



## Article

# Epidemiological Role of *Dictyophara europaea* (Hemiptera: Dictyopharidae) in the Transmission of ‘*Candidatus Phytoplasma solani*’

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**Abstract:** Bois noir, an economically important disease of grapevine yellows that causes significant economic losses in wine production, is associated with ‘*Candidatus Phytoplasma solani*’ and transmitted to grapevines by cixiids *Hyalesthes obsoletus* and *Reptalus panzeri*. Polyphagous planthopper *Dictyophara europaea*, commonly found in natural habitats, harbors phytoplasmas from distinct groups and is an alternative vector in the open epidemiological cycles of the Flavescence dorée phytoplasma in grapevine in European vineyards. This study addresses the role of *D. europaea* in the transmission cycle(s) of ‘*Ca. P. solani*’ among wild habitats, natural reservoir plants, and the vineyard agroecosystem using MLSA and transmission trials with naturally infected adults to grapevine and *Catharanthus roseus*. The infection rates of *D. europaea* ranged from 7% to 13% in diverse locations, while reservoir herbaceous plants were infected in the amount of 29%. A total of 13 CaPsoL MLSA genotypes were detected in *D. europaea* (7) and plants (8). Nine of them corresponded to previously identified genotypes. Two new genotypes were found in *D. europaea* (tuf-b1/S1/V14/Rqg50-sv1 and tuf-b1/S18/V14/Rqg50-sv1) and one in *Convolvulus arvensis* (tuf-b1/S1/V2-TA/Rqg31-sv1), whereas one was shared by two hosts, *Crepis foetida* and *Daucus carota* (tuf-b1/S1/V2-TA/STOL-sv1). Naturally infected *D. europaea* successfully transmitted the tuf-b1/S1/V2-TA/STOL type to five grapevines and six periwinkles, tuf-b1/S1/V2-TA/Rqg31 to one grapevine, and tuf-b1/S1/V2-TA/Rqg50 to one periwinkle, indicating that *D. europaea* is an intermediate vector in CaPsoL epidemiological cycles.

**Keywords:** grapevine yellows; stolbur phytoplasma; *Dictyophara europaea*; insect vector; molecular epidemiology; stolbur reservoir plants; multilocus (MLSA) genotypes



**Citation:** Cvrković, T.; Jović, J.; Krstić, O.; Marinković, S.; Jakovljević, M.; Mitrović, M.; Toševski, I.

Epidemiological Role of *Dictyophara europaea* (Hemiptera: Dictyopharidae) in the Transmission of ‘*Candidatus Phytoplasma solani*’. *Horticulturae* **2022**, *8*, 654. <https://doi.org/10.3390/horticulturae8070654>

Academic Editors: Katarzyna Kmiec, Elżbieta Mielniczuk and Katarzyna Golan

Received: 19 June 2022

Accepted: 13 July 2022

Published: 19 July 2022

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## 1. Introduction

‘*Candidatus Phytoplasma solani*’ (‘*Ca. Phytoplasma solani*’; CaPsoL) [1], formerly known as stolbur phytoplasma, belongs to the 16S rRNA XII-A subgroup. It is endemic to Europe and the Mediterranean region, inhabiting a diverse host range of herbaceous and woody plants, and is the causative agent of diseases on a variety of cultivated plants, including solanaceous crops, grapevine, maize, and lavender [2–7]. Bois noir (BN), a disease belonging to the grapevine yellows (GY) complex, induced by ‘*Ca. Phytoplasma solani*’ is widespread in central Europe, the Mediterranean region, and the Middle East. It is known to cause severe damages in vineyards, with infection rates reaching 50–80 percent in some areas and significant economic losses in wine production [8,9]. The molecular typing of CaPsoL strains associated with various hosts (plant or insect) or of different geographic origins is largely used in research studies of BN epidemiology, including disease spread, outbreaks, and increases in the incidence [6,10–13]. The multilocus sequence analysis

(MLSA) approach, based on (i) two housekeeping genes, *tuf* and *secY*, and (ii) two variable membrane proteins, which are under selection pressure and are presumably involved in host recognition, *vmp1* and *stamp* [2,6,14,15], is used for the characterization of CaPsoI in epidemiological studies [7,10–12,16,17].

The principal vector of ‘*Ca. Phytoplasma solani*’ to grapevine is *Hyalesthes obsoletus* Signoret, 1865 (Hemiptera: Cixiidae), a polyphagous cixiid (or oligophagous cryptic complex of three species) [18] feeding and acquiring ‘*Ca. P. solani*’ primarily on bindweed (*Convolvulus arvensis* L.), nettle (*Urtica dioica* L.), chaste tree (*Vitex agnus-castus* L.), and stinking hawk’s beard (*Crepis foetida* L.) [12,18–20]. *Reptalus panzeri* (Löw, 1883) (Hemiptera: Cixiidae), another common cixiid planthopper, was later identified as an additional vector of BN to grapevine in southeastern Europe [11].

*Dictyophara europaea* (Linnaeus, 1767) (Hemiptera: Dictyopharidae), also known as the European lantern fly, is a polyphagous planthopper species found throughout the Western Palaearctic and is commonly associated with a variety of plant species and ecosystems [21]. It is frequently found, particularly in the late summer dry season, in association with drought-tolerant deciduous plant species such as *Clematis vitalba* L., which commonly occupy vineyard ecosystems [22]. This planthopper is known to harbor a number of different phytoplasmas from phylogenetically distant 16S rRNA groups, including 16SrII, 16SrV, and 16SrXII [23–25] and is a proven vector of Flavescence dorée (FD; 16SrV-C subgroup) transmission from natural reservoir *C. vitalba* to grapevine in southeastern Europe [23,26]. Moreover, a recent study in northern Italy that included the experimental testing of eight alternative CaPsoI vectors in grapevine suggested that *D. europaea* could transmit CaPsoI to grapevine [27]; however, its epidemiological role remains unknown. Considering all the above-mentioned findings, the primary goal of our study was to determine the role of this dictyopharid planthopper in the ‘*Ca. P. solani*’ transmission cycle(s) among wild habitats, natural reservoir plants, and vineyard agroecosystem and to provide insight into the influence of these associations in BN dynamics within the vineyard.

This was investigated through a survey on the occurrence and infection incidence of *D. europaea* and tentative reservoir plants in natural habitats and vineyard surroundings, as well as by combining molecular epidemiology data based on (MLSA) multilocus sequence analysis on four genes (*tuf*, *secY*, *vmp1*, and *stamp*) of ‘*Ca. P. solani*’ isolates and the experimental transmission of the pathogen by *D. europaea*.

## 2. Materials and Methods

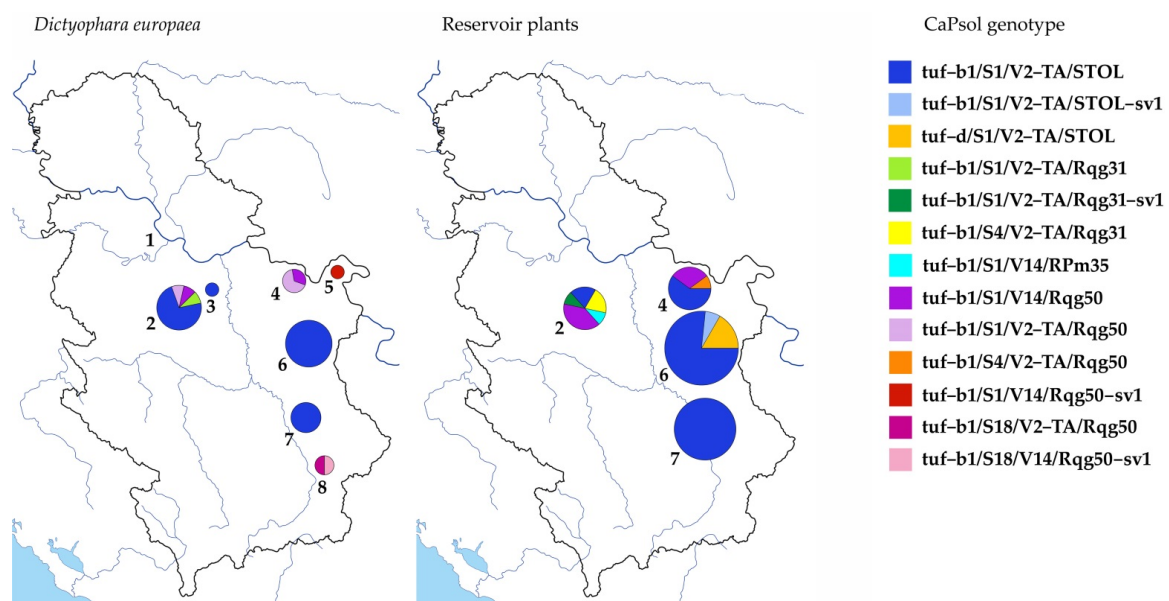
### 2.1. Study Sites

During a four-year period, from 2016 to 2019, a field survey was carried out in a total of eight different locations in central, eastern, and south-eastern Serbia (Table 1). Three of the selected locations were in the surroundings of vineyard agroecosystems previously found to be affected by grapevine yellows disease [22,24]. The remaining five locations had a variety of habitat types, including stony and semi-mesic meadows, abandoned pastures, and xerothermic ruderal slopes (Table 1). The composition of the vegetation was distinct to each location, with a wide variety of plant covers and an intricate spatial layout.

**Table 1.** Surveyed locations of *D. europaea* populations associated with diverse habitat types, with information on collection date, the number of collected /phytoplasma-infected specimens and ‘*Ca. Phytoplasma solani*’ MLSA genotyping.

Region	Location (GPS) <sup>1</sup>	Habitat Type	Date of Collection	No. of Analyzed/CPs-Positive Specimens (%)	‘ <i>Ca. P. solani</i> ’ MLSA Genotype <sup>2</sup>			
					<i>tuf</i> Sequence	<i>secY</i> Sequence	<i>vmp1</i> Profile	<i>stamp</i> Sequence
Northern Serbia	1. Dobanovci (44°50.890' N 20°10.089' E)	Semi-mesic meadow	August 2017	15/0	-	-	-	-
Central Serbia	2. Topola (44°13.705' N 20°41.137' E)	Vineyards region	July 2016	27/3 (11%)	tuf-b1	S1	V2-TA	STOL (2)
			August 2016	14/1 (7%)	tuf-b1	S1	V2-TA	Rqg31
			August 2017	75/7 (9%)	tuf-b1	S1	V2-TA	STOL (5)
	3. Krnjevo (44°25.787' N 21°02.764' E)	Vineyards region	September 2018	10/1 (10%)	tuf-b1	S1	V2-TA	STOL
	4. Donji Milanovac (44°31.501' N 22°02.500' E)	Abandoned pasture	July 2017	26/3 (12%)	tuf-b1	S1	V2-TA	Rqg50
					tuf-b1	S1	V14	Rqg50 (2)
Eastern Serbia	5. Brza Palanka (44°29.201' N 22°27.381' E)	Moderate hillside meadow	July 2018	12/1 (8%)	tuf-b1	S1	V14	Rqg50-sv1
			August 2016	48/5 (10%)	tuf-b1	S1	V2-TA	STOL (5)
			August 2017	23/3 (13%)	tuf-b1	S1	V2-TA	STOL (3)
			August 2018	34/4 (12%)	tuf-b1	S1	V2-TA	STOL (4)
Southern Serbia	7. Jasenovik (43°21.909' N 22°01.341' E)	Stony meadow	August 2017	48/5 (10%)	tuf-b1	S1	V2-TA	STOL (5)
	8. Vlasotince (42°56.283' N 22°05.543' E)	Xerothermic ruderal slope	July 2017	25/2 (8%)	tuf-b1	S18	V14	Rqg50-sv1
					tuf-b1	S18	V2-TA	Rqg50
Total				357/35 (10%)				

<sup>1</sup> The number preceding the location name corresponds to the number denoting the location in Figure 1. <sup>2</sup> Genotype designations are in accordance with previously published nomenclature for the *tuf* gene [17,28], the *secY* gene [7,28], and *vmp1* RFLP profiles [6,11], while *stamp* genotypes are indicated by their corresponding reference strains [11,15] or as SNP variants (sv) when they include a single-nucleotide mutation. The number of isolates at a location with the same MLSA genotype, if more than one, is indicated in brackets next to the *stamp* genotype.



**Figure 1.** Map of surveyed localities in Serbia for occurrence of fulgoroid planthopper *Dictyophara europaea* and its tentative host plants naturally harboring ‘*Candidatus Phytoplasma solani*’. For each location diversity and frequencies of ‘*Candidatus Phytoplasma solani*’, multilocus genotypes based on *tuf*, *secY*, *vmp1*, and *stamp* genes are presented for *Dictyophara europaea* populations and tentative reservoir plants. Pie chart sections represent the proportion of specific multilocus genotypes. Maps were generated with d-maps.com ([https://d-maps.com/carte.php?num\\_car=27550&lang=en](https://d-maps.com/carte.php?num_car=27550&lang=en), accessed on 29 May 2022).

## 2.2. Survey of *D. europaea* and Reservoir Plants

Insect collections were carried out from mid-July to mid-September from 2016 to 2018, in accordance with previously observed data on adult flight periods [22]. Due to the adults' polyphagous feeding behavior, specimens were collected using entomological sweep nets and mouth aspirators from dominant broadleaf weedy plants in each locality. Collected specimens were placed in 2 mL plastic vials (Sarstedt) filled with 96% ethanol and transported to a laboratory in mobile refrigerators at 10 °C, where they were identified at the species level using taxonomic keys [21,29]. The insects were stored in ethanol at −20 °C after identification until DNA extraction.

In all studied areas, no plants expressed symptoms of phytoplasma infection. The most abundant plant species from the four selected locations were sampled in early September to determine their status as 'Ca. *P. solani*' reservoir plants and inoculum sources (Table 2). Each plant's leaf samples were stored in a separate plastic bag and transported in the field refrigerator at 10 °C. Each sample was processed for DNA extraction upon arrival at the laboratory and then stored in a freezer at −20 °C for further analysis.

**Table 2.** 'Candidatus *Phytoplasma solani*' identification in surveyed asymptomatic weeds (frequency and genotype) collected in September 2018/2019.

Location (GPS) <sup>1</sup>	Plant Species	No. of Analyzed/CPs-Positive Specimens (%)	'Ca. <i>P. solani</i> ' MLSA Genotype <sup>2</sup>			
			<i>tuf</i> Sequence	<i>secY</i> Sequence	<i>vmp1</i> Profile	<i>stamp</i> Sequence
2. Topola	<i>Salvia pratensis</i>	20/2 (10%)	tuf-b1	S1	V2-TA	STOL (2)
	<i>Convolvulus arvensis</i>	20/8 (40%)	tuf-b1	S1	V14	Rqg50 (4)
			tuf-b1	S4	V2-TA	Rqg31 (2)
			tuf-b1	S1	V2-TA	Rqg31-sv1
			tuf-b1	S1	V14	Rpm35
	<i>Clematis vitalba</i>	6/0	-	-	-	-
4. Donji Milanovac	<i>Convolvulus arvensis</i>	12/4 (33%)	tuf-b1	S1	V14	Rqg50 (3)
			tuf-b1	S4	V2-TA	Rqg50
	<i>Cichorium intybus</i>	6/2 (33%)	tuf-b1	S1	V2-TA	STOL (2)
	<i>Daucus carota</i>	6/1 (17%)	tuf-b1	S1	V2-TA	STOL
	<i>Sorghum halepense</i>	6/2 (33%)	tuf-b1	S1	V2-TA	STOL (2)
	<i>Elymus repens</i>	6/1 (17%)	tuf-b1	S1	V2-TA	STOL
6. Rogljevo	<i>Crepis foetida</i>	20/12 (60%)	tuf-b1	S1	V2-TA	STOL (8)
			tuf-b1	S1	V2-TA	STOL-sv1
			tuf-d	S1	V2-TA	STOL (3)
	<i>Picris hieracioides</i>	16/6 (38%)	tuf-b1	S1	V2-TA	STOL (6)
	<i>Cichorium intybus</i>	6/2 (33%)	tuf-b1	S1	V2-TA	STOL (2)
	<i>Daucus carota</i>	16/7 (44%)	tuf-b1	S1	V2-TA	STOL (4)
			tuf-b1	S1	V2-TA	STOL-sv1
			tuf-d	S1	V2-TA	STOL (2)
	<i>Sorghum halepense</i>	6/2 (33%)	tuf-b1	S1	V2-TA	STOL (2)
	<i>Elymus repens</i>	6/1 (17%)	tuf-b1	S1	V2-TA	STOL
	<i>Achillea millefolium</i>	6/0	-	-	-	-
<i>Falcaria vulgaris</i>	6/0	-	-	-	-	
<i>Clematis vitalba</i>	6/0	-	-	-	-	
7. Jasenovik	<i>Crepis foetida</i>	18/10 (56%)	tuf-b1	S1	V2-TA	STOL (10)
	<i>Picris hieracioides</i>	20/8 (40%)	tuf-b1	S1	V2-TA	STOL (8)
	<i>Erigeron annuus</i>	6/1 (17%)	tuf-b1	S1	V2-TA	STOL
	<i>Daucus carota</i>	6/1 (17%)	tuf-b1	S1	V2-TA	STOL
	<i>Cynodon dactylon</i>	8/1 (13%)	tuf-b1	S1	V2-TA	STOL
	<i>Carlina vulgaris</i>	6/0	-	-	-	-
	<i>Senecio erucifolius</i>	6/0	-	-	-	-
Total		246/71 (29%)				

<sup>1</sup> The number preceding the location name corresponds to the number denoting the location in Figure 1. <sup>2</sup> Genotype designations are in accordance with previously published nomenclature for the *tuf* gene [17,28], the *secY* gene [7,28], and *vmp1* RFLP profiles [6,11], while *stamp* genotypes are indicated by their corresponding reference strains [11,15] or as SNP variants (sv) when they include a single-nucleotide mutation. The number of isolates at a location with the same MLSA genotype, if more than one, is indicated in brackets next to the *stamp* genotype.

## 2.3. DNA Extraction from Insects, Herbaceous Hosts, and Experimental Plants

Total DNA was extracted from 357 individual insect specimens using a previously reported nondestructive extraction method [13]. Under a stereomicroscope Leica MZ7.5 (Leica Microsystems, Wetzlar, Germany), each *D. europaea* specimen was perforated between the hind legs and mesothorax and put into 2 mL tubes (Sarstedt, Nümbrecht, Germany) containing 800 µL of extraction buffer (20 mM TRIS, 10 mM EDTA, 0.5% SDS)

and 8  $\mu$ L of proteinase K. After overnight incubation at 56 °C, the specimens were removed and stored in 96% ethanol, and DNA was extracted from the solution using a chloroform–isopropanol procedure. In the final extraction stage, isolated DNA was dissolved in 150  $\mu$ L of TE buffer (10 mM TRIS, 1 mM EDTA; pH 7.6).

Using a previously reported CTAB procedure [30], total DNA was extracted from the leaf samples of field-collected plants and of experimental plants (grapevines and periwinkles). The DNA pellets were resuspended in 100  $\mu$ L of TE buffer. The DNA extracted from insects and plants was stored at –20 °C until further analysis.

#### 2.4. ‘*Ca. P. solani*’ Identification

The initial identification of ‘*Ca. P. solani*’ in field-collected *D. europaea* adults was conducted using the ‘*Ca. P. solani*’-specific Stol11 nested PCR protocol with the Stol11F2/R1 and Stol11F3/R2 primer pair [31,32], following a previously published protocol [33]. The same ‘*Ca. P. solani*’ detection protocol was utilized for all field samples and experimental plants from the transmission trials. DNA extracts of ‘*Ca. P. solani*’-infected grapevine and insects from Serbia [11,12] were used as positive controls in all amplification reactions.

#### 2.5. Experimental Transmission of ‘*Ca. P. solani*’ with Field-Collected *Dictyophara europaea*

To determine whether *D. europaea* naturally harboring CaPsol could act as a natural vector within an ecosystem and given that its vector role for FD phytoplasma has previously been established [23,26], we undertook transmission trials with CaPsol-infected specimens. Experiments were carried out with adults from two locations where *D. europaea* populations were dense and naturally carried phytoplasma at a rate of around 10%: (i) Topola, in the grape-growing region of central Serbia, with a rate of CaPsol-infected specimens of 9%, and (ii) Rogljevo in eastern Serbia, with a rate of CaPsol-infected specimens of 11% (Table 1). In the middle of August 2018, a total of 576 *D. europaea* adults were collected using entomological nets and mouth aspirators in Topola and Rogljevo.

During an inoculation access period (IAP) lasting 48 h at 24  $\pm$  1 °C (16/8 h light/dark period), 12 experimental grapevine seedlings and 12 periwinkle plants per each locality were exposed to 12 *D. europaea* adults presumably naturally infected with ‘*Ca. P. solani*’ at the rate of ~10% (Table 3).

**Table 3.** Experimental transmission of ‘*Ca. P. solani*’ to grapevine and *Catharantus roseus* by naturally infected *Dictyophara europaea*.

Location of <i>D. europaea</i> Collection	Experimental Plant	No. of <i>D. europaea</i> Specimens per Plant	No. of CPs-Transmitted/Replicate Plants	<i>tuf/secY/vmp1/stamp</i> Genotype of Transmitted Isolates (Number of Isolates)
Rogljevo, Eastern Serbia	<i>Vitis vinifera</i> cv. Chardonnay	12	3/12	tuf-b1/S1/V2-TA/STOL (3)
	<i>Catharantus roseus</i>	12	4/12	tuf-b1/S1/V2-TA/STOL (4)
Topola, Central Serbia	<i>Vitis vinifera</i> cv. Chardonnay	12	2/12	tuf-b1/S1/V2-TA/STOL (2)
	<i>Catharantus roseus</i>	12	3/12	tuf-b1/S1/V2-TA/STOL (2) tuf-b1/S1/V2-TA/Rqg50 (1)
Total			12/48	

Furthermore, four grapevine seedlings and four periwinkles that had not been exposed to insects were used as control plants. The insects’ feeding was visually confirmed, and after two days, all individuals were removed from the experimental plants.

The experimental plants were kept in the growth chamber under the same conditions as during the IAP and monitored for symptom development. Ten months later, the leaves of all test and control plants were sampled for the identification of ‘*Ca. P. solani*’ using the Stol11 protocol.

### 2.6. Multilocus Sequence Analysis (MLSA) of ‘*Ca. P. solani*’ Isolates

Field-collected weeds and insects, and experimentally infected grapevines and periwinkles were subjected to molecular typing to assess the epidemiological relevance of ‘*Ca. P. solani*’ isolates associated with *D. europaea*. Isolates were characterized by sequence and/or RFLP analysis of the following four phytoplasma genomic loci: (i) the two housekeeping genes—*tuf*, which encodes translation elongation factor Tu, and *secY*, encoding the preproteins translocase subunit SecY—and (ii) two membrane protein genes—antigenic membrane protein gene *stamp* and variable membrane protein *vmp1* gene of ‘*Ca. P. solani*’.

The *tuf* gene of ‘*Ca. P. solani*’ was amplified using nested PCR with primer Tuf1f/r, followed by TufAYf/r, as described previously [2].

The *secY* gene was amplified using nested PCR with primers PosecF1 and PosecR1, followed by PosecF3 and PosecR3 [34], while the *stamp* gene was amplified using the StampF/R0 and StampF1/R1 primer pairs [15]. The nested PCR amplicons were sequenced in both directions with the primers used for *tuf* gene amplification, reverse primer PosecR3 for *secY*, and forward primer StampF1 for the *stamp* gene. The sequencing was performed by Macrogen Inc., and the sequences were deposited in the GenBank database under accession numbers ON804249–ON804271, ON804278–ON804304, and ON804313–ON804341 for the *stamp*, *tuf*, and *secY* genes, respectively (Table S1). The sequences were compared to those previously published [11,17,20,35,36] using the BLASTn algorithm (<https://blast.ncbi.nlm.nih.gov>, accessed on 26 February 2022).

Using NETWORK software (Network 10.2.0.0, Forster P.&M., Colchester, England, [www.fluxus-engineering.com](http://www.fluxus-engineering.com), accessed on 18 May 2022), a phylogenetic network was constructed to assess the evolutionary relatedness and resolve the genealogy of the *stamp* genotypes of all ‘*Ca. P. solani*’ types involved in the epidemiological cycles in the studied locations. The network was constructed using the strains found in this study and the Balkan reference strains [11,16,20,35,37,38]. Gene genealogies were inferred by performing median joining (MJ) calculations [39] with parameter  $e = 0$  and maximum parsimony (MP) post-processing to obtain a network containing all the shortest trees.

A fragment of the *vmp1* gene encoding a putative ‘*Ca. P. solani*’ membrane protein was amplified using the StolH10F1/R1 primer pair [14], followed by the TYPH10F/R inner primer pair [34]. The nested products of all characterized isolates were digested with the *RsaI* restriction enzyme, and isolates expressing the V2 profile were also digested with *TaqI* and *AluI* [11,20]. The digested products were separated using automated capillary electrophoresis QIAxcel Advanced System (Qiagen) according to conditions described previously [13], and restriction profiles were compared with the reference controls of the V2-TA, V3, V4, V7-A, V14 and V17 *vmp1* profiles [11,20].

Primer pairs that were used for amplification and sequencing of each analyzed gene region are listed in Supplementary Table S2.

## 3. Results

### 3.1. ‘*Ca. Phytoplasma solani*’ Identification in *Dictyophara europaea* and Natural Reservoir Plants

The survey of *D. europaea* adults and reservoir plants naturally harboring ‘*Ca. P. solani*’ was undertaken in eight sites, three of which were located in the grape-growing regions of central and eastern Serbia. Over the course of four years, specimens of *D. europaea* and weeds were collected from mid-July to mid-September (from 2016 to 2019). There were 357 specimens of *D. europaea* collected from the various regions that were studied, and 35 (10%) of them tested positive for CaPsol. To be more specific, infection rates ranged from 7% to 13% in seven diverse localities, while none of the individuals collected in Dobanovci (in the Srem region) tested positive for CaPsol (Table 1).

In vineyard region of Topola in central Serbia (Table 1 and Figure 1, location 2), *Salvia pratensis* L. (Lamiaceae) was the most abundant plant, evenly covering the surface of the meadow, intermixed with field bindweed (*Convolvulus arvensis* L. (Convolvulaceae)) and climbing shrub (*Clematis vitalba* L. (Ranunculaceae)) at the edges of the meadow. In this location, the majority of *D. europaea* specimens were obtained from *S. pratensis*, while the

incidence of insect infection was 9% (Table 1). In total, 2 of 20 *S. pratensis* and 8 of 20 *C. arvensis* carried CaPsol. In contrast, none of the six examined *C. vitalba* was infected with CaPsol (Table 2).

The vegetation in the grape-growing region of Šumadija, at the location of Krnjevo (Table 1 and Figure 1, location 3), consisted of various plant species, with nearly equal proportions of grasses and deciduous plants. Only ten *D. europaea* were collected as a result of survey at this location, one of which tested positive for CaPsol infection (Table 1).

In the abandoned pasture on Danube River at Donji Milanovac (Table 1 and Figure 1, location 4), *C. arvensis* intermixed with various grass plants covered most of the surface [40]. CaPsol infection was detected in four of twelve asymptomatic bindweeds, two of six *Sorghum halepense* and *Cichorium intybus*, and one of six *Daucus carota* and *Elymus repens* (Table 2). The infection rate of *D. europaea* in this location was 12% (Table 1).

In a moderate hillside meadow at location Brza Palanka (Table 1 and Figure 1, location 5), near Danube River in eastern Serbia, we collected 12 *D. europaea*, one of which was infected with CaPsol (Table 1).

The ruderal hillside at the vineyard region of Rogljevo in eastern Serbia (Table 1 and Figure 1, location 6) had plant coverage that consisted of *C. foetida* and *P. hieracioides* as dominant plants, followed by various Asteraceae, Poaceae, and Apiaceae plants. Climbing shrub *C. vitalba* was found along the meadow's edges. The *D. europaea* population was dense, with 105 specimens collected in 2016–2018, of which 12 (11%) were positive for CaPsol infection (Table 1). CaPsol was detected in 12 of 20 *C. foetida*, 7 of 16 *D. carota*, and 6 of 16 *P. hieracioides*. CaPsol was also found in two of six *S. halepense*, and *C. intybus*, and one of six *E. repens*. None of the analyzed *C. vitalba*, *Achillea millefolium*, and *Falcaria vulgaris* tested positive for CaPsol (Table 2).

In the Jasenovik village, near the town of Niš in southern Serbia, specimens of *D. europaea* were collected from the stony meadow (Table 1 and Figure 1, location 7), where *Crepis foetida* L. and *Picris hieracioides* L. (Asteraceae) covered approximately 30% of the surface. Other members of the Asteraceae family were also abundant in this location, as well as a variety of legume plant species. CaPsol was found in 10 of the 18 *C. foetida* and 8 of the 20 *P. hieracioides* plants sampled. Among the other reservoir plants collected, *Cynodon dactylon*, *Daucus carota*, and *Erigeron annuus* tested positive for CaPsol at rates of 13–17%, while *Carlina vulgaris* and *Senecio erucifolius* were not infected (Table 2). We collected 48 *D. europaea* at this location, 5 of which (10%) carried CaPsol (Table 1).

At a xerothermic ruderal slope near the town of Vlasotince, also in eastern Serbia (Table 1 and Figure 1, location 8), we collected 25 *D. europaea*, 2 of which tested positive for CaPsol infection (Table 1).

Finally, none of the 15 *D. europaea* specimens collected in the semi-mesic meadow at the location of Dobanovci in Vojvodina (northern Serbia, location 1), tested positive for CaPsol infection (Table 1).

### 3.2. 'Ca. *Phytoplasma solani*' Genotypes Associated with *Dictyophara europaea* and Reservoir Plants

All 'Ca. *P. solani*' isolates identified in *D. europaea*, reservoir plants, and experimental test plants were characterized through the multilocus typing of four genes: *tuf*, *secY*, *vmp1*, and *stamp*. The sequence analyses of the *tuf* gene region of CaPsol strains identified in *D. europaea* from eight locations revealed that all were infected with the *tuf*-b1 genotype (Table 1). Among plant isolates, *tuf*-b1 was also the most prevalent genotype, detected in 93% of infected plants. Beside *tuf*-b1, at the location of Rogljevo, ruderal hillside in a traditional vine growing district of east Serbia, the *tuf*-d type was detected in 25% of infected *C. foetida* and 28% of infected *D. carota* (Table 2). This type was recently identified in sugar beet, parsley, and parsnip from northern Serbia [38].

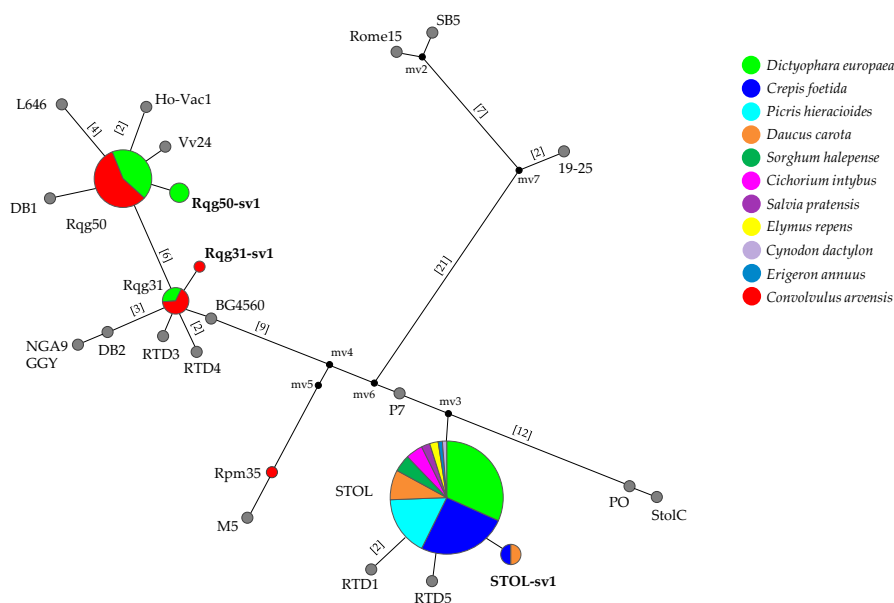
The *secY* genotyping revealed the presence of three genotypes in *D. europaea* and reservoir plants (Tables 1 and 2). The most prevalent was the S1 genotype (STOL; AM992086) [15,28], which is common in and endemic to Europe and is associated with various crops and

reservoir plants [1,7,11,15,17]. This genotype was detected in all *D. europaea* isolates from six locations (except Vlasotince) and was prevalent (96%) in reservoir plants from all natural habitats. Two CaPsol-positive *D. europaea* collected at the xerothermic ruderal slope near Vlasotince carried the *secY* S18 genotype corresponding to isolate LOZA2 (FN813282) [7,15], which was previously identified in grapevine sampled in eastern Serbia in 2006. In 25% of *C. arvensis* CaPsol-infected plants from vineyards region in the locality of Topola and in 25% of CaPsol-infected *C. arvensis* from the abandoned pasture of Donji Milanovac, the S4 genotype was detected (GGY; AM992093) [15,28]. This genotype is also endemic to Europe and is associated with various crops and reservoir plants.

The *vmp1* RFLP typing using *RsaI*, *TaqI*, and *AluI* endonucleases resulted in the identification of two different profiles, namely, V2-TA, and V14, both previously identified and designated as such [6,11] (Tables 1 and 2). Most of the CaPsol isolates of *D. europaea* (86%) and reservoir plants (89%) belonged to the V2-TA genotype. This profile was detected in plants and insects from all surveyed locations. Beside V2-TA, profile V14 was detected in one of eleven *D. europaea* from Topola, two of three *D. europaea* from Donji Milanovac, one of two *D. europaea* from Vlasotince, and in one specimen collected in Brza Palanka, as well as in five of eight and three of twelve infected bindweeds from Topola and Donji Milanovac, respectively.

The sequence analysis of the *stamp* gene marker resulted in the identification of seven different CaPsol genotypes among all *D. europaea* and reservoir plant isolates (Tables 1 and 2; Figure 2). Four genotypes (STOL, Rqg50, Rqg31, and Rpm35) were identical to previously recorded reference strains [11,15]. The STOL genotype was the most abundant and present in *D. europaea* and the weeds from four locations. In Krnjevo, Rogljevo, and Jasenovik, this was the only genotype present in *D. europaea*, while in the vineyard region of Topola, this genotype was detected in 72% of infected insects. The STOL genotype was found in all reservoir plants with the exception of *Convolvulus arvensis*, as expected, given that this plant harbors specific '*Ca. P. solani*' genotypes due to its association with *Hyalesthes obsoletus* as a primary vector, driving the pathogen transmission and host-plant-associated epidemiology [12,20,36,40]. Genotype Rqg50 was found in CaPsol-infected *C. arvensis* and *D. europaea* isolates from Topola and Donji Milanovac. All isolates associated with *C. arvensis* and *D. europaea* from Donji Milanovac carried this genotype, as well as 50% of *C. arvensis* and 18% of *D. europaea* isolates from Topola. This genotype was also identified in one of two CaPsol-positive *D. europaea* from Vlasotince. Genotype Rqg31 was sporadically found in isolates of *D. europaea* and bindweed from Topola, while the Rpm35 type was found only in one isolate of *C. arvensis* from the same location. The new *stamp* genotype (Rqg50-sv1), an SNP variant of Rqg50, was detected in a single specimen of *D. europaea* from Vlasotince and Brza Palanka. One of eight CaPsol-positive *C. arvensis* isolates from Topola harbored new *stamp* genotype Rqg31-sv1, which is an SNP variant of Rqg31. One of twelve CaPsol-positive *C. foetida* and one of seven CaPsol-positive *D. carota* from Rogljevo had a unique *stamp* genotype (STOL-sv1) representing an SNP variant of the STOL genotype.





**Figure 2.** Median-joining network inferred from *stamp* genotypes of ‘*Candidatus Phytoplasma solani*’ strains detected in naturally infected *Dictyophara europaea* and reservoir plants in this study and those previously identified in the Balkans [11,16,20,35,37,38]. Genotypes PO and StoIC originating from

France [15] were used for stabilization of the network. Genotypes are represented by circles and paired genotypes are connected by a line. Circle sizes are proportional to the strain frequency found in this study and colors correspond to the associated host as shown in the figure legend. Each line connecting the circles is one mutational difference. For more than one mutational difference between strains, numbers of SNP mutations are presented in brackets; black dot vertices represent median vectors (mv). Gray circles represent reference strains not detected in this study. Strain and vector abbreviations are indicated. Newly detected strains designated in this study are in bold.

All isolates carrying the *tuf-d* genotype were affiliated to the *stamp* gene STOL genotype, while in other ‘*Ca. Phytoplasma solani*’ isolates of the same host plant the *tuf-b1* type of the *tuf* gene occurred syntopically, i.e., coexisting on location.

### 3.3. Multilocus Sequence Analysis

Overall, thirteen *tuf/secY/vmp1/stamp* comprehensive ‘*Ca. Phytoplasma solani*’ genotypes were detected in the surveyed localities (Figure 1). The isolates of *D. europaea* harbored seven CaPsol genotypes, while eight were detected in natural plant reservoirs. Among them, nine corresponded to previously identified genotypes [10,11,17,38]. New MLSA genotypes *tuf-b1/S1/V14/Rqg50-sv1* and *tuf-b1/S18/V14/Rqg50-sv1* were found in *D. europaea*. New MLSA genotype *tuf-b1/S1/V2-TA/Rqg31-sv1* was found in *C. arvensis*, whereas *tuf-b1/S1/V2-TA/STOL-sv1* was shared by two hosts, *C. foetida* and *D. carota*.

### 3.4. Transmission Trials

To determine whether *D. europaea* could be a natural vector of ‘*Ca. P. solani*’ and to elucidate its epidemiological role, transmission trials using naturally infected adults were performed with specimens collected in Topola and Rogljevo, where CaPsol was previously detected in 11 out of 116 (9%) and in 12 out of 105 (11%) individuals, respectively (Table 1).

The survival rate of insects was above 90% after 48 h on both grapevine seedlings and periwinkles. Five weeks after inoculation, four *C. roseus* test plants exposed to *D. europaea* from Rogljevo and three *C. roseus* inoculated by *D. europaea* from Topola developed symptoms of phytoplasma infection in the form of intense leaf yellowing and virescence.

Approximately four months after the experiments ended, grapevine seedlings exposed to both populations of *D. europaea* developed the first symptoms of phytoplasma infection.

Symptoms appeared as interveinal discoloration and lamina reddening in three grapevine seedlings inoculated with *D. europaea* from Rogljevo and three grapevine seedlings inoculated with *D. europaea* from Topola. The subsequent analysis of plant DNA using the Stol11 protocol confirmed that all grapevine and periwinkle plants with symptoms were infected with CaPsoI. The 18 symptomless grapevine plants and 17 periwinkles tested negative for phytoplasma presence (Table 3).

All CaPsoI-positive grapevine and *C. roseus* isolates were subjected to genotyping using MLSA. Overall, three different '*Ca. P. solani*' genotypes based on *tuf/secY/vmp1/stamp* typing were found in the experimental plants. All three grapevines and four periwinkles infected by *D. europaea* individuals from Rogljevo harbored the *tuf-b1/S1/V2-TA/STOL* type. The *D. europaea* population from Topola transmitted the same genotype to two grapevine seedlings and two periwinkles. Moreover, individuals from Topola also transmitted the *tuf-b1/S1/V2-TA/Rqg31* genotype to single grapevine and *tuf-b1/S1/V2-TA/Rqg50* to one periwinkle (Table 3).

#### 4. Discussion

The European lantern fly (*D. europaea*) is a common polyphagous phloem-feeding planthopper found throughout the Western Palaearctic, with records of its range expanding to Northwestern China [41]. It is a univoltine species frequently found on ruderal, xerothermic, and sunny hillsides in association with dicotyledonous herbs, grasses, and shrubs [21,29]. The long period of adult activity in ecologically diverse ecosystems, which lasts from the end of June to the beginning of October (reviewed in [22]), combined with this planthopper's polyphagous feeding behavior, enables it to come into contact with various phytoplasmas invading diverse plant species.

Even though the presence of phytoplasmas from three distinct groups was found in *D. europaea*, including 16SrII, 16SrV, and 16SrXII [23–27], the role of this planthopper has only been confirmed in the FD phytoplasma (16SrV-C subgroup) epidemiological cycle, in which *D. europaea* acquires phytoplasma from naturally infected *C. vitalba* and transmits it to grapevine [23,26].

Our study utilized a combination of molecular and experimental approaches to identify and link relevant constituents in the epidemiological transmission routes of CaPsoI, clarifying the epidemiological role of this planthopper in the spread of BN in Serbian vineyards. The multilocus typing of four epidemiologically informative genes, *tuf*, *secY*, *vmp1*, and *stamp* [2,6,14,15], revealed the complexity of CaPsoI epidemiology, as indicated by the number of detected pathogen genotypes and the number of hosts shared by the same genotype.

The most frequent CaPsoI comprehensive genotype was *tuf-b1/S1/V2-TA/STOL*. It was the only genotype found in the isolates of *D. europaea* as well as potential reservoir plants, including *C. foetida*, *P. hieracioides*, *S. pratensis*, *E. annuus*, *D. carota*, *C. intybus*, *S. halepense*, and *E. repens*, in the localities of Rogljevo and Jasenovik. This genotype is regarded as the historical reference strain for CaPsoI, and it originates from pepper in Serbia [1]. It is the most common and widespread genotype across the Balkan peninsula, and it has previously been linked to three distinct epidemiological cycles of the *tuf-b1* CaPsoI strain: (i) the *R. panzeri*-vectored disease cycle, causing maize redness (MR) disease in maize, BN disease in grapevine, and 'stolbur' disease in potato [4,11,36,42]; (ii) the '*Ca. P. solani*' disease cycle driven by *H. obsoletus* associated with *C. foetida* in BN-affected vineyards in eastern Serbia [12]; and (iii) the '*Ca. P. solani*' disease cycle driven by *H. obsoletus* from *Vitex agnus-castus* in the coastal zone of Montenegro [20].

Aside from the dominant STOL genotype, two other *stamp* genotypes of epidemiological importance in the Balkans, Rqg50 and Rqg31, were detected in *D. europaea* and asymptomatic *C. arvensis* in areas where field bindweed was the dominant reservoir plant. Overall, six MLSA genotypes were found in *D. europaea* and *C. arvensis* from these locations (Topola and Donji Milanovac), one of which (*tuf-b1/S1/V14/Rqg50*) was shared by both *D. europaea* and field bindweeds. The Rqg50 and Rqg31 *stamp* genotypes have

previously been proven to be vectored to grapevine and potato plants by *H. obsoletus* associated with *C. arvensis* [12,36] and have repeatedly been found in *C. arvensis*, its reservoir plant [12,20,40].

Regarding the *tuf* gene variability, in the location of Rogljevo in eastern Serbia, 3 of 12 *C. foetida* and 2 of 7 *D. carota* positive for CaPsol were found to be infected with the *tuf-d* type. This finding represents the first detection of the *tuf-d* genotype in plants of the natural environment and, even more, in asymptomatic plants which could serve as a reservoir for this genotype. The *tuf-d* type has previously been explicitly linked to the epidemic outbreaks of rubbery taproot disease in sugar beet (RTD) in northern Serbia, Croatia, Hungary, and Slovakia [38], but without connections to the source plants of the outbreaks. Since in our study this *tuf* type was found in tentative reservoir plants alongside *tuf-b1* and was always associated with the STOL genotype, its epidemiological implications are uncertain. Although more research is needed, it appears that *tuf-d* is an SNP variant of *tuf-b* with shared ecological links with host plants.

In our transmission trials, all three epidemic genotypes identified in *D. europaea* and reservoir plants were transmitted to periwinkles and/or grapevines (Table 3). Naturally infected *D. europaea* from both locations, Topola and Rogljevo, transmitted the *tuf-b1/S1/V2-TA/STOL* CaPsol type to periwinkles and grapevines. In addition to this genotype, insects from Topola were capable of transmitting *tuf-b1/S1/V2-TA/Rqg31* to grapevine seedlings and *tuf-b1/S1/V2-TA/Rqg50* to periwinkle.

Vector feeding habits and mobility, as well as the synchrony of host plant and vector phenology, determine whether the relationship between vector and the plant source of phytoplasma infection is well balanced or whether one plant serves as the predominant source for phytoplasma acquisition [43]. The seasonal occurrence and temporal dynamics of *D. europaea* in natural habitats in Serbia are well documented [22]. Due to the high mobility of adults, in late summer, when herbaceous vegetation is drying, they aggregate on the plants that are better adapted to drought conditions (e.g., *S. pratensis*) or migrate to shrubs growing close to the meadows (e.g., *C. vitalba*) [22]. Based on the MLSA matching of CaPsol in *D. europaea* and reservoir host plants, it could be assumed that deciduous plants susceptible to dry conditions, such as *C. foetida*, *P. hieracioides*, and *S. pratensis*, together with *C. arvensis*, the major and most widely distributed natural host of CaPsol, are the main reservoirs for CaPsol acquisition throughout the season.

Furthermore, given that *C. vitalba* has previously been found infected with CaPsol in Italy [44] and that *D. europaea* aggregates on *C. vitalba* in the late summer, acquiring FD phytoplasma and transmitting it to grapevine [23,26], it should be expected that *C. vitalba* could act as a reservoir plant for CaPsol, also in Serbia. Interestingly, none of the twelve analyzed plants from two localities where *D. europaea* populations were the most abundant was found to be infected with this phytoplasma. In the vineyards of Tuscany, *C. vitalba* and other weedy plants were found to be CaPsol-infected with the PO *stamp* genotype [44] belonging to Cluster I [28,45]. This genotype, as all other genotypes from *stamp* Cluster I, was never found in the Balkans and is characteristic of Western Europe. We can only speculate that *C. vitalba* is not susceptible to CaPsol genotypes from *stamp* Cluster II and III, which are widely distributed in Serbia [11–13,36,38] and Southeastern Europe [16,20,35,37], but further investigations considering more samples of climbing shrub from a wider area need to be pursued.

In previous research in China, cultivated plants of the *Salvia* genus (*S. miltiorrhiza*) were heavily affected by CaPsol, with infection rates ranging from 50 to 70 percent and severe plant damage [46,47]. Moreover, results from France indicated that cultivated crop *Salvia sclarea* is a host plant for CaPsol and its vector *H. obsoletus* and could be responsible for the increased incidence of CaPsol in lavender, causing lavender decline [48]. The presence of *Salvia pratensis* in natural habitats in Serbia, the aggregation of *D. europaea* on its lush leaves during the dry season [22], and the presence of the STOL genotype, which is widely distributed and experimentally transmitted to grapevine, indicate that this plant could

have a significant role in CaPsol epidemiology and BN outbreaks as its reservoir plant and feeding/aggregation host for polyphagous vectors.

Our results from the field-collected data and from the laboratory-controlled transmission assays provide experimental and molecular evidence that *D. europaea* is involved in pathogen dispersal within natural habitats and between natural habitats and cultivated crops. Combined with the life-history strategy of this planthopper, which involves long-living adults with a polyphagous feeding behavior, there are clear implications that this planthopper is an intermediate vector in the epidemiological cycles of diverse CaPsol-induced diseases and their natural plant reservoirs.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae8070654/s1>, Table S1: List of CaPsol isolates characterized on *tuf*, *secY*, *vmp1*, and *stamp* genes and their corresponding profiles or GenBank accession numbers sorted according to the hosts; Table S2: Primer pairs used for identification and characterization of CaPsol isolates.

**Author Contributions:** Conceptualization, T.C., J.J. and I.T.; methodology, T.C., J.J. and I.T.; validation, T.C.; formal analysis, T.C., J.J., O.K., S.M., M.J. and M.M.; investigation, T.C., J.J., O.K., S.M., M.J., M.M. and I.T.; data curation, T.C., J.J. and I.T.; visualization, T.C. and J.J.; writing—original draft preparation, T.C.; writing—review and editing, T.C., J.J., O.K., S.M., M.J., M.M. and I.T. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research study was funded by Ministry of Education, Science and Technological Development of the Republic of Serbia, contract No. 451-03-68/2022-14/200010.

**Data Availability Statement:** DNA sequences are available in the GenBank database, and accession numbers are listed in Table S2. All other relevant data are within the paper and its Supplementary Materials.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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