) of Croatia: *VS* Vukovar-Srijem, *OB* Osijek-Baranja, *BP* Brod-Posavina, *PS* Požega-Slavonia, *VP* Virovitica-Podravina, *KK* Koprivnica-Križevci, *ME* Međimurje, *VŽ* Varaždin, *KZ* Krapina-Zagorje, *SM* Sisak-Moslavina, *ZG* Zagreb, *KA* Karlovac, *IST* Istria and *ZD* Zadar

[b] Abbreviations in bold correspond to the four Croatian grapevine growing regions according to the Law on wine (2019): *CUP* Croatian Uplands, *SLD* Slavonia and Croatian Danube, *ISK* Croatian Istria and Kvarner and *DAL* Dalmatia

9.18%). Other detected MLST genotypes were relatively scarce, sporadically distributed and represented by one (15 MLST genotypes) or a few (10 MLST genotypes) samples (Table 2; Fig. 5).

Phylogenetic analyses of concatenated nucleotide sequences of *secY*, *stamp*, and *vmp1* genes

Five main clusters are identified in the phylogenetic tree generated by the analysis of the concatenated

representative nucleotide sequences of the *secY, stamp*, and *vmp1* genes here named *secY/stamp/vmp* cluster 1 to 5 (Fig. 6). Each *secY/stamp/vmp* cluster could be distinguished from one another by their affiliation to *vmp1* genotype. Overall, the *secY/stamp/vmp* cluster 5 was characterised by the V14 (tuf-b1) genotype, cluster 4 by V2-TA (tuf-b1/tuf-b2) genotype, cluster 3 by V4 (tuf-b1) genotype, cluster 2 by V17 and the newly reported V28 (tuf-b1) genotype and cluster 1 by V3/V23/V18 (tuf-a/tuf-b2) genotypes. As demonstrated above, the clusters were grouped also according to the tuf type and can therefore be hypothesized that they are a part of a specific epidemiological cycle associated with nettle (*secY/stamp/vmp* cluster 1) or bindweed (*secY/stamp/vmp* clusters 2, 3, and 5*). SecY/stamp/vmp* cluster 4 encompassed the strains belonging to the tuf-b1 and tuf-b2 type and could not be related to a particular transmission cycle as previously described (Fig. 6).

Discussion

First molecular evidence of CPs phytoplasmas infecting Croatian grapevines was recorded in the publications of Škorić et al. (1998) and Šeruga et al. 2000). Through the national yearly survey programme, it was soon confirmed that this etiological agent is widely distributed in the country. The diversity of CPs isolates in Croatia was assessed by combining the data sets of four epidemiologically informative genes (*tuf, secY, vmp1*, and *stamp*) from various hosts infected with CPs. Altogether, diversity of CPs associated MLST genotypes was highest in Uplands (CUP) and Slavonia and Danube (SLD) regions, moderate in Istria and Kvarner (ISK) and low in Dalmatia (DAL) (Fig. 5; Table 1).

Our results demonstrated the presence of CPs types tuf-a, tuf-b1, and tuf-b2, indicating that strains involved in BN disease in Croatia can be presumably associated with both main natural epidemic cycles within the BN pathosystem including the bindweed and nettle, as affiliation of these host plants and particular tuf genotype was previously well documented in several studies (Langer and Maixner 2004; Aryan et al. 2014). Regarding the *tuf* gene variability throughout the regions, in Istria and Dalmatia only tuf-a and tuf-b1 genotypes were detected. This is somewhat expected since the same genotypes were recorded in southwest Slovenia (Mehle et al. 2022) bordering Istria region and central Italy (Landi et al. 2019; Murolo et al. 2020) which also shares a maritime border with Dalmatia to the east. In Uplands and Slavonia and Danube regions all three tuf types were detected. On the basis of data from other studies conducted in the region, where tuf-b1 type is typically found in the Balkans/Eastern Europe area (Cvrković et al. 2014; Atanasova et al. 2015) and tuf-a and tuf-b2 are dominant types in most of the north-western European regions (Johannesen et al. 2012; Aryan et al. 2014), tuf strain presence in Uplands and Slavonia and Danube regions can be attributed to the particular geographical position between the two aforementioned areas. The higher frequency of tuf-b2 (50%) in Slavonia and Danube is also in agreement with data recorded in southeast Slovenia bordering this region (Mehle et al. 2022). Overall, distribution of tuf strains is probably a predominant result of specific pedoclimatic condition influencing weed composition in and around vineyards.

Due to their role in the host adaptation to both plants and insects, *stamp* and *vmp1* genes are valuable indicators of the CPs population structure (Cimerman et al. 2009; Fabre et al. 2011; Pacifico et al. 2009). These complex interactions with hosts are manifested in increased polymorphism of these two genes which enables tracking the migration of pathogen strains and deciphering of complex CPs transmission pathways. Moreover, *stamp* and *vmp1* phylogenetic clusters can be distinctly affiliated with bindweed or nettle related cycles (Figs. 3 and 4), (Murolo and Romanazzi 2015; Plavec et al. 2015; Kosovac et al. 2016; Quaglino et al. 2016).

As expected, *stamp* was revealed to be a highly discriminative marker with 13 genotypes identified amongst which ST59 from *H. obsoletus* was described here for the first time. Prevalence of the ST6 genotype in Croatia is in agreement with the data from Austria and Slovenia, where it was found in grapevine, *H. obsoletus* and *U. dioica* (Aryan et al. 2014; Mehle et al. 2022). The ST46 genotype was found in grapevine and *U. dioica* in southeast Slovenia (Johannesen et al. 2012; Mehle et al. 2022) and grapevine in Italy (Accesion No.MT777487). As in

(See figure on next page.)

Fig. 2 Unrooted phylogenetic tree constructed by parsimony analysis of the *secY* gene sequences from CPs representative isolates identified in this study and nucleotide sequences representative of *secY* sequence variants previously described (Table 2 and Additional file 2: Table S2). Representative *secY* sequence variants are given/displayed with the accession number. Numbers on main branches correspond to bootstrap values as percentages (500 replicates). The *secY* genotypes are identified and shown to the right of the tree. Genotypes were designated following established nomenclature (SEE-ERANET nomenclature), including designation of previously identified and published CPs genotypes. Grapevine samples are not marked. Samples from other hosts found infected with CPs are denoted in the following manner: insects (black dot), other host plants (black triangle)

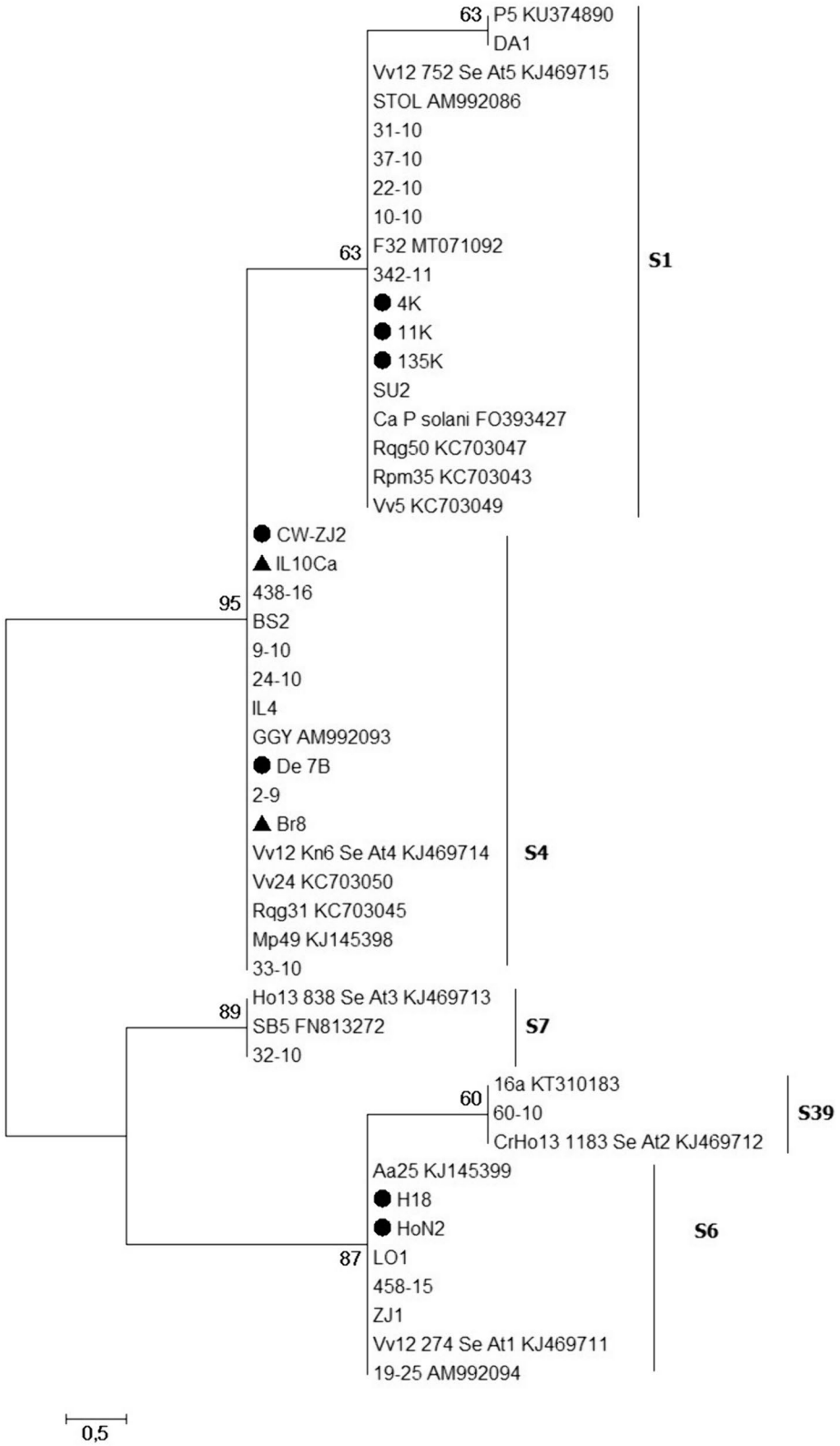

Fig. 2 (See legend on previous page.)

Croatia, the ST19 genotype has been reported for grapevines in Slovenia and Italy (Contaldo et al. 2021; Mehle et al. 2022). When inspecting the *stamp* genotype in relation to the number of hosts shared by the same genotype within our samples, the most heterogeneous was ST9. The ST9 genotype was found in *V. vinifera, C. arvensis, R. pseudoacacia*, and *H. obsoletus* and also previously recorded in Serbia in *Reptalus quinquecostatus* (Cvrković et al. 2014). Particularly interesting was the genotype ST4 identified in *H. obsoletus*, *A. altissima*, and *C. wagneri*. This diversity of hosts for ST4 is in accordance with data from other countries where ST4 was detected, amongst other hosts, in *Anaceratagallia ribauti* in Austria (Aryan et al. 2014), *R. panzeri* in Serbia (Cvrković et al. 2014), tomato in Italy (Contaldo et al. 2021), and grapevine in Slovenia, Italy, Hungary, and Germany (Fabre et al. 2011; Pierro et al. 2018; Contaldo et al. 2021). *Stamp,* the single CPs ortholog of the gene encoding AMP protein of "*Ca*. P. asteris" (Suzuki et al. 2006, Kakizawa et al. 2006), has a presumed important role in the interactions with insect vector and is exposed to positive selection pressure resulting in the protein sequence (Fabre et al. 2011). In this light, the variability of *stamp* gene hitherto found is not surprising and the number of new genotypes will surely grow. Although we detected fewer *vmp1* (8) than *stamp* genotypes (12), upon analysis of our data it appears that the *vmp1* gene has a very important role in the adaptation of phytoplasmas to the changing vineyard insect vector fauna. Indeed, the prevalent genotype V18 was found in both grapevine and *H. obsoletus,* and was recorded as most frequent in Slovenia and Austria in the same hosts (Aryan et al. 2014; Mehle et al. 2022). Furthermore, it is worth noting that in this study we identified one new vmp1 genotype V28. Of other major vmp1 genotypes, V3 has been reported for grapevine in France and Italy (Pacifico et al. 2009), grapevine in Slovenia and was found in grapevine and *H. obsoletus* in Austria (Mehle et al. 2022). The later hosts were also found infected in Montenegro (Kosovac et al. 2016). As in Croatia, the V4 genotype was found in diverse set of hosts in neighbouring countries: grapevine in France and Italy (Pacifico et al. 2009; Conigliaro et al. 2020), grapevine, *R. panzeri*, and *R. quinquecostatus* in Serbia (Cvrković et al. 2014), grapevine and *H. obsoletus* in Austria (Aryan et al. 2014; Mehle et al. 2022), *H. obsoletus* in North Macedonia (Atanasova et al. 2015) and grapevine and *H. obsoletus* in Montenegro (Kosovac et al. 2016).

The significance of vmp1 marker is corroborated with concatenation analysis which enabled identification of 5 *secY/stamp/vmp1* clusters that could be distinguished primarily on their *vmp1* gene affiliation. Concatenated nucleotide sequences of *secY/stamp/vmp1* gene have been employed in order to increase the number of variant sites and to minimise the stochastic errors which arise due to limited information contained in a single gene (Leigh et al. 2008). As previously suggested by Pierro et al. (Pierro et al. 2018), certain *secY/stamp/vmp1* clusters probably share some common biological features related to particular plant and insect hosts.

As *secY* is a conserved housekeeping gene (Lee et al. 2006, 2010), the number of *secY* genotypes detected here (five) was expectedly smaller as opposed to those of variable surface protein genes. All of the detected *secY* genotypes had been previously reported in other studies. Our analyses showed that among detected genotypes, isolates were mainly associated with CPs strains carrying the *secY* genotypes S1 (23.46%) and S6 (34.69%). Both genotypes were also recorded in grapevines and *H. obsoletus* in Slovenia and Austria, with S6 again being the dominant genotype (Mehle et al. 2022). Precisely these variants were recently linked to the severe symptoms and a higher phytoplasma titres in infected grapevines. Specifically, the new study by Pierro et al. (2022) demonstrated the possible relationship between SecY protein structure and the phytoplasma strain virulence which could also explain the prevalence of S6 genotype in this area. Genotype S6 was detected in grapevine and *H. obsoletus*, while S1 genotype has much wider distribution and was detected in various hosts in addition to grapevine and *H. obsoletus*, including *R. panzeri* and *D. europea* (Fabre et al. 2011; Aryan et al. 2014; Cvrković et al. 2014, 2022).

The tremendous complexity of the BN epidemiological network in Croatian grapevine-growing regions is mirrored in the 28 CPs MLST genotypes identified in a relatively modest number of genotyped samples (Table 2). As previously demonstrated (Plavec et al. 2015), *H. obsoletus*

(See figure on next page.)

Fig. 3 Unrooted phylogenetic tree constructed by parsimony analysis of the *vmp1* gene sequences from CPs representative isolates identified in this study and nucleotide sequences representative of *vmp1* sequence variants previously described (Table 2 and Additional file 2: Table S2). Representative *vmp1* sequence variants are given/displayed with accession number. Numbers on main branches correspond to bootstrap values as percentages (500 replicates). The *vmp1* genotypes are identified and shown to the right of the tree. Genotypes were designated following established nomenclature (SEE-ERANET nomenclature), including designation of previously identified and published CPs genotypes. Grapevine samples are not marked. Samples from other hosts found infected with CPs are denoted in the following manner: insects (black dot), other host plants (black triangle)

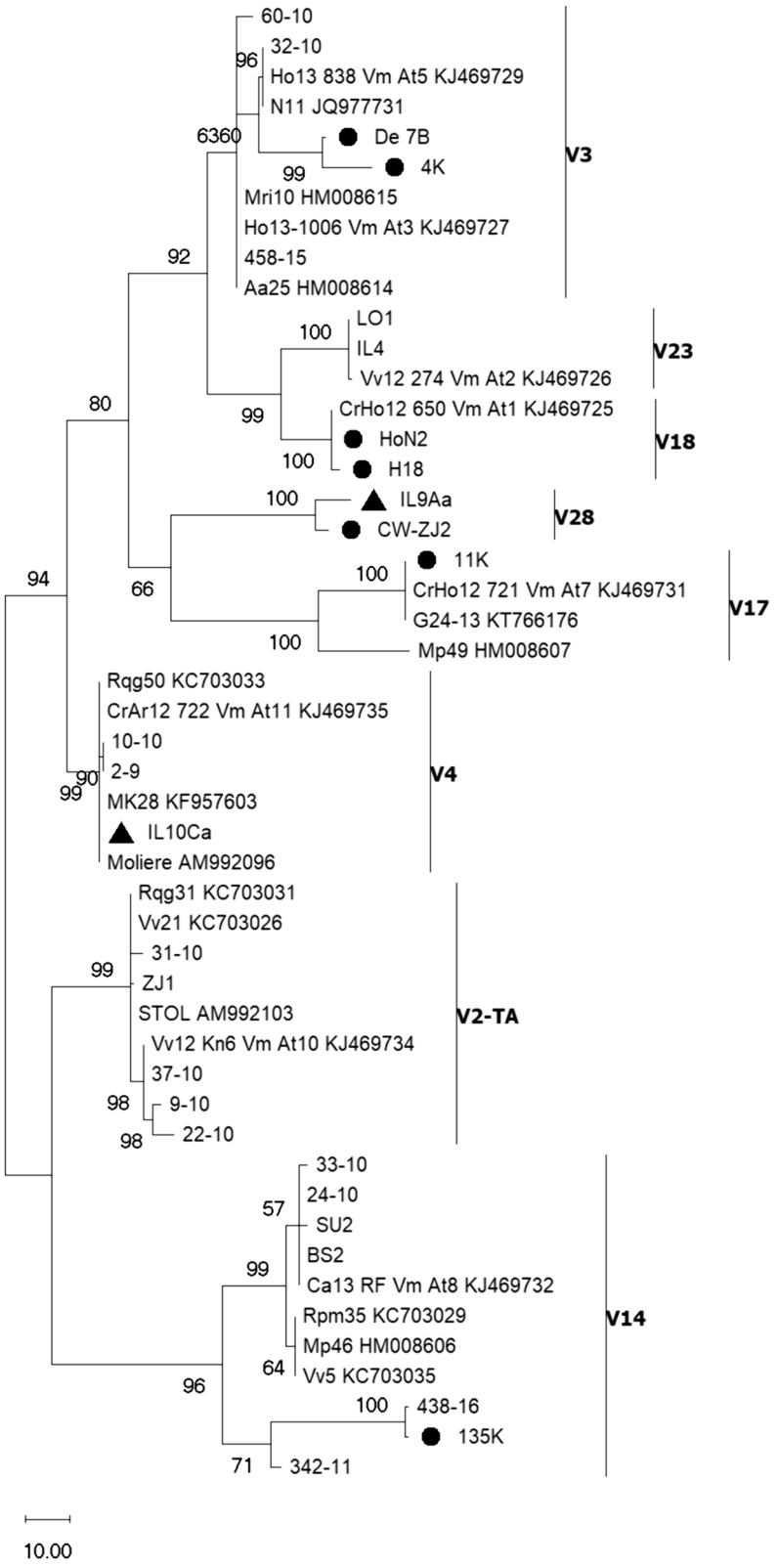

Fig. 3 (See legend on previous page.)

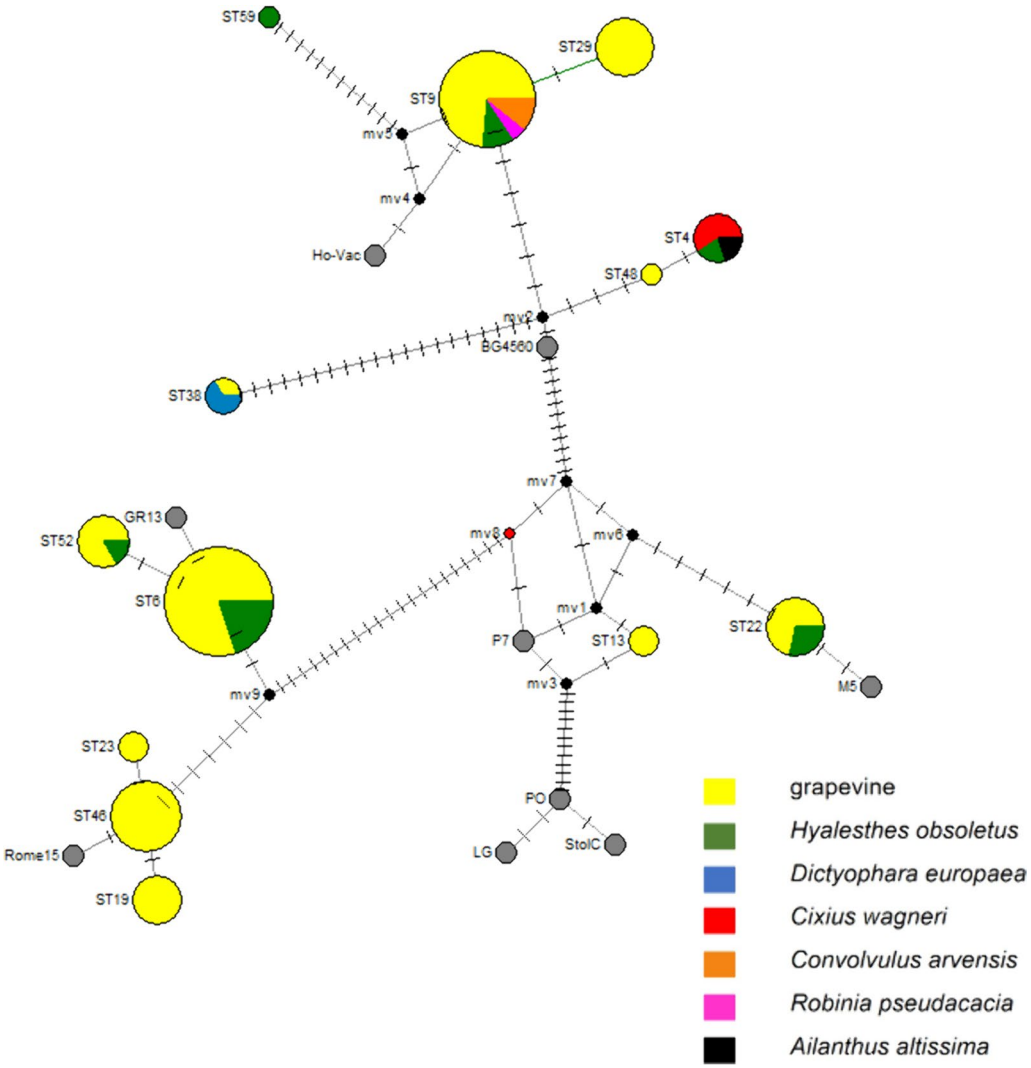

Fig. 4 Median-joining network inferred from *stamp* gene sequences of CPs strains detected in this study and of strains previously identified and published (Aryan et al. 2014; Atanasova et al. 2015; Cvrković et al. 2014; Fabre et al. 2011; Johannesen et al. 2012; Kosovac et al. 2016) (Table 2 and Additional file 2: Table S2). Genotypes are represented by circles and each SNP mutation is represented by a hatch mark. Circle sizes are proportional to strain frequency found in this study and host association(s) of genotypes is represented by different colour(s) of circles as shown in the figure legend. Grey circles represent reference strains not detected in this study while black dots represent median vectors. Network of a total of 22 *stamp* genotypes is presented. Genotypes were designated following established nomenclature (SEE-ERANET nomenclature), including designation of and newly identified genotype detected in this study (ST59)

plays a major role in the CPs epidemiology of the Croatian vineyards. This finding is corroborated with 7 MLST genotypes (CPsSqt1, CPsSqt3, CPsSqt8, CPsSqt12, CPsSqt14, CPsSqt21, and CPsSqt25) detected in *H. obsoletus* analysed here. Moreover, the prevalent MLST genotype CPsSt21 (S6-ST6-V18-tuf-b2) in Croatian vineyards was also found in *H. obsoletus* and grapevine thus confirming a key role of this vector in the spread of BN disease in the country. This MLST genotype is also the one considered as an emerging strain (CPsM4_At1) massively propagated by high *H. obsoletus* populations developing on *U. dioica* in Austria (Aryan et al. 2014) and was recently confirmed as prevalent MLST genotype in northeast Slovenia (Mehle et al. 2022). Though abundantly present in Uplands and Slavonia and Danube regions this major MLST genotype however has never been detected in Istria. Reasons for such clear distribution could be found in the *H. obsoletus* population study of Johannesen and Riedle-Bauer (2014) where *H. obsoletus* populations were assigned to different origins. In this study *H. obsoletus* population from Austria and a part of population from Slovenia were assigned to Pannonian

Table 2 Overview of CPs collective genotypes with GenBank accession numbers of the representative samples for each collective CPs genotype identified in this and previous study (Plavec et al. 2015)

CPs phytoplasma collective genotypes (28) this study				**Accession no. of representative sequences**			**Corresponding multilocus genotypes from other studies**
Multilocus genotypes: *secY/stamp/vmp1/tuf*	Multilocus genotype name (no of isolated genotypes)	Representative sample (other samples)	Pathogen host[a]	*secY*	*stamp*	*vmp1*	Multilocus genotype name [X]*contains vmp1 and/or tuf restriction profile, not sequence*
S1/ST4/V17/tuf-b1	CPsSqt1 (1)	*11 K*	*Ho*	OQ968500	OQ872373	OR165158	
S1/ST9/V4/tuf-b1	CPsSqt2 (9)	*10–10 (38–9, 42–9, 43–9, 59–10, 227–14, 247–14, 393–15, 611–16)*	*Vv*	OQ954504	OQ872374	OR165156	[c]Rqg50g[x], [b]CPsM4_At6, [b]CPsM4_At9, [b]CPsM4_At12
S1/ST9/V14/tuf-b1	CPsSqt3 (3)	*135 K (LO5, DA1)*	*Vv, Ho*	OQ968501	OQ920911	OR165159	[c]Vv5g[x], [b]CPsM4_At7
S1/ST9/V17/tuf-b1	CPsSqt4 (1)	1690	Vv	same as OQ968500	OQ920912	same as OR165158	[b]CPsM4_At10
S1/ST13/V2-TA/tuf-b1	CPsSqt5 (1)	*31–10*	*Vv*	OQ954505	OQ872375	OR165150	[c]STOLg[x]
S1/ST13/V14/tuf-b1	CPsSqt6 (1)	*SU2*	*Vv*	OQ954506	OQ920913	OR165140	
S1/ST22/V2-TA/tuf-b1	CPsSqt7 (1)	*37–10*	*Vv*	OQ954507	OQ872376	OR165151	[b]CPsM4_At13
S1/ST22/V14/tuf-b1	CPsSqt8 (1)	*342–11 (264–14, JS4, 16 K)*	*Vv, Ho*	OQ954508	OQ920909	OR165141	[c]Rpm35g[x]
S1/ST52/V2-TA/tuf-b1	CPsSqt9 (1)	*22–10*	*Vv*	OQ954509	OQ920915	OR165152	-
S1/**ST59**/V3/tuf-b1	CPsSqt10 (1)	*4 K*	*Ho*	OQ954502	OQ872377	OR165160	-
S4/ST4/**V28**/tuf-b1	CPsSqt11 (3)	*IL9Aa,CW-ZJ2 (CW-K)*	*Aa, Cw*	OQ954503	OQ915043	OR165139	-
S4/ST9/V4/tuf-b1	CPsSqt12 (6)	*IL10Ca (5–10, Br8, ZJ7, IL10Rp, 1 K)*	*Vv, Ca, Rp, Ho*	KJ573589	KJ573597	OR165163	-
S4/ST22/V4/tuf-b1	CPsSqt13 (1)	*2–9*	*Vv*	OQ968494	OQ920908	OR165157	-
S4/ST22/V14/tuf-b1	CPsSqt14 (2)	*438–16 (Ho1-1)*	*Vv, Ho*	OQ968495	OQ920910	OR165142	-
S4/ST29/V2-TA/tuf-b1	CPsSqt15 (4)	*9–10 (4–9, 308–13, IL7)*	*Vv*	KP274913	KP274914	OR165153	-
S4/ST29/V14/tuf-b1	CPsSqt16 (2)	*JS2 (24–10)*	*Vv*	OQ968496	OQ915044	OR165143	[c]Vv24g[x]
S4/ST29/V23/tuf-b2	CPsSqt17 (1)	*IL4*	*Vv*	OQ968497	OQ915045	OR165155	-
S4/ST38/V3 /tuf-b2	CPsSqt18 (1)	*De 7B*	De	OQ968502	OQ915038	same as OR165145	-
S4/ST38/V14 /tuf-b2	CPsSqt19 (1)	*BS2*	*Vv*	OQ968498	OQ920914	OR165144	-
S4/ST48/V14/tuf-b1	CPsSqt20 (1)	*33–10*	*Vv*	OQ968499	OQ915039	same as OR165144	-

Table 2 (continued)

CPs phytoplasma collective genotypes (28) this study				Accession no. of representative sequences			Corresponding multilocus genotypes from other studies
S6/ST6/V18/tuf-b2	CPsSqt21 (25)	*H18 (31–9, 33–9, 40–9, 8–10, 11–10, 13–10, 38–10, 40–10, 41–10, GVu1, GVu2, GBr2, GBr4, 305–12, 344–12, 290–13, 380–15, 533–16, 764–16, IL11, H17, H21, H24, Ho2-4)*	*Vv, Ho*	KJ573585	KJ573595	OR165162	[b]CPsM4_At1
S6/ST23/V3/tuf-a	CPsSqt22 (2)	*458–15 (7–9)*	*Vv*	KP274912	KP274915	OR165145	[b]CPsM4_At3, [d]S6/ST23/N3/tuf-a
S6/ST52/V23/tuf-b2	CPsSqt23 (3)	*LO1 (37–9, ZJ1)*	*Vv*	OQ968503	OQ915040	OR165154	[b]CPsM4_At2
S6/ST52/V2-TA/tuf-b2	CPsSqt24 (1)	*ZJ1*	*Vv*	OQ968504	OQ920916	OR165149	-
S6/ST52/V18/tuf-b2	CPsSqt25 (1)	*HoN2*	*Ho*	OQ968505	OQ920917	OR165161	-
S7/ST19/V3/tuf-a	CPsSqt26 (4)	*32–10 (39–9, 434–12, 385–15)*	*Vv*	OQ968506	OQ915041	OR165146	[b]CPsM4_At5
9/ST19/V3/tuf-a	CPsSqt27 (1)	*18–9*	*Vv*	OQ968508	OQ920918	OR165148	-
S39/ST46/V3/tuf-a	CPsSqt28 (12)	*60–10 (18–9, 19–9, 29–9, 7–10, 653–15, NM7, PO1, 510–16, 719–16, 785–16, 1073–17)*	*Vv*	OQ968507	OQ915042	OR165147	[b]CPsM4_At4

[a] *Vv* V. vinifera, *Ca* C. arvensis, *Rp* R. pseudoacacia, *Aa* A. altissima, *Ho* H. obsoletus, *Cw* C. wagneri, *De* D. europaea

[b] Aryan et al. (2014)

[c] Cvrković et al. (2014)

[d] Johannesen et al. (2012)

origin, while the other part of *H. obsoletus* population was assigned to Adriatic origin. It was therefore suggested in the study of Mehle et al. (2022) that the area of these Pannonian populations is affiliated to the prevalent MLST genotype CPsSt21 (S6-ST6-V18-tuf-b2). Our results corroborate such a scenario in Croatia – the absence of S6-ST6-V18-tuf-b2 genotype in Istria and its prevalence in the continental regions could be interpreted by geographic distribution of *H. obsoletus* population of different origins and their specific affiliation to the host plants and to the specific CPs strain (Johannesen and Riedle-Bauer 2014).

The second in number of detected MLST grapevine-associated BN genotypes CPsSqt28 (S39/ST46/V3/tuf-a) corresponded to the Austrian CPsM4_At4 also found in *H. obsoletus* and in *H. obsoletus* infected *Catharanthus roseus* (Aryan et al. 2014) (Table 2). This MLST genotype was recorded both in Istria and Uplands region indicating another important BN pathosystem involving the main vector. However, the third most frequent MLST genotype identified CPsSqt2 (S1/ST9/V4/tuf-b1) represents an alternative epidemic route and corresponds to the genotype Rqg50g, previously associated with the *R. panzeri*-vectored disease cycle in Serbia (Cvrković et al. 2014). Even though this MLST genotype was recorded in Uplands region, it was predominantly found in Slavonia and Danube region which shares the border with Serbia. This is again in accordance with the regional distribution of CPs strains. STOLg, here denoted CPsSqt5 (S1/ST13/V2-TA/tuf-b1) was frequently found in grapevine in the Balkans as a major genotype (Cvrković et al. 2014; Atanasova et al. 2015). During our study CPsSqt5 (S1/ST13/V2-TA/tuf-b1) was detected only in one *V. vinifera* plant from the far east of Croatia (Table 1), corroborating its role in the Balkans associated with isolates from south-eastern Europe (Fabre et al. 2011; Quaglino et al. 2013). This single finding here could also indicate the current shift in the genotype composition due to the different

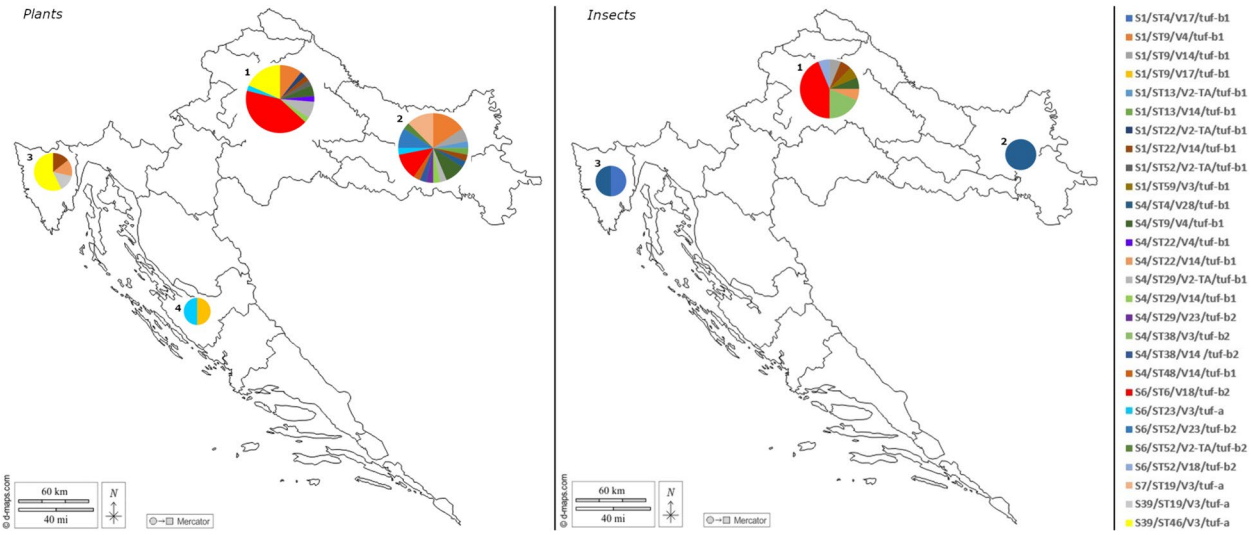

Fig. 5 Map of the four surveyed grapevine growing regions in Croatia for occurrence of CPs in grapevine, tentative reservoir plants, and insects: 1. Croatian Uplands (CUP), 2. Slavonia and Croatian Danube (SLD), 3. Croatian Istria and Kvarner (ISK), and 4. Dalmatia (DAL). For each grape growing region diversity and frequencies of CPs multilocus genotypes based on *tuf, secY, vmp1*, and *stamp* genes are presented according to the data listed in the Table 1 in a form of pie chart sections. Map is generated by using the template map from https://d-maps.com/carte.php?num_car=5359&lang=en) (accessed on 14 February 2023)

factors such as climate conditions. In this research, we sporadically detected several MLST genotypes previously reported in neighbouring countries such as CPsSqt8 (S1/ST22/V14/tuf-b1) known as Rpm35g and of epidemiological importance in Serbia (Cvrković et al. 2014) and Montenegro (Kosovac et al. 2016), along with CPsSqt3 (S1/ST9/V14/tuf-b1) detected in Serbia (Vv5; Cvrković et al. 2014) and Austria (CPsM4_At7; Aryan et al. 2014), then CPsSqt16 (S4/ST29/V14/tuf-b1) also detected in Serbia (Vv24g; Cvrković et al. 2014), Montenegro (tuf-b/Vv24/V14; Kosovac et al. 2016) and North Macedonia (Atanasova et al. 2015), further CPsSqt22 (S6/ST23/V3/tuf-a) detected in Austria (CPsM4_At3; Aryan et al. 2014) and Croatia/Slovena (S6/ST23/N3/tuf-a; Johannesen et al. 2012) and CPsSqt26 (S7/ST19/V3/tuf-a) found also in Austria (CPsM4_At5; Aryan et al. 2014). Especially interesting was the genotype CPsSqt11 (S4/ST4/V28/tuf-b1) which encompasses a new vmp1 genotype V28 and was restricted to *A. altissima* and *C. wagneri*. In the case of FDp, it was recently shown that specific sequence of the adhesin genes *VmpA* and *VmpB* carried by vectotype variants are correlated with the transmission with specific vectors (Malembic-Maher et al. 2020). Functional studies demonstrated that *vmpA* of FDp is encodes surface proteins acting in the phytoplasma adhesion to insect cells (Arricau-Bouvery et al. 2018, 2021; Canuto et al. 2023). The genotype V28 of CPs could reflect the adaptation of the genotype CPsSqt11 to *C. wagneri* insect vector.

Although *H. obsoletus* is a principal vector of CPs in Croatia, our survey revealed that its population did not correlate with the distribution and high number of CPs positive plants. Even though this finding could be a result of the limited insect sample number, the number of MLST genotypes detected point out to different CPs epidemiological pathway(s) involving yet unfamiliar participants and existing simultaneously with the main pathosystem.

Our study confirmed the presence of CPs infected *D. europaea* for which the vector role was recently demostrated (Quaglino et al. 2019; Cvrković et al. 2022). Moreover, in recent years there has been an increase in number of infected *D. europaea* (Additional file 1: Table S1) suggesting that the role of *D. europaea* in the transmission cycle(s) of CPs in Croatia should be more thoroughly addressed. Interestingly, MLST genotype detected in our two analysed specimens CPsSqt18 (S4/ST38/V3/tuf-b2) was different from MLST genotypes detected in Serbia (Cvrković et al. 2022). In comparison, all Serbian isolates were of tuf-b1 genotype while our isolates are of tuf-b2 type. This could be the result of specific, locally determined interactions of hosts and vectors that favour adaptive mutations which was previously recorded even for confined areas (Murolo and Romanazzi 2015).

Previous research identified the presence of other insects from the Cixiidae family in Croatian vineyards (Plavec et al. 2015). While two representatives of this family, *R. quinquecostatus* and *R. panzeri* were the most abundant species recorded in Serbian vineyards (Cvrković et al. 2014), in Croatian grapevine-growing

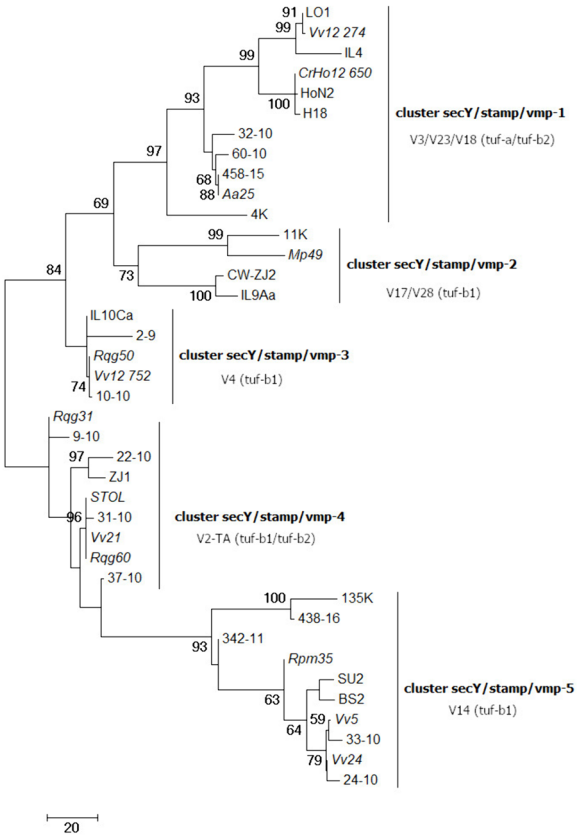

Fig. 6 Unrooted phylogenetic tree obtained from concatenated sequences of multiple (*secY/stamp/vmp1*) genes of representative CPs isolates identified in this study and nucleotide sequences of *secY/stamp/vmp1* sequence variants previously described and identified (Table 2 and Additional file 2: Table S2). Maximum parsimony analysis was performed. Numbers on main branches correspond to bootstrap values as percentages (1000 replicates). The *secY/stamp/vmp1* clusters are identified and shown to the right of the tree including the affiliation of the cluster members to the *vmp1* and *tuf* genotype

regions, they were sporadically present and were rarely found infected (Plavec et al. 2015). On the other hand, their "cousin" *R. cuspidatus* was detected more frequently and was found infected with CPs in several instances (Additional file 1: Table S1; Plavec et al. 2015). While some research already identified this species as a possible phytoplasma vector (Mehle et al. 2011), detailed studies, to our knowledge, have not been conducted.

More intriguing was the detection of *C. wagneri* infected with CPs. In all three positive specimens the same genotype was detected, even though the specimens were collected in distant locations of the country. *C. wagneri* is already a proven vector of '*Candidatus* Phlomobacter fragariae', a bacterium responsible for strawberry marginal chlorosis (SMC) disease (Danet et al. 2003). It was also previously found to be infected by the SBR bacterium (γ-3 proteobacterium) and transmit the pathogen to sugar beet (Bressan et al. 2008). However, although *C. wagneri* was found sharing several plant "delicacies" such as strawberry and sugar beet with *H. obsoletus*, it was never found infected with CPs and transmission of CPs to these plants was attributed exclusively to *H. obsoletus* (Fos et al. 1992; Danet et al. 2003; Bressan et al. 2008). The ability of *C. wagneri* to transmit these two phloem-restricted bacteria, its polyphagous nature, together with our findings of specimens harbouring CPs suggests that it could be considered as a possible participant in a CPs cycle. In any case, the biology, host plant associations and feeding preferences of this cixiid and its possible alternative vector role should be considered and more thoroughly investigated.

In light of recent BN epidemiological studies (Trivellone et al. 2005; Riedle-Baue et al. 2008; Cvrković et al. 2014, 2022; Kosovac et al. 2016), it is easy to speculate the active role of different, non host-specific vectors or vectors tied to new host/source plants with different ecological dynamic in the dissemination of BN strains. Findings of CPs infected insects from the genus *Ciixius* as well as findings of *R. cuspidatus* and intriguing number of infected *D. europae* raise additional questions and draw attention to the need to conduct new vector-searching studies to address the role of possibly other vectors as new key players in BN epidemics in Croatia.

Conclusions

The current MLST approach has shown to be a valuable tool for assessment of the variability of CPs strains in the grapevine pathosystem. The extensive *tuf, secY, stamp*, and *vmp1* genes-based analyses overall identified 28 different comprehensive genotypes with one being prevalent and widespread in nine counties across central and north-western Croatia. While *H. obsoletus-U. dioica* associated cycle still seems to be the most represented the number and diversity of detected genotypes (including two new for *stamp* and *vmp1*) linked to alternative vectors and new reservoir plants, suggests the presence of several other independent epidemiological transmission routes. The data obtained in the present study reveals considerable genetic variability and wide geographical distribution of CPs BN-involved genotypes in Croatia. Moreover, this genetic characterization of CPs strains is necessary for assigning future outbreaks to genotypes with epidemiological relevance and ultimately for successful control of the disease.

Methods

Sampling—plants and insects

Different grapevine varieties with typical GY symptoms (i.e. colour alteration – yellowing/reddening, downward curling of the leaves, berry shrivel, uneven or total lack of

cane lignification) were collected from mid-June to mid-September throughout the years 2009–2020 in grapevine-growing regions of the country as a part of a national survey programme. Parallel to the grapevine sampling, wild plants reported as potential CPs hosts, together with known and presumed CPs vectors belonging to suborder Auchenorrhyncha, were randomly collected inter-row or at vineyards borders. Insects were collected with sweep nets and mouth aspirators, placed in 2 mL plastic tubes containing 96% ethanol and stored at 4 °C. They were subsequently identified to the species level using taxonomic keys by Holzinger et al. (Holzinger et al. 2003) and Biedermann and Niedringhaus (Biedermann 2004). In total, 3336 grapevine, 89 weed and other plant samples, along with 267 insects were collected and tested for the presence of CPs (Additional file 1: Table S1).

Reference phytoplasma strains

CPs reference strains GGY, STOL, Charente 1, and 19–25 were maintained in Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don) by successive graft inoculations in the phytoplasma collection at INRA centre in Bordeaux, France.

DNA extraction

Total nucleic acids were extracted from 1 g of leaf midribs of grapevine or other plants according to the previously described CTAB extraction protocol (Maixner et al. 1995; Šeruga et al. 2003) for a minor part of the samples. For most of the samples, DNA extraction was performed as described in Plavec et al. 2019. Nucleic acids from individual insects were extracted with the commercial kit OmniPrep™ (G-Biosciences, St. Louis, MO, USA) according to the manufacturer's instructions. The resulting DNA pellets were resuspended in 1 × TE buffer (10 mM Tris–HCl, 0.1 mM EDTA) and stored at -20 °C until further analyses.

Phytoplasma detection

Specific detection of CPs associated with BN disease was carried out through TaqMan triplex real-time PCR assay (Pelletier et al. 2009), which enables simultaneous detection of FD and BN phytoplasma in the infected grapevine. Real-time PCR experiments were performed in 96 well plates in two dilutions and in duplicates using 7300 Real Time PCR System (Applied Biosystems, Waltham, MA, USA) and Mx3000P real-time PCR (Agilent, Santa Clara, CA, USA). CPs identification in weed and insect samples was also performed according to the Pelletier et al. (2009) without the internal control. From CPs positive samples, representative isolates were chosen from most CPs foci in the country for further analyses by MLST (Table 1).

Multilocus sequence typing (MLST) of phytoplasma strains

The molecular characterization of selected CPs strains was performed on *tuf, secY, vmp1*, and *stamp* genes. Fragments of these genes were amplified in nested PCR employing the GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) according to the previously described PCR conditions using the following primers: gene *tuf*-fTUF1/rTUF1 (Schneider et al. 1997) and fTUFAY/rTUFSTOL (Schneider et al. 1997/ Foissac unpublished); gene *secY*-POSecF1/POSecR1 and POSecF3/POSecR, with POSecN2 for sequencing (Fialová et al. 2009); gene *stamp*-StampF/StampR0 and StampF1/StampR1 (Fabre et al. 2011); and gene *vmp1*-StolH10F1/StolH10R (Cimerman et al. 2009) and TYPH10F/TYPH10R (Fialová et al. 2009). Amplified products were separated by electrophoresis in 1% agarose gel, stained with GelRed solution (Olerup SSP, West Chester, PA, USA) and visualised under UV light (UViTech, Cambridge, England, United Kingdom). Phytoplasma-free periwinkle and *Vitis vinifera* DNA, together with water controls, were included in all PCR assays as negative controls.

Sequencing and phylogenetic analyses

The *tuf, secY, vmp*1, and *stamp* gene amplicons were directly sequenced in both directions by a commercial services Macrogen Inc. (Seoul, Republic of Korea; Amsterdam, Netherlands) or Genewiz Europe (Leipzig, Germany). Raw chromatograms were assembled and edited with the SequencherTM 4.7 software (Gene Codes Corporation, Ann Arbor, MI, USA) or Geneious (Biomatters Ltd., Auckland, New Zealand) and then aligned with ClustalX 2.0 (Thompson et al. 1997).

Phylogenetic analyses were conducted by MEGA 7 software (Kumar et al. 2016) (maximum parsimony method (MP), Subtree-Pruning-Regrafting (SPR) algorithm, bootstrap 500 replicates). After phylogenetic analysis, nucleotide sequences were attributed to sequence variants by their comparison with sequences deposited in GenBank (Additional file 2: Table S2) following the previously designed nomenclature (SEE-ERANET, X. Foissac, INRA, Bordeaux, France; Foissac et al. 2013). A representative CPs isolate for every *tuf, secY, stamp*, and *vmp1* sequence variant identified was included in a separate phylogenetic analysis generating unrooted phylogenetic trees. From *stamp* sequences detected in this study and the reference strains, median-joining (MJ) network was constructed using software NETWORK v. 10.2.0.0. (www.fluxus-engieering.com). Median-joining (MJ) calculation (Bandelt et al. 1999) was performed by keeping the parameter $\varepsilon = 0$ and with maximum parsimony (MP) post processing.

The MLST genotype (*tuf/secY/stamp/vmp1)* was determined by combining the sequence variant of all four genes for each sample. One representative nucleotide sequence

for each genotype (sequence variant) was deposited in NCBI GenBank. For each *tuf, secY*, *vmp1*, and *stamp* genotype, one representative nucleotide sequence was deposited in NCBI GenBank. Accession numbers of representative *secY*, *stamp*, and *vmp1* nucleotide sequences and reference sequences used in the study are listed in Additional file 2: Table S2. Accession numbers are given in Table 2. Finally, the sequences of representative isolates from *secY/stamp/vmp1* genes were aligned and concatenated using Geneious (Biomatters Ltd., Auckland, New Zealand) and phylogenetic analysis was performed with MEGA 7 (MP, SPR algorithm, bootstrap 1000 replicates).

Abbreviations

Amp	Antigenic membrane protein
BN	Bois noir
CPs	*Candidatus* Phytoplasma solani
CPsSqt	*Candidatus* Phytoplasma solani sequence type
CUP	Croatian Uplands
DAL	Dalmatia
EDTA	Ethylenediaminetetraacetic acid
ENA	European Nucleotide Archive
FD	Flavescence dorée
GY	Grapevine yellows
ISK	Istria and Kvarner
MJ	Median-joining
MLST	Multilocus sequence typing
MP	Maximum parsimony
NCBI	National Center for Biotechnology
SEE-ERANET	South East European—European Research Area Networks
SLD	Slavonia and Danube
SNP	Single Nucleotide Polymorphism
SPR	Subtree-Pruning-Regrafting
TE	Tris-EDTA
Vmp	Variable membrane protein A

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00261-w.

Additional file 1: Table S1. Number of CPs positive samples detected in analysed grapevine, other plants and insects collected throughout grapevine growing regions in Croatia.

Additional file 2: Table S2. Origin and accession numbers of CPs reference nucleotide sequences for the *stamp*, *secY*, and *vmp1* genes used in this study for phylogenetic analysis available in GenBank (NCBI) and ENA (EMBL-EBI) databases.

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Compliance with ethical standards
This research fully complies with Ethical Standards applicable for this journal and the relevant national and international ethics related rules and professional codes of conduct.

Authors' contributions
MM and JP conceived and designed the study. GI collected samples and carried out field investigation. JP and MM performed experiments and analysed data. JP wrote the original draft. MM, DŠ, and XF revised and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Representative nucleotide sequences were deposited in NCBI GenBank and are available according to given accession numbers. Accession numbers of representative *secY, stamp,* and *vmp1* nucleotide sequences from this study are listed in Additional file 2: Table S2.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests or conflict of interest.

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