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Multiplex PCR for Discriminating Host Plant Associations of *Hyalesthes obsoletus* (Hemiptera: Cixiidae), a Key Vector and Driver of '*Ca.* Phytoplasma solani' Epidemiology

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Abstract: Given the ecological and epidemiological specialization of *Hyalesthes obsoletus*, the principle vector of '*Candidatus* Phytoplasma solani', the primary objective of this study was to develop molecular tools for discriminating three host plant associations of the vector populations: (i) *Convolvulus arvensis–Urtica dioica* (*Ca–Ud*), (ii) *Vitex agnus-castus* (*Vac*), and (iii) *Crepis foetida* (*Cf*). The genetic diversity of the nearly full-length mitochondrial *COI* gene (1467 bp) was analyzed and compared among previously reported and newly collected individuals of the three host plant associations on a wide geographic range. Multiplex PCR was designed and evaluated for discriminating *H. obsoletus* host plant associations based on the size of amplified fragments: 1084 bp for the *Cf* association, 645 bp for the *Ca–Ud* association, and 355 bp for the *Vac* association. Examples of the epidemiological value of combining data on the genetic characteristics of the vector and the pathogen are provided. The method is intended to facilitate an accurate identification of the vector's phylogenetic lineage, natural host plant preference, and epidemiological transmission routes of '*Ca.* P. solani'. When applied to *H. obsoletus* specimens collected from cultivated plants within an agroecosystem and combined with '*Ca.* P. solani' genotyping, the method should provide valuable information on disease epidemiology, source(s) of emergence, and transmission routes.

Keywords: barcoding; host plant; insect vector; molecular identification; mt*COI* gene; stolbur

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1. Introduction

The use of molecular methods to describe, determine, or precisely identify biological species has been one of the most notable advantages of their incorporation into taxonomical or applied biological studies (e.g., [1–4]). Molecular determination and identification of species type material (e.g., holotype, paratype/s, syntype/s) (e.g., [5,6]), cryptic species characterized by the absence of stable morphological traits for identification, and ecological host races or biotypes resulting from ecological specialization have increased the value of fundamental and applied taxonomic studies and natural world research [7,8]. This is due to the fact that molecular methods can acquire an enormous amount of data—much more than those available in the morphological characteristics of the species, i.e., select the species-specific ones, and define them as the reliable and repeatable tool for identification. Combined with other characteristics of the species in question (morphology, biology, geography, ecology, etc.), molecular methods are especially useful and valuable [9]. When dealing with the need for a precise, specific, and reliable determination and identification of agricultural pests, beneficial organisms, or endangered species, molecular methods have frequently proven to be both reliable and the most cost-effective option (e.g., [10–14]). In addition, the overall limited availability of traditional taxonomists' expertise on the identification of specific groups of organisms or species has been cited repeatedly as one of the primary reasons for the use of molecular identification methods, DNA barcoding,

molecular taxonomy, or even better, an integrative taxonomy, which can lead to the most precise molecular tools for identification [1,9,15].

The cixiid planthopper *Hyalesthes obsoletus* Signoret, 1865 (Hemiptera: Cixiidae) is a primary natural vector of '*Candidatus* (*Ca.*) Phytoplasma solani' ('*Ca.* P. solani')—a major phytoplasma pathogen of agricultural crops in Europe, previously known under its trivial name, "stolbur phytoplasma" [16–18]. '*Ca.* P. solani' affects many economically important plants, causing significant yield losses and increases in agricultural production costs [19], while also occurring in plants in natural environments bordering agricultural fields as reservoirs and inoculum sources [20–24]. '*Ca.* P. solani' primarily affects all major grape-growing regions in Europe and the Middle East, the lavender fields of France, the maize fields of Serbia, and various solanaceous crops in Western, Central, and Southeastern Europe, Asia Minor, and Transcaucasia [17,25–37]. In the epidemiological context of '*Ca.* P. solani' transmission and emergence, *H. obsoletus* is a major vector of the most prevalent phytoplasma disease of grapevine, the Bois noir (BN) [16,21,38], the vector of lavender decline [33], and has also been shown to vector '*Ca.* P. solani' to sugar beet, potato, pepper, and natural reservoir plants serving as the vector's host plant [38–41]. In the last two decades, several other cixiid planthoppers have been identified as vectors of '*Ca.* P. solani', namely *Pentastiridius leporinus* (Linnaeus, 1761), *Reptalus panzeri* (Löw, 1883), and *Reptalus artemisiae* (Becker, 1865) (previously known as *R. quinquecostatus* (Dufour, 1833) [42]) [26,27,29,43–47].

Efforts to design the tools for the molecular identification of '*Ca.* P. solani' planthopper vectors have so far been made only in three cases: for the differentiation of congeneric species with clearly discriminative morphological features on the male genitalia but a lack of distinctive traits for nymphs and females [10,11], and for the identification and discrimination of three species from distinct genera [48] whose outer morphological features allow differentiation, including both males and females (e.g., [49,50]). The former case develops molecular identification tools for four *Reptalus* species: *R. artemisiae*, *R. cuspidatus* (Fieber, 1876), *R. panzeri*, and *R. quinquecostatus* (Dufour, 1833) (previously known as *R. melanochaetus* (Fieber, 1876) [42]) [11], and three *Hyalesthes* species (*H. obsoletus*, *H. luteipes* Fieber, 1876, and *H. scotti* Ferrari, 1882) [10], while the latter case develops tools for molecular identification and the discrimination of cixiid vectors in sugar beet fields (*H. obsoletus*, *R. artemisiae*, and *P. leporinus*) [48]. In all three cases, methods were designed to allow scientists and plant protection practitioners to conduct epidemiological surveys, trace transmission pathways, obtain information on the host plant feeding preferences of immature stages of the vectors, and develop more effective control and management strategies for '*Ca.* P. solani'-induced plant diseases.

The need for the molecular identification of *H. obsoletus* individuals and/or populations is more demanding and complex, due to a number of previously established facts: (i) *H. obsoletus* is a major vector of '*Ca.* Phytoplasma solani', and a key vector of BN disease of grapevine [16,17,20,21,30,32,34,38,51–53]; (ii) *H. obsoletus* populations show clear signs of host plant-associated specialization and genetic differentiation, leading to the formation of host races [54,55] or cryptic species [56]; (iii) cryptic species of *H. obsoletus* are morphologically indistinguishable, but delineated by host plant choice and often found in sympatry [38,56], and iv) *H. obsoletus* host associations show signs of driving segregated epidemiological pathways of '*Ca.* P. solani' transmission, i.e., separated epidemiological cycles and routes of pathogen dissemination [21,38,47]. In Southeastern Europe, the distribution center of *H. obsoletus* and the area of most '*Ca.* P. solani'-inflicted crop diseases, specific host plant associations of the vector, and vector-based transmission routes are determined. These are represented by three phylogenetic plant-associated lineages of vector populations that are ecologically delineated by host plant preference as follows: (i) *Convolvulus arvensis* and *Urtica dioica*, (ii) *Vitex agnus-castus*, and (iii) *Crepis foetida* [56]. In addition, even though the geographic overlapping of the host plant-associated vector populations is evident, there are additional signs of geographic segregation of *H. obsoletus* populations associated with *V. agnus-castus* in Greece and in Montenegro [56]. All three cryptic species, or host plant

associations, are evidenced as vectors of '*Ca.* P. solani' (reviewed in [47]). Moreover, the genetic divergence of '*Ca.* P. solani' strains vectored by *H. obsoletus* [20] was the first indication of the ecological segregation of vector populations associated with the pathogen's reservoir plants. Strains of '*Ca.* P. solani' were initially identified as genetically distinct using *tuf* gene typing of isolates associated with *U. dioica* (tuf-a type) and *C. arvensis* (tuf-b type) [20], and later verified using several other epidemiologically informative genes (*secY*, *vmp1*, and *stamp*) that led to the discovery of more complex strain diversification [57–60].

Given the ecological and epidemiological specialization of *H. obsoletus* populations, the primary objective of our study was to develop and evaluate molecular methods of discrimination for the three host plant-associated vector populations (i.e., cryptic species). We intended to design a method that would be reliable, simple, and cost-effective enough to be used routinely. Furthermore, we aimed to link epidemiological data on the genetic characteristics of vector populations to the specific strains of '*Ca.* P. solani' that they harbor, as well as to combine this with information on host plant association and geographic area of origin.

2. Materials and Methods

2.1. Hyalesthes obsoletus Specimens Used for mtDNA COI Genotyping

For this study we used previously reported DNA material of *H. obsoletus* individuals originating from plant-specialized populations across a wide geographical area [56] as well as newly collected specimens. From the previously reported material, we used the DNA of selected specimens collected in association with one of the following host plants: *C. arvensis*, *U. dioica*, *V. agnus-castus*, and *C. foetida.* In total, 55 individuals were chosen for mitochondrial (mtDNA) cytochrome oxidase subunit I (*COI*) gene characterization and as the basis for the development of a protocol for specific identification of *H. obsoletus* host plant association (Table 1). The specimens were chosen to represent the majority of the *COI-COII* gene region diversity identified in our earlier study [56], as well as the geographic variability. They were selected to represent 26 of the 29 *COI-COII* haplotypes identified in our study, out of a total of 43 up-to-date known haplotypes [56]. These are the *H. obsoletus* haplotypes identified in association with their natural host plants in Southeastern Europe and Turkey, i.e., the geographic area where at least two out of three host-specialized *H. obsoletus* lineages have been shown to co-occur in sympatry. The remaining three haplotypes were excluded from analyses because they were collected from crop plants. The other 14 *COI-COII* haplotypes not included in analyses originate from either the Western European BB *COI-COII/16S-ND1*phylogeographic haplogroup or Israel.

During 2018 and 2019, additional surveys and a sampling of *H. obsoletus* populations were conducted to gain a more comprehensive understanding of haplotype diversity and to further examine the genetic segregation of plant-specialized populations in Greece versus Montenegro. The majority of the sampling was performed on the western coast of mainland Greece and on the Peloponnese, with a focus on populations associated with *V. agnus-castus*. In addition, specimens were collected from *U. dioica* in the southern Peloponnese, *V. agnus-castus* on Krk island in the northern Adriatic Sea (Croatia), and *Convolvulus cantabrica* in Southeastern Serbia, a novel potential host plant of *H. obsoletus* (Table 2). Six to twenty specimens were collected per location and host plant, and screened for *COI* genetic diversity. Unique specimens carrying distinct haplotypes per location were chosen for genetic comparison. In total, nine new localities were surveyed, resulting in the addition of 20 extra *H. obsoletus* individuals for *COI* gene diversity analysis. Newly collected specimens were kept in 96% ethanol and stored at −20 °C until DNA extraction. Total DNA was extracted from each individual insect specimen using the previously described non-destructive sodium dodecyl sulfate (SDS) extraction method [56].

Table 1. List of 55 *Hyalesthes obsoletus* specimens analyzed for genetic diversity of near full-length mitochondrial *COI* marker gene sorted according to affiliated haplotype and information on their previously determined affiliation to *COI-COII* (*16S-ND1*) haplotype, and country, locality, and host plant association of population origin [56].

***COI* Marker Gene (1467 bp)**			**Associated Haplotype *COI-COII* (*16S-ND1*) [1]**	**Country**	**Locality**	**Host Plant Association [2]**
Haplotype	**GenBank Acc No**	**Frequency**				
H1	MK172874	5	A (B)	Montenegro	Podgorica	*Ca*
				Greece	Kilkis	*Ca*
			W (L)	Greece	Kilkis	*Ca*
			W (B)	Greece	Profitis	*Ud*
			X (B)	Greece	Kilkis	*Ca*
H2	MK172875	13	E (C)	Serbia	Topola	*Ca*
				Serbia	Boljetin	*Ud*
				Serbia	Knjaževac	*Ud*
				Serbia	Bačka Topola	*Ud*
				Serbia	Srednjevo	*Ud*
				Serbia	Grnčar	*Ud*
				Montenegro	Podgorica	*Ud*
				Romania	Petrevo selo	*Ca*
			π (C)	North Macedonia	Strumica	*Ca*
			ξ (Q)	Serbia	Zaječar	*Ud*
			ω (C)	Serbia	Bačka Topola	*Ud*
			R (C)	Serbia	Gakovo	*Ud*
			α (C)	Montenegro	Podgorica	*Ud*
H3	MK172876	1	β (C)	Montenegro	Podgorica	*Ud*
H4	MK172877	1	F (C)	Serbia	Gakovo	*Ud*
H5	MK172878	7	E (C)	Serbia	Vranje	*Ud*
				North Macedonia	Strumica	*Ud*
				North Macedonia	Strumica	*Ca*
				Greece	Filadelfio	*Ud*
				Greece	Profitis	*Ud*
			ψ (C)	North Macedonia	Strumica	*Ca*
			ρ (C)	Greece	Profitis	*Ud*
H6	MK172879	1	S (K)	Turkey	Erzincan	*Ca*
H7	MK172880	1	η (N)	Montenegro	Ulcinj	*Vac*
H8	MK172881	1	θ (N)	Montenegro	Ulcinj	*Vac*
H9	MK172882	1	Z (N)	Montenegro	Kamenari	*Vac*
H10	MK172883	2	Z (N)	Montenegro	Kamenari	*Vac*
			γ (N)	Montenegro	Bar	*Vac*
H11	MK172884	4	Y (M)	Greece	Appolonia	*Vac*
				Greece	Arethousa	*Vac*
				Greece	Vrasna	*Vac*
				Greece	Larisa	*Vac*
H12	MK172885	1	σ (M)	Greece	Lesbos	*Vac*
H13	MK172886	12	J (H)	Serbia	Deligrad	*Cf*
				Serbia	Porečka reka	*Cf*
				Serbia	Negotin	*Cf*
				Serbia	Tamnič	*Cf*
				Serbia	Pirot	*Cf*
				Serbia	Jasenovik	*Cf*
			M (H)	Serbia	Negotin	*Cf*
				Serbia	Tamnič	*Cf*
				Romania	Calafat	*Cf*
				Bulgaria	Vidin	*Cf*
			λ (H)	Serbia	Vranjska banja	*Cf*
			μ (H)	Serbia	Deligrad	*Cf*
H14	MK172887	1	U (H)	Serbia	Porečka reka	*Cf*
H15	MK172888	1	V (H)	Serbia	Porečka reka	*Cf*
H16	MK172889	1	J (H)	Turkey	Kırşehir	*Cf*
H17	MK172890	1	J (H)	Turkey	Erzincan	*Cf*
H18	MK172891	1	J (H)	Turkey	Erzincan	*Cf*

[1] Data on *COI-COII* (*16S-ND1*) haplotypes associated with each analyzed *H. obsoletus* specimen are retrieved from our previous work [56]; [2] host plant association of the specimen: *Ca*—*C. arvensis*, *Ud*—*U. dioica*, *Vac*—*V. agnus-castus*, and *Cf*—*C. foetida*.

Table 2. List of 20 newly collected specimens of *Hyalesthes obsoletus* analyzed for genetic diversity of near full-length mitochondrial *COI* marker gene sorted according to affiliated haplotype and information on their country, region, location, and host plant of collection.

COI Marker Gene (1467 bp)		Country	Region	Locality	GPS Coordinates	Host Plant Association [1]
Haplotype	GenBank Acc No					
H2	MK172875	Greece	Peloponnese (south)	Sparta, Mystras	N37 04.548 E22 21.769	*Ud*
		Greece	Peloponnese (south)	Taygetus, Artemisia	N37 05.818 E22 13.719	*Ud*
H7	MK172880	Croatia	Primorje–Gorski Kotar	Krk, Stara Baška	N44 58.138 E14 39.797	*Vac*
H11	MK172884	Greece	Epirus	Igoumenitsa	N39 32.495 E20 17.245	*Vac*
		Greece	Western Greece	Menidi #	N39 03.643 E21 06.175	*Vac*
		Greece	Peloponnese (west coast)	Giannitsochori	N37 23.729 E21 42.232	*Vac*
		Greece	Peloponnese (west coast)	Kyparissia	N37 15.958 E21 41.007	*Vac*
		Greece	Peloponnese (south coast)	Kalamata	N37 03.761 E22 08.862	*Vac*
H19	OQ372231	Greece	Peloponnese (west coast)	Kyparissia	N37 15.958 E21 41.007	*Vac*
H20	OQ372232	Greece	Peloponnese (west coast)	Kyparissia	N37 15.958 E21 41.007	*Vac*
H21 *	OQ372233	Serbia	Southeastern Serbia	Pirot	N43 07.878 E22 26.923	*C. cantabrica*
H22	OQ372234	Greece	Epirus	Igoumenitsa	N39 32.495 E20 17.245	*Vac*
H23	OQ372235	Greece	Epirus	Igoumenitsa	N39 32.495 E20 17.245	*Vac*
H24	OQ372236	Greece	Epirus	Igoumenitsa	N39 32.495 E20 17.245	*Vac*
H25	OQ372237	Greece	Western Greece	Menidi	N39 03.643 E21 06.175	*Vac*
H26	OQ372238	Greece	Western Greece	Menidi	N39 03.643 E21 06.175	*Vac*
H27	OQ372239	Greece	Western Greece	Menidi	N39 03.643 E21 06.175	*Vac*
H28	OQ372240	Greece	Peloponnese (south)	Taygetus, Artemisia	N37 05.818 E22 13.719	*Ud*
H29	OQ372241	Greece	Peloponnese (south)	Taygetus, Artemisia	N37 05.818 E22 13.719	*Ud*
H30	OQ372242	Greece	Peloponnese (south)	Taygetus, Artemisia	N37 05.818 E22 13.719	*Ud*

[1] Host plant association of the specimen: *Ud*—*U. dioica*, *Vac*—*V. agnus-castus*, and *C. cantabrica*—*Convolvulus cantabrica*; * haplotype affiliated to CB haplotype on the *COI-COII*/*16S-ND1* gene regions; # locality of the H11 haplotype carrying '*Ca.* P. solani' *stamp* genotype GR-Ho-Vac (OQ377809).

Several mitochondrial markers, including the *COI-COII* and *16S-ND1* gene regions as well as *COI* gene fragments, have been utilized in previous studies for genetic identification or phylogenetic analyses of *H. obsoletus* [10,38,54,56,60–62]. However, we have chosen a nearly full-length *COI* gene for this study because it encompasses a barcoding region that is widely used for species molecular identification [1,7,8,15,63], and the gene's length (approximately 1550 bp) is sufficient for the analysis of a longer fragment of a single gene to provide the required amount of data for primer design and method development (e.g., [64]). In addition, we aimed to correlate previously obtained information on the diversity of *COI-COII* and *16S-ND1 H. obsoletus* haplotypes [56] with the diversity of the *COI* gene, so that future research can rely on these results regardless of the marker employed.

2.2. PCR Amplification and Sequencing of COI Gene

The nearly full-length *COI* gene was amplified from the DNA extracts of 75 individuals of *H. obsoletus* using a combination of four previously published [64,65] and three newly designed primers, resulting in overlapping amplicons that allowed us to read each nucleotide position at least twice. LCO1490 [65] and PAT (i.e., TL2-N-3014) primers [64] were used to amplify ~1500 bp-long fragment, LCO1490 and HCO2198 primer pair [65] was used to generate ~700 bp-long amplicons on the 5′-end of the gene, and CO1-RLR (i.e., C1-J-2195) and PAT primers [64] were used to amplify ~900 bp-long fragment on the 3′-end. After obtaining the initial sequences of the nearly complete *COI* gene of *H. obsoletus*, the identities of primer sequences and their binding positions were compared. In response, we designed three new primers for use in amplification and/or sequencing reactions. HCO2198-Ho (5′-TAAACTTCTGGATGACCAAAGAATCA-3′) was designed to be used as a reverse primer in place of HCO2198 [65] in amplification reactions, whereas HCOf-Ho (5′-TGATTCTTTGGTCATCCAGAAGTTTA-3′) was designed as the reverse

complement of HCO2198-Ho for sequencing reactions. The third primer, PAT-Ho (5′-TTCATAGCACTTTTCTGCCATTTTA-3′), was designed to replace PAT [64] in both amplification and sequencing reactions. All three newly designed primers are identical to the *COI* sequence reads of *H. obsoletus* haplotypes obtained in this study or to the sequences of the *tRNA-Leu* region between the *COI* and *COII* genes in haplotypes obtained in our earlier study [56].

Polymerase chain reactions (PCR) for all primer pairs were performed under the same conditions in a 20-µL reaction volume containing high yield reaction buffer A with 1.5 mM $MgCl_2$ (1×), an additional 2.25 mM $MgCl_2$, 0.6 mM of each dNTP, 0.5 µM of each primer, 1 U of FastGene *Taq* DNA polymerase (NIPPON Genetics Europe, Dueren, Germany) and 1 µL of template DNA. Amplification was conducted in a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) applying the following thermal steps: initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 60 s, annealing at 52 °C for 60 s, and elongation at 72 °C for 90 s (150 s for longer LCO1490/PAT-Ho fragments); final elongation was performed at 72 °C for 7 min. The obtained amplicons were sequenced using the primers LCO1490 and HCOf-Ho for forward reads, and HCO2198-Ho and PAT-Ho for reverse reads. Sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands). Sequences were edited using FinchTV v.1.4.0 (http://www.geospiza.com) and aligned with ClustalW [66] within the MEGA 7 software [67]. Haplotypic nucleotide sequence data were deposited in the NCBI GenBank database under the accession numbers MK172874-91 (Table 1) and OQ372231-42 (Table 2).

2.3. COI Sequence Comparison and Haplotype Network Reconstruction

The mitochondrial *COI* gene diversity among different *H. obsoletus* haplotypes was determined by comparing the nucleotide variability and substitutions at each aligned position (Figure 1). Mean genetic distances and diversities (overall, within, and between groups) were assessed based on best-fit substitution model analyses as determined in MEGA 7 (following the lowest BIC scores, i.e., Bayesian information criterion, considered to best describe the substitution pattern) [67] among haplotypes of the *H. obsoletus sensu lato* grouped according to their associated host plant: *C. arvensis–U. dioica* (*Ca–Ud*), *V. agnus-castus* (*Vac*), or *C. foetida* (*Cf*). The haplotype originating from *C. cantabrica* was grouped within the *Ca–Ud* haplogroup based on *Convolvulus* being its host plant and initial haplogroup determination using concatenated *COI-COII* and *16S-ND1* gene regions according to published protocols [54,60]. TCS version 1.21 [68] was used to infer haplotype networks using statistical parsimony [69] with a confidence limit of 91% (connection limit = 22) to assess the evolutionary relatedness and genealogy of the *H. obsoletus* host plant-associated haplotypes. In addition, connections between haplotypes were validated by performing TCS network and median-joining (MJ) calculation [70], keeping the parameter e = 0 using the software PopART version 1.7 (http://popart.otago.ac.nz) (accessed on 6 February 2023). Finally, the *H. obsoletus COI* gene haplotypes were compared to a previously published reference sequence to establish their relationship [10,38,48,62,71,72].

2.4. Design of Multiplex PCR Tool for Specific Identification of Hyalesthes obsoletus Host Plant Associations

Based on the mt*COI* sequence variability (Figure 1), three forward primers were designed with binding positions in distinct regions of the gene, in accordance with variable nucleotide positions identified as host plant-specific for each *H. obsoletus* haplogroup. In addition, a single reverse primer containing nucleotide sequences corresponding to all three host-associated haplogroups was designed at the 3′-end of the gene. Primer 3 software v. 0.4.0 (https://bioinfo.ut.ee/primer3-0.4.0/) (accessed on 6 February 2023) was used to evaluate the characteristics (melting temperature, stability, self-complementarity, etc.) of the primers [73,74].

Primer specificity and efficiency were initially tested and evaluated in a separate single-primer set (singleplex) reaction before being incorporated into a multiplex PCR assay

as a mixture of primers. Conditions of multiplex amplification protocol were adjusted to enable the specific, selective, and reliable amplification of targeted host plant associations of *H. obsoletus*. More than a thousand *H. obsoletus* samples from the three host plant associations (*Ca–Ud*, *Vac*, and *Cf*), originating from our earlier research and surveys in Serbia, North Macedonia, Montenegro, Bulgaria, Romania, Greece, and Turkey [21,30,38,40,56], as well as newly collected specimens, were utilized to assess the specificity of the primers and amplification protocol.

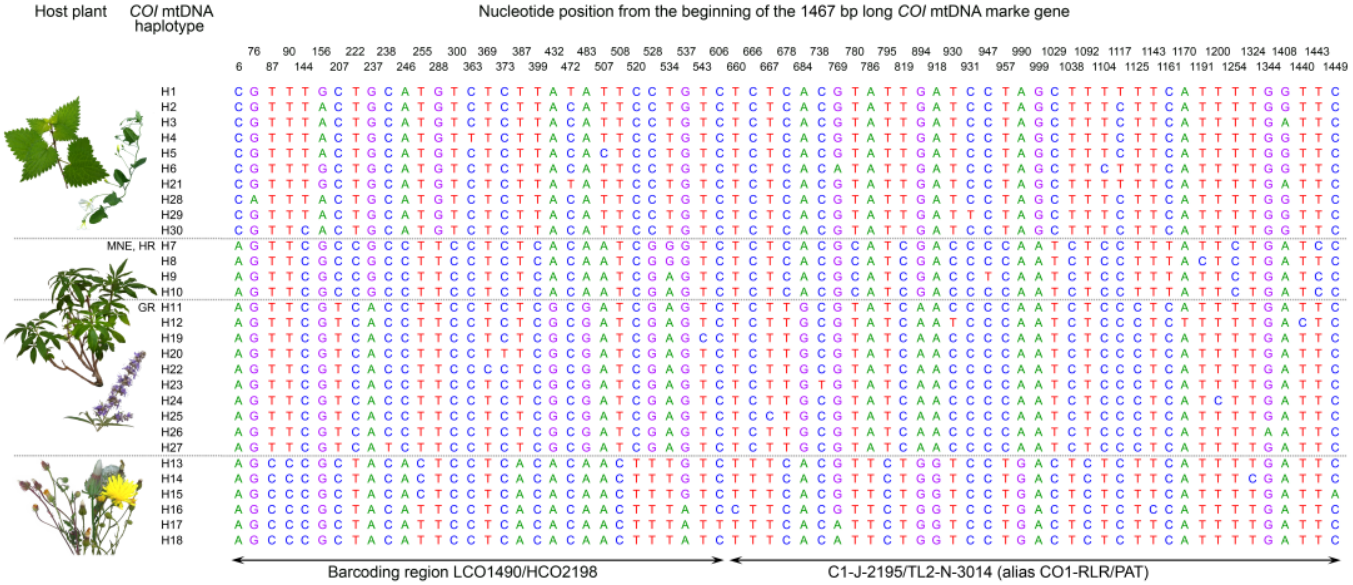

Figure 1. Positions of 67 variable sites within the 1467-bp long *COI* mtDNA marker gene fragment of the 30 *Hyalesthes obsoletus* haplotypes associated with *C. arvensis–U. dioica* (H1–H6, H21, and H28–H30), *V. agnus-castus* (H7–H12, H19, H20, and H22–27), and *C. foetida* (H13–H18). Due to the additional geographic divisions within *V. agnus-castus* associated haplotypes [56], these are also designated according to their country of origin: "MNE" for Montenegro, "HR" for Croatia, and "GR" for Greece. Bases are denoted according to the beginning of the *COI* sequence reads. Variable nucleotide positions within the range of the *COI* barcoding region delineated by LCO1490 and HCO2198 primer annealing positions [65] and a 3′ segment of the *COI* region originally employed for identification of *Hyalesthes* spp. [10] delineated by C1-J-2195 and TL2-N-3014 primers [64] are indicated by arrows.

2.5. Evaluation of the Strain Diversity of 'Ca. Phytoplasma solani' in Hyalesthes obsoletus and Shared Host Plants

Epidemiological data on the association of '*Ca.* P. solani' *stamp* strain(s) with the specific host plant association of *H. obsoletus* were retrieved from our previous studies [21,23,24,30,38,40] and combined with the geographical origin of insect and plant samples to provide an overview of the utility value of the presented method of vector identification to be used for the epidemiological discrimination of '*Ca.* P. solani' routes of transmission. The host-specific origin of these *H. obsoletus* specimens carrying '*Ca.* P. solani' *stamp* strains was determined using the multiplex PCR identification protocol. To supplement the data on '*Ca.* P. solani' occurrence in plant-specialized *H. obsoletus*, specimens collected on *V. agnus-castus* in Greece were tested for '*Ca.* P. solani' presence and strain genotype using '*Ca.* P. solani'-specific *stamp* gene amplification [59].

In addition, we conducted a case study in 2018 and 2019 to evaluate the multiplex PCR protocol for *H. obsoletus* host plant haplogroup identification in combination with *stamp* genotyping of the associated '*Ca.* P. solani' strain they harbor. We assessed the use of this information for identifying the '*Ca.* P. solani' epidemiological cycle, i.e., the underlying epidemiology, by analyzing *H. obsoletus* specimens within an agroecosystem. For the case study, we chose a vineyard in the Podunavlje district of Central Serbia that

was infected with BN (Krnjevo, GPS: N44 25.722 E21 02.907). Each year, sampling occurred at the beginning and middle of July. Using sweep-nets and mouth aspirators, *H. obsoletus* specimens were collected from the groundcover bordering the vineyard and the first two rows of the vineyard. Collections were also made from patches of *U. dioica* some 300 m outside the vineyard. Specimens were preserved in 96% ethanol, and DNA was extracted as previously described. All isolates were analyzed by multiplex PCR for host plant-specific *H. obsoletus* identification and by '*Ca.* P. solani'-specific *stamp* gene amplification [59] for pathogen identification and strain genotyping.

All of the '*Ca.* P. solani' *stamp* gene sequences obtained in this study were compared to the available genotypes in NCBI GenBank using the BLASTn algorithm (https://blast.ncbi.nlm.nih.gov) (accessed on 6 February 2023), as well as by haplotype network reconstruction with reference strains [32–34,59,75–77] using the median-joining (MJ) calculation [70], with the parameter e = 0 in the software PopART. The new *stamp* genotype identified in our study, GR-Ho-Vac, originating from a *H. obsoletus* specimen associated with *V. agnus-castus* in Greece, was submitted to NCBI GenBank under accession number OQ377809.

3. Results

3.1. Haplotype Diversity and Differentiation of Hyalesthes obsoletus Host Plant Associations

The nearly complete *COI* gene sequences of 1476 bp were obtained for 75 *H. obsoletus* individuals associated with one of the three host-plant associations: *Ca–Ud* (including *C. cantabrica*), *Vac*, and Cf. A total of 30 haplotypes that originated from a wide geographic area and all host plant associations were identified. Among previously reported *H. obsoletus* specimens [56], eighteen haplotypes were identified [78], six per host plant association (Table 1). The analysis of newly collected specimens revealed the existence of 12 additional haplotypes (Table 2). In total, nine haplotypes were identified in specimens associated with *C. arvensis-U. dioica*; a single haplotype was collected on *C. cantabrica*; four haplotypes were found in association with *V. agnus-castus* in Montenegro and Croatia; ten with *V. agnus-castus* in Greece; and six haplotypes were identified in association with *C. foetida*.

Nucleotide variability analyses confirmed the genetic segregation according to host-plant specialization in each of the 30 *COI* haplotypes, as revealed by haplotype network reconstruction (Figure 2). Three distinct clusters of *H. obsoletus* host plant-associated haplotypes, as well as two sub-clusters of geographically separated haplotypes associated with *V. agnus-castus* from Montenegro and Croatia, and from Greece, were identified. The topology of the *COI* phylogenetic network matched that which was previously obtained using concatenated *COI-COII* and *16S-ND1* gene regions [56], but it also revealed further geographic variation in all clusters. For example, *Cf*-associated *H. obsoletus* haplotypes from Turkey (H16, H17, and H18) formed a separate haplogroup from those originating in Serbia, Romania, and Bulgaria (Figure 2). On the *COI* gene, the geographic separation of *Ca–Ud*-associated AA/AB and EC haplogroups was also confirmed, with the former group being affiliated to H1 and its deriving haplotypes and the latter group being affiliated with H2 and its deriving haplotypes. The occurrence of the CB haplotype (*COI* haplotype H21) of *H. obsoletus* belonging to the AB haplogroup in association with *C. cantabrica* in Southeastern Serbia was a new discovery. This haplotype was affiliated to the group of H1 derived haplotypes in accordance with the compatible phylogeographic informativeness of the *COI* gene with concatenated *COI-COII* and *16S-ND1* gene regions (Figure 2). The AB group of *H. obsoletus* haplotypes had not previously been identified in Serbia, despite extensive surveys [56]. Therefore, the occurrence of this haplotype could be the result of insect vector's further host plant adaptation or geographic range expansion, although neither is currently supported by evidence and therefore will require more comprehensive research in the future.

Comparing the *COI* haplotype diversity identified in our study with data on previously (partially) *COI*-genotyped *H. obsoletus* specimens [10,48,62,71,72] allowed us to identify geographic ranges for some of them that were distinct from our own. The H1 haplotype, which was found in Montenegro and Greece (Table 1), is also present in Northern Italy

(FN179291 and GU552999), Southeastern France (isolate Ho-Ss5, LT841328), and Germany (ON210854), whereas the H2 haplotype from Serbia, Romania, North Macedonia, and Montenegro is also found in South Russia, Rostov Oblast (GU553002), and confirmed for Romania (GU553001). The *Vac*-associated haplotypes of the Montenegrin phylogeographic sub-group were also confirmed to occur in the coastal area of Herzegovina (Bosnia and Herzegovina), where *H. obsoletus* specimens were collected on *V. agnus-castus* [72] and affiliated to either the H7 or H10 haplotype based on the available 3′-end of the *COI* gene sequence (KY320569).

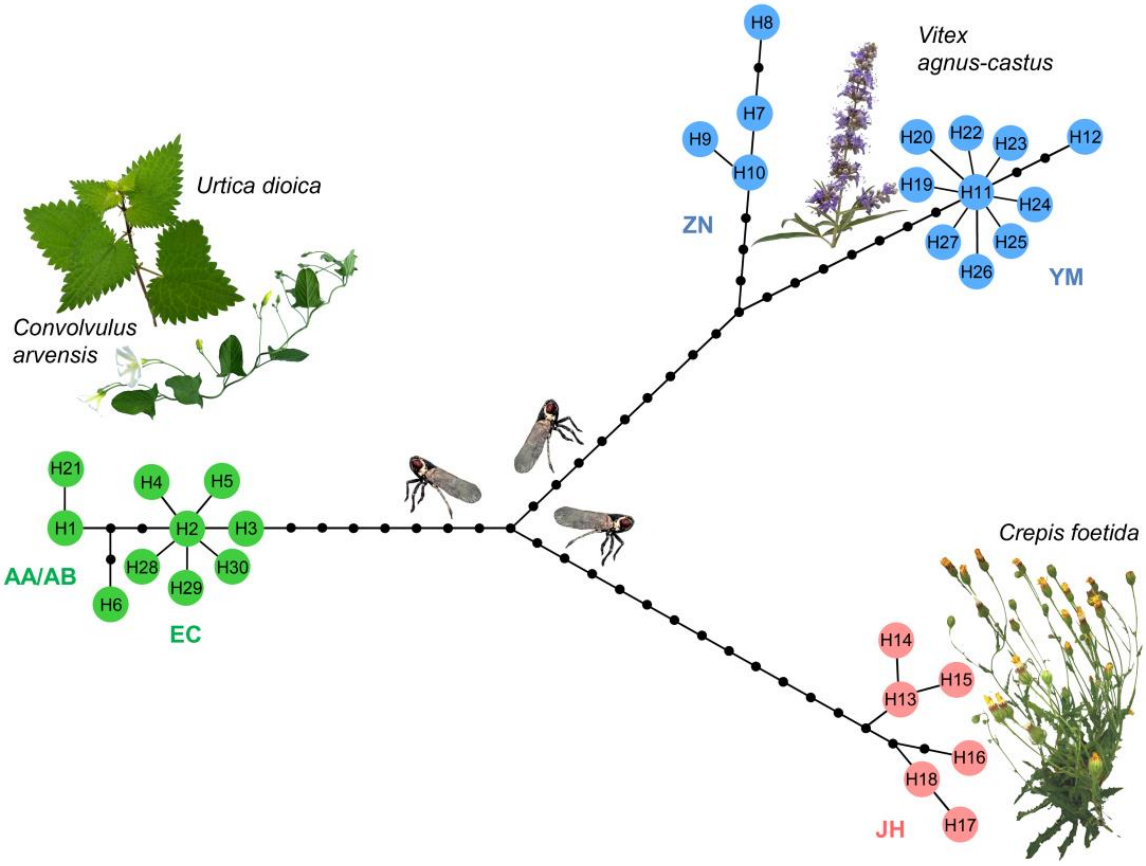

Figure 2. The phylogenetic haplotype network obtained using statistical parsimony algorithm [69] in TCS program [68] on 1467 bp of mitochondrial *COI* gene of the 30 *Hyalesthes obsoletus* haplotypes identified in this study. Haplotype colors correspond to the host plant associations. Black dot vertices represent missing or unsampled haplotypes. Haplotypes designation (H1–H30) corresponds to those in Tables 1 and 2. Below each group of haplotypes, their affiliation to previously established nomenclature of concatenated *COI-COII* and *16S-ND1* haplogroups (AA/AB, EC, ZN, YM, JH) [54,56,60] is denoted.

The overall mean genetic distance among 30 *COI* haplotypes was 1.4%, while within *Ca–Ud*-associated and *Cf*-associated haplogroups was 0.2% for each group, and 0.5% for the group of *Vac*-associated haplotypes. The mean genetic distance between groups ranged from 1.7% for the *Ca–Ud* and *Cf* associations to 2.2% for the *Vac* and *Cf* associations (Table 3). Comparing the 1476 bp-long *COI* gene sequence of all identified haplotypes within *H. obsoletus sensu lato* revealed 67 variable, 20 singleton, and 47 parsimony-informative sites. Out of these, 23 were haplogroup-specific and thus informative for discriminating *Ca–Ud*, *Vac*, and *Cf* host plant associations of *H. obsoletus* (Figure 1).

Table 3. Mean mitochondrial *COI* gene distances and diversities based on best-fit substitution model analyses (Tamura 3-parameter + G) among 30 haplotypes of the *Hyalesthes obsoletus sensu lato* grouped according to their associated host plant: *C. arvensis–U. dioica* (*Ca–Ud*), *V. agnus-castus* (*Vac*), or *C. foetida* (*Cf*).

Host Plant Associated Group	Mean Genetic Distance (SE)					Mean Genetic Diversity (SE)		Coefficient of Differentiation (SE)
	Overall	Within Group	Between Group			Overall	Between Group	
			Ca–Ud	*Vac*	*Cf*			
Ca–Ud	0.014 (0.002)	0.002 (0.001)	–	(0.003)	(0.004)	0.014 (0.002)	0.011 (0.002)	0.773 (0.035) *
Vac		0.005 (0.001)	0.019	–	(0.004)			
Cf		0.002 (0.001)	0.017	0.022	–			

SE, standard error obtained by a bootstrap procedure (500 replicates); * the coefficient of differentiation was 0.868 (0.025) when the grouping was extended to include haplotypes of *H. obsoletus* associated with *Vac* in Montenegro and Greece as separate groups.

3.2. Multiplex PCR for Discriminating Host Plant Associations of *Hyalesthes obsoletus*

The position of primer sequences for specific amplification of plant-specialized *H. obsoletus* was determined by intra- and inter-group variability (Figure 1), i.e., the position of host plant group-specific nucleotide substitutions. The primers were designed to match the substitutions specific to each host plant group of *H. obsoletus* haplotypes at the 3′-end of the primer while positioning them in distinct regions of the *COI* gene (Table 4). The forward primers were designed for specific amplification of *Cf*, *Ca–Ud*, and *Vac*-associated *H. obsoletus* haplogroups; their binding sites corresponded to 68 bp, 506 bp, and 797 bp positions counting from the beginning of sequence reads, respectively. For universal amplification of all *H. obsoletus* haplotypes among the three host plant associations, the HoR reverse primer was designed to match all sequences at 1128-bp position (Table 4, Figure 3).

Table 4. Primer sequences and conditions for specific and selective multiplex PCR amplification of *Hyalesthes obsoletus* host plant associations.

Host Plant Association	Primer Name	Primer Sequence 5′ → 3′	Primer Position [1]	HoR-Primed PCR Fragment Size	Multiplex PCR Reaction Conditions
C. foetida	HoCfF	TTAATAACGACCAAATTTACAAC	68–90 bp	1084 bp	94 °C/60 s, 56 °C/60 s, 72 °C/120 s, 30 cycles; high yield reaction buffer, 2.5 mM $MgCl_2$, 0.5 mM each dNTP, 0.5 µM each primer, 1 U *Taq* DNA polymerase
C. arvensis/ U. dioica	HoF	TTTTACTACTTTCACTACCAGTC	506–528 bp	645 bp	
V. agnus-castus	HoVacF	ACATATTTACAGTAGGAATAGAC	797–819 bp	355 bp	
Universal for *H. obsoletus*	HoR	CATTTTTGRTTTATTGATAGTCCT	1128–1151 bp	–	

[1] Bases according to the beginning of the *COI* sequence reads (MK172874-91 and OQ372231-42).

The reaction conditions for multiplex PCR were similar to those described previously for *COI* amplification, with the exception that the annealing temperature was increased to 56 °C to enable specificity and that the elongation step was 120 s for a total of 30 cycles (Table 4). The host plant association of *H. obsoletus* was determined by the size of the amplified fragments: 1084 bp for the *Cf* association, 645 bp for the *Ca–Ud* association, and 355 bp for the *Vac* association (Figure 4). The multiplex PCR method enabled the precise identification of all *H. obsoletus* specimens retrieved from our previous studies and subsequently analyzed [21,30,38,40,56]. No cross-amplifications between the haplogroups of diverse host plant associations were recorded. In particular, among over 700 specimens from our previous study on the host plant association of *H. obsoletus* [56], all of them produced selective amplification products that corresponded to their original host plant of collection. All 57 *H. obsoletus* specimens harboring '*Ca.* P. solani' from BN-affected vineyards in North Macedonia [30] and all 38 '*Ca.* P. solani'-infected specimens from potato

fields in Serbia [40] produced a 645 bp fragment. Among twenty-six *H. obsoletus* individuals collected from BN-affected vineyards in Montenegro [21], twenty-two produced 645 bp fragments and four produced 355 bp fragments, indicating the co-occurrence of *Ca–Ud*-association and *Vac*-association. Similarly, among 54 individuals from vineyards under BN infection in Serbia [38], 38 produced fragments of 1084 bp and the remaining specimens yielded fragments of 645 bp, confirming both *Ca–Ud* and *Cf*-association.

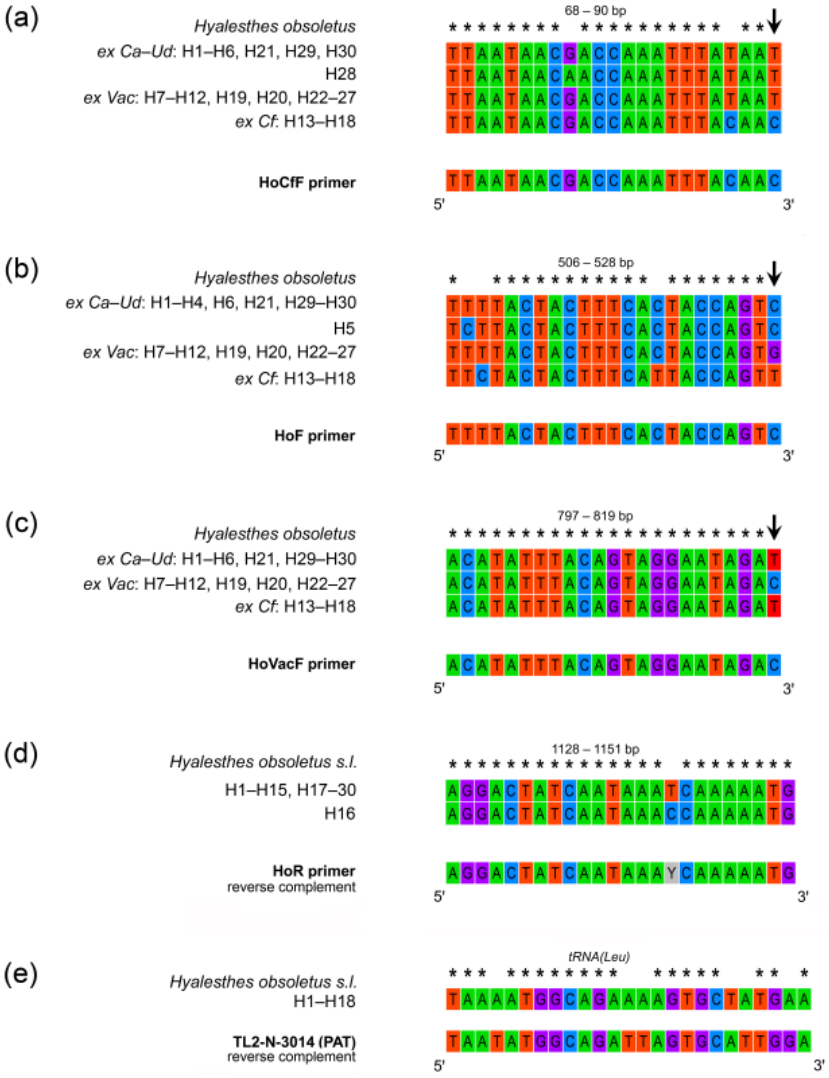

Figure 3. Primer positions and nucleotide variation on mtDNA *COI* gene and flanking *tRNA(Leu)* in *Hyalesthes obsoletus* haplotypes associated with *C. arvensis–U. dioica* (*ex Ca–Ud*: H1–H6, H21, H28–H30), *V. agnus-castus* (ex *Vac*: H7–H12, H19, H20, and H22–27), and *C. foetida* (*ex Cf*: H13–H18). Primer annealing sites and sequence variability exploited for primer design are presented as follows: (**a**) HoCfF, forward primer specific for selective amplification of *H. obsoletus ex Cf*; (**b**) HoF, forward primer specific for selective amplification of *H. obsoletus ex Ca–Ud*; (**c**) HoVacF, forward primer specific for selective amplification of *H. obsoletus ex Vac*; (**d**) HoR, reverse primer universal for amplification of all host plant associations of *H. obsoletus*; (**e**) sequence variability of *tRNA(Leu)* in *H. obsoletus* haplotypes associated with all three host plant groups compared with the sequence of TL2-N-3014 primer [64] formerly used for *Hyalesthes* spp. identification [10]. Asterisks (*) indicate identical nucleotides. The arrows indicate nucleotide substitutions at the 3′ end of the primer binding site which allow for specificity in amplification. Data on *tRNA(Leu)* sequence variability are taken from our previous work [56].

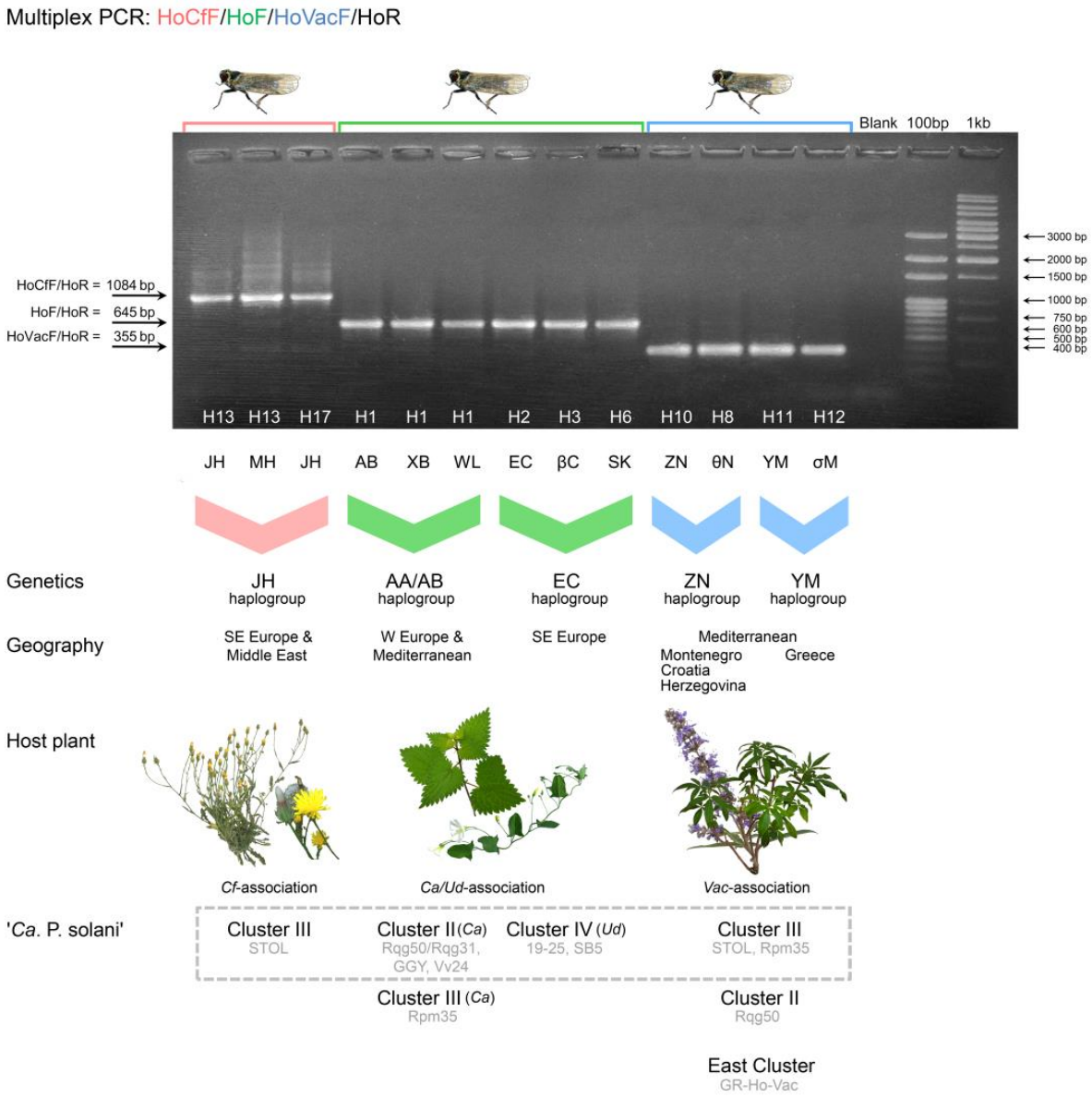

Figure 4. A multiplex PCR assay for the specific identification of *Hyalesthes obsoletus* host plant associations using the HoCfF/HoF/HoVac/HoR primer mix and specific protocol. The size of the amplified fragment determines the host plant association: 1084 bp for *Cf*-association, 645 bp for *Ca/Ud*-association, and 355 bp for *Vac*-association. Molecular weight markers: 100-bp DNA ladder (Solis BioDyne, Tartu, Estonia) and GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Waltham, MA, US). The *COI* and concatenated *COI-COII* and *16S-ND1* haplotypes of presented individuals are designated on and below the agarose gel. The genetics of *H. obsoletus* (i.e., haplogroups according to [54,56,60]), geography, host plants, and '*Ca.* Phytoplasma solani' *stamp* strains are given below the gel. Data on the '*Ca.* Phytoplasma solani' strains are retrieved from our previous works [21,23,24,30,38,40] and from the case study presented herein. *Stamp* Clusters II, III, and IV are designated as explained in previous studies [32,33,59], while "East Cluster" refers to '*Ca.* Phytoplasma solani' strains from Azerbaijan [32] and Thailand [77] that formed a joint cluster with the new strain from *H. obsoletus* associated with *V. agnus-castus* collected in Greece in this study. The cluster of most frequently found '*Ca.* Phytoplasma solani' genotypes associated with each *H. obsoletus* haplogroup and host plant are denoted within the grey hashed square.

For the case study, we collected 175 *H. obsoletus* specimens from a BN-infected vineyard in Central Serbia, 140 specimens from the vineyard's border, and 35 from patches of

U. dioica outside the vineyard. All specimens were tested with multiplex PCR for host plant association identification. In 87 individuals, the *Ca–Ud*-associated 645 bp fragment was amplified, while in 88 individuals, the *Cf*-associated 1084 bp long fragment was amplified.

3.3. Linking 'Ca. Phytoplasma solani' Epidemiology with Host Plant-Specialized Hyalesthes obsoletus

We were able to make plausible links between host plant association, genetics of the vector, geographic area of origin, and pathogen strain by summarizing the data from previous studies on the occurrence of '*Ca*. P. solani' in *H. obsoletus* [21,30,38,40] (Figure 4). The *H. obsoletus* specimens from Serbia in which a 645 bp fragment was amplified by multiplex PCR for host association identification harbored the '*Ca*. P. solani' *stamp* strains of either Cluster II (genotypes Rqg31/Rqg50 and Vv24) or Cluster III (Rpm35), whereas specimens from North Macedonia with the same product length harbored either strains of Cluster IV (SB5 and 19–25 and their deriving genotypes M1–M3) or of Cluster II (Rqg50 and GGY). This indicated that in Serbia, there is a '*Ca*. P. solani' transmission route originating from *C. arvensis*, whereas in North Macedonia, there are transmission routes originating from both *C. arvensis* and *U. dioica*. In contrast, specimens of *H. obsoletus* from Serbia that yielded an amplification of a 1084 bp fragment via multiplex PCR harbored only the STOL *stamp* genotype of the Cluster III, indicating the *C. foetida* transmission route. Furthermore, among *H. obsoletus* specimens from Montenegrin vineyards, three types of epidemiological associations were linked: (i) a 645 bp fragment of multiplex PCR amplification and '*Ca*. P. solani' *stamp* strains of Cluster II (Rqg31/Rqg50) or Cluster III (Rpm35), indicating a *C. arvensis* source of transmission, (ii) a 645 bp multiplex PCR fragment and *stamp* strains of Cluster IV (SB5 and 19–25), indicating a *U. dioica* transmission route, and (iii) a 355 bp fragment amplification and infection with '*Ca*. P. solani' strains of Cluster III (STOL) or Cluster II (Rqg50), indicating a *V. agnus-castus* transmission route.

As a case study, the presence of '*Ca.* P. solani' was identified in 54 of the 175 (31%) *H. obsoletus* specimens collected from the BN-affected vineyard. The *stamp* gene Cluster III genotype STOL was found in 37 individuals, all of whom amplified the *Cf*-associated 1084 bp-long fragment in multiplex PCR. Cluster II (Rqg31/Rqg50) and Cluster IV (19–25) genotypes were identified in fourteen and three *H. obsoletus* individuals, all of which amplified the *Ca–Ud*-associated 645 bp fragment. Consequently, these results indicated the presence of three transmission cycles and two host plant-group associations of the vector (*Ca–Ud* and *Cf*) within and beyond the studied vineyard. The results agreed with the *stamp* genotyping of symptomatic grapevine isolates from the same vineyard that revealed a majority of grapevines infected with the STOL genotype and a smaller proportion with the Rqg50 genotype (data not shown).

Concerning the experimentally demonstrated *V. agnus-castus*-sourced route of transmission in Montenegrin vineyards [21], indications include *H. obsoletus* specimens producing a 355 bp fragment in specific multiplex PCR and the STOL or Rpm35 genotypes of the *stamp* Cluster III being the most frequently found in association with either the vector or the reservoir plant, or being transmitted by the *Vac*-associated *H. obsoletus*. Additionally, among plant-specialized *H. obsoletus* collected on *V. agnus-castus* in Greece, we found a single specimen harboring '*Ca.* P. solani'. The GR-Ho-Vac strain from this specimen showed a maximum identity of 97.5% with the available sequences in the GenBank database (12/474 nucleotide substitutions). The strain was affiliated through haplotype network reconstruction to the *stamp* cluster, herein designated as "East" (Figure 4), which gathered the strains from Azerbaijan (isolates AZ13-H40 and AZ13-H26, accession numbers LT899749 and LT899744) [32] and Thailand (isolate TH-NKT-P26, accession number MW464312) [77] next to the strain found in *H. obsoletus* collected on *V. agnus-castus* in Greece (data not shown), thus indicating their common origin or adaptation to similar host(s). Given that 16 species of *Vitex* are naturally occurring in Thailand [79] and *V. agnus-castus* is present along the Caspian Sea coast in Azerbaijan and has been discussed as a plausible source of new diversified *stamp* genotypes [32], the natural reservoir plant may be the link between the isolates in this cluster and an indication of a specific transmission route.

4. Discussion

The planthopper *Hyalesthes obsoletus* is a major, widespread vector and driver of the '*Ca.* P. solani' epidemiology. This claim is supported by numerous evidences of its ecological specialization (e.g., [20,25,55]), followed by genetic differentiation of host plant-associated populations [54–56,60] toward plants that serve as common reservoirs of '*Ca.* P. solani' [20,21,38]. Evidences of ongoing processes of host plant specialization [33,61,62,80] and speciation [56] leading to the formation of host races or cryptic species within the vector's populations in sympatry are simultaneously evidences of host plant-specialized routes of '*Ca.* P. solani' transmission at the level that host races or cryptic taxa are driving the diversification of the pathogen, the emergence of new strains, and the separate epidemiological cycles (e.g., [33,61]). Adults of *H. obsoletus* are frequently found on diverse natural and cultivated plants, leading to the assumption that it is a polyphagous species (reviewed in [56]). However, it is the strict host plant preferences of nymphs and adults during mating and oviposition that drive the specialization processes of both vector and the pathogen, as well as the resulting transmission route and epidemiological cycle. The prevailing perception of *H. obsoletus* as a polyphagous species, with a few exceptions (e.g., [33,38,54,56,60,80]), highlights the importance of our findings and the need to treat the vector according to its true role, i.e., as an ecologically adapted host plant-specialized key vector and driver of '*Ca*. P. solani' epidemics, and as a species complex with the potential for further shifts in host plant use and adaptations to new environmental conditions.

Molecular tools and genetic data are now available for the accurate, cost-effective, and rapid identification of host plant associations of *H. obsoletus* (i.e., cryptic species) using multiplex PCR, as well as for comparison and identification of further diversification, differentiation, and specialization by phylogenetic analyses of the most widely used barcoding fragment of the *COI* gene [1,7,15,63]. This study's analysis of the *COI* gene encompassed a 630 bp (without primers) of the barcoding region and the entire second portion of the gene at its 3′-end, facilitating future comparisons of *H. obsoletus* genetic diversity regardless of the primers used, length, or position of the amplified fragment. As intended, the multiplex PCR method for discriminating host plant associations of *H. obsoletus* should enable accurate identification of the vector's phylogenetic lineage, natural host plant preference and epidemiological transmission routes of '*Ca.* P. solani'. When applied to specimens collected on cultivated plants within an agroecosystem and combined with '*Ca.* P. solani' genotyping, the method should provide valuable information on disease epidemiology and source(s) of emergence and transmission.

In addition to defining the intended applications and expectations of the presented multiplex PCR method, we consider it essential to highlight its limitations as well. The limitations are defined by the biological and genetic characteristics of *H. obsoletus* as a complex of cryptic, recently evolved species [56]. Due to shallow genetic differentiation in host-associated haplogroups or species caused by ecological adaptation, the number of segregating nucleotide positions that can be used for discrimination is limited (e.g., [12]). This is especially true for host races or morphologically indistinguishable (cryptic) species (e.g., [5,55]). Hence, the method of multiplex PCR identification of *H. obsoletus* host plant associations presented here could not be used to discriminate *H. obsoletus sensu lato* from its morphologically distinct congeners (a total of 32 species excluding *H. obsoletus* [81]). In contrast to methods previously developed for molecular identification and differentiation of morphologically distinct cixiid planthoppers, including *H. obsoletus* [10,11,48], the current method could not be used for the differentiation of *H. obsoletus sensu lato*, i.e., host plant associations, from other *Hyalesthes* species that may co-occur together, such as *H. luteipes*. However, the latter species has distinctive morphological features compared to *H. obsoletus sensu lato*, and thus this is not a true limitation but rather a notion that specimens should be examined through a stereo microscope and identified by external morphological characteristics to the genus level prior to the molecular identification of host associations. Likely, *H. obsoletus* has distinct external morphological features of shiny black body coloration and a white collar-like pronotum [10], while other *Hyalesthes* species commonly found in

or surrounding agroecosystems in Europe are of brownish body coloration and yellowish pronotum (*H. luteipes*, *H. scotti*, and *H. philesakis* Hoch, 1985). Notably, the method described for the molecular identification of three *Hyalesthes* species [10] is inapplicable to the identification and differentiation of *H. obsoletus sensu lato* from its congeners, which is to be expected given the limited knowledge of *H. obsoletus*' genetic variability at the time the method was developed.

The need for the reliable and precise identification of *H. obsoletus* host associations as vectors driving '*Ca.* P. solani' transmission and epidemiology is becoming more apparent as the number of '*Ca.* P. solani' epidemic outbreaks grows [33,53,61], as does the number of cultivated plants affected in a wider geographical area [32,36,77,82], and disease management and control strategies become more demanding [83]. As with the pathogen's molecular identification and genotyping, the molecular identification of *H. obsoletus* host associations should be the method of choice in studies elaborating the '*Ca.* P. solani' transmission routes or epidemiology. We provided an overview of the epidemiological utility of combining data on the genetic characteristics of the vector and the pathogen based on the diversity information currently available. This will hopefully encourage and persuade more researchers to apply the molecular methods for identification of *H. obsoletus* host plant associations, which will undoubtedly result in a more comprehensive understanding of the epidemiology and origin of '*Ca.* P. solani'.

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Data Availability Statement: DNA sequences are available in the GenBank database, accession numbers are listed in Tables 1 and 2. All other relevant data are within the paper.

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