*Plant Pathology* (2016) Doi: 10.1111/ppa.12524

# Molecular characterization of elm yellows phytoplasmas in Croatia and their impact on *Ulmus* spp.

Z. Katanić[a], L. Krstin[a], M. Ježić[b], M. Zebec[c] and M. Ćurković-Perica[b]*

[a]*Department of Biology, University of J. J. Strossmayer in Osijek 31000, Osijek;* [b]*Division of Biology, Faculty of Science, University of Zagreb 10000, Zagreb; and* [c]*Department of Forest Genetics, Dendrology and Botany, Faculty of Forestry, University of Zagreb, 10000 Zagreb, Croatia*

'*Candidatus* (*Ca.*) Phytoplasma ulmi', the causal agent of elm yellows, was found widely distributed across elm populations in Croatia, infecting *Ulmus laevis* and *Ulmus minor*. Especially high prevalence of the infection, approximately 75%, was detected in *U. laevis*, but more than half of the trees were symptomless. '*Candidatus* Phytoplasma solani' and '*Ca.* Phytoplasma asteris' were also detected. The latter could possibly represent a new 16SrI subgroup, most closely related to 16SrI-B. Diversity of '*Ca.* Phytoplasma ulmi' in Croatia was determined by sequencing of the 16S rRNA gene, ribosomal protein genes *rpl22* and *rps3*, *secY* and *secY-map* genes, in 62 phytoplasma isolates. Phylogenetic analysis indicated that Croatian isolates share a common origin and are closely related to strains of '*Ca.* Phytoplasma ulmi' from southeastern Europe. However, comparative sequence analysis revealed mutations at positions where variability has never been detected before, including positions within sequences unique to '*Ca.* Phytoplasma ulmi' in the 16S rRNA gene, *rpl22*, *rps3* and *secY*. New genotypes were identified based on the sequenced genes. This study points to a significantly higher genetic diversity than previously reported, and a necessity to revise the formal description of this phytoplasma species and to include newly discovered characteristics.

*Keywords:* '*Candidatus* Phytoplasma ulmi', elm yellows, genetic diversity, phylogeny

## Introduction

Phytoplasmas are non-helical, wall-less prokaryotes that are pathogenic to many plant species worldwide. They are members of the class Mollicutes and have a provisional status as genus '*Candidatus* (*Ca.*) Phytoplasma' (IRPCM, 2004). They colonize plant phloem and are transmitted by phloem-feeding insect vectors of the order Hemiptera (Hogenhout *et al.*, 2008). Because phytoplasmas cannot be cultivated *in vitro*, their identification and characterization is mainly based on molecular analysis of phytoplasma genes, particularly the highly conserved 16S rRNA gene. Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA is used for classification of phytoplasmas into 16Sr groups and subgroups (Lee *et al.*, 1998). The dissimilarity of this sequence is used as one of the criteria for the description of novel '*Ca.* Phytoplasma' species, along with distinct plant host and insect vector range, for those which share high sequence similarity (>97·5%; IRPCM, 2004). Other more variable genetic markers are also used for finer differentiation of closely related phytoplasmas (Lee *et al.*, 2004a,b; Arnaud *et al.*, 2007; Jović *et al.*, 2011; Quaglino *et al.*, 2013).

The 16SrV phytoplasma group is a large and diverse group divided into five subgroups (16SrV-A to 16SrV-E). Members of this group are associated with diseases in various plants including elm, grapevine, alder, blackberry, cherry, *Spartium* sp., *Ziziphus* sp. and *Eucalyptus* sp. (Lee *et al.*, 2004b). Many diseases associated with the 16SrV phytoplasma group are economically important, for example flavescence dorée (FD) phytoplasmas causing grapevine yellows in European grapevine (Angelini *et al.*, 2001; Lee *et al.*, 2004b; Arnaud *et al.*, 2007). In contrast, alder yellows phytoplasma (AldY), which belongs to the 16SrV-C subgroup, was found to be widespread among European alders, but infection was often symptomless (Arnaud *et al.*, 2007; Holz *et al.*, 2016).

The elm yellows disease, also referred to as elm phloem necrosis and elm witches' broom disease, is a disease of elms (*Ulmus* spp.) caused by elm yellows phytoplasma '*Ca.* Phytoplasma ulmi' (16SrV-A subgroup; Lee *et al.*, 2004b). However, occasional infection of elms with other phytoplasmas, such as '*Ca.* Phytoplasma asteris', '*Ca.* Phytoplasma solani' and '*Ca.* Phytoplasma trifoli', has been documented (Lee *et al.*, 1995; Jacobs *et al.*, 2003; Credi *et al.*, 2006). Proposal of the 16SrV-A subgroup, associated with elm yellows, as a novel candidate species named '*Ca.* Phytoplasma ulmi' with EY1 as a reference strain, was based on phylogenetic analyses of the 16S rRNA gene, ribosomal protein genes *rpl22* and *rps3* and *secY* gene sequences (Lee *et al.*, 2004b). The 16S rDNA sequence similarity within the 16SrV group is very high, but heterogeneity within the group was more evident from analysis of the *rpl22*, *rps3* and *secY* gene (encoding a translocase protein) sequences. Members of the 16SrV-A subgroup were found to represent a separate, rather homogeneous,

*E-mail: mirna.curkovic-perica@biol.pmf.hr

© 2016 British Society for Plant Pathology 

Plant Pathology

strain cluster. When compared to previously described species, '*Ca*. Phytoplasma ulmi' shows specificity to particular natural plant hosts (elms) and insect vectors and these were used as additional criteria for its description as a separate species. Furthermore, signature sequences unique to '*Ca*. Phytoplasma ulmi' have been identified in all three analysed genes (Lee *et al*., 2004b).

Severity and outcome of elm yellows disease can vary significantly among elm species. European and Asian elms and their interspecific hybrids, in which symptomless infections often occur, are considered less susceptible to '*Ca*. Phytoplasma ulmi' infection compared to American elms (Sinclair *et al*., 2000; Carraro *et al*., 2004). If present, symptoms on European elms are leaf yellowing and drying, reduced growth and stunting, and development of witches' broom. Even tree decline can occur in European elms, but overall tree mortality is lower when compared to American elms (Braun & Sinclair, 1979; Sinclair *et al*., 2000). Nevertheless, the presence of this pathogen has been proven so far in several European countries including Italy (Mäurer *et al*., 1993; Marcone *et al*., 1997; Sfalanga *et al*., 2002; Arnaud *et al*., 2007), France (Mäurer *et al*., 1993; Boudon-Padieu *et al*., 2004; Arnaud *et al*., 2007), Germany (Mäurer *et al*., 1993), the Czech Republic (Navrátil *et al*., 2009) and Serbia (Jović *et al*., 2011), in wych elm (*U. glabra*), European white elm (*U. laevis*) and field elm (*U. minor*) trees as well as in some introduced Asian elm species.

Genetic variability of elm yellows phytoplasma in Europe has recently been suggested to be much higher than initially assumed. Genetically diverse isolates with differences in unique regions of 16S rDNA, *rpl22*, *rps3* and *secY* reported by Lee *et al*. (2004b) were described in Serbia (Jović *et al*., 2008, 2011) and in the Czech Republic (Navrátil *et al*., 2009), and existence of more than one phylogenetic lineage of '*Ca*. Phytoplasma ulmi' was revealed based on analysis of these genes and the *map* gene encoding methionine aminopeptidase (Arnaud *et al*., 2007; Jović *et al*., 2011). However, the full extent of diversity, distribution and impact of elm yellows phytoplasma in Europe is still unclear (EFSA PLH Panel, 2014). To the authors' knowledge, there is no available information on phytoplasma infection of naturally occurring elm species (*U. glabra*, *U. laevis* and *U. minor*) in Croatia. Therefore, the objectives of this study were to: (i) detect, identify and determine the prevalence of phytoplasmas in these three elm species in Croatia, (ii) determine the genetic diversity of '*Ca*. Phytoplasma ulmi' isolates and their phylogenetic relationships with previously described members of this '*Ca*. Phytoplasma' species, and (iii) estimate the impact of phytoplasma infections on natural *Ulmus* spp. populations.

## Materials and methods

### Plant samples

During June and July 2012, a total of 139 leaf samples of *U. glabra*, *U. laevis* and *U. minor* were collected from six sites across Croatia: Nova Kapela and Donji Miholjac (Eastern Croatia); Đurđevac, Jastrebarsko and Kalnik (Central Croatia); and Cetina (Southern littoral Croatia) (Table 1). Occurrence of symptoms, such as leaf yellowing and drying, premature leaf fall and drying of branches, was recorded in the field. Trees both with and without symptoms were sampled from each site. If symptoms were present, five leaves were taken from the part of the crown where symptoms were visible. If symptoms were not present, five leaves were taken randomly from different parts of the crown. All five leaves collected from each single tree

Table 1 Detection and identification of phytoplasmas infecting *Ulmus* species from six sites in Croatia

| Site | | Plant host | No. of samples | No. of phytoplasma-infected/samples with symptoms[a] | '*Ca*. Phytoplasma' species[a] |
|---|---|---|---|---|---|
| Eastern Croatia | Nova Kapela | *U. laevis* | 10 | 5/4 | '*Ca*. Phytoplasma ulmi' |
| | | *U. minor* | 13 | 1/1 | '*Ca*. Phytoplasma asteris' |
| | Donji Miholjac | *U. laevis* | 18 | 14/6 | '*Ca*. Phytoplasma ulmi' |
| | | *U. minor* | 2 | 0 | – |
| Central Croatia | Đurđevac | *U. laevis* | 15 | 15/8 | '*Ca*. Phytoplasma ulmi' |
| | | *U. minor* | 10 | 2/2 | '*Ca*. Phytoplasma ulmi' |
| | Kalnik | *U. glabra* | 23 | 1/1 | '*Ca*. Phytoplasma solani' |
| | Jastrebarsko | *U. laevis* | 16 | 13/8 | '*Ca*. Phytoplasma ulmi' |
| | | *U. minor* | 9 | 1/1 | '*Ca*. Phytoplasma ulmi' |
| Southern littoral Croatia | Cetina | *U. laevis* | 20 | 12/1 | '*Ca*. Phytoplasma ulmi' |
| | | *U. minor* | 3 | 0 | – |
| Total[b] | | *U. laevis* | 79 | 59/27 | '*Ca*. Phytoplasma ulmi' |
| | | *U. minor* | 37 | 4[c]/4 | '*Ca*. Phytoplasma ulmi'; '*Ca*. Phytoplasma asteris' |
| | | *U. glabra* | 23 | 1/1 | '*Ca*. Phytoplasma solani' |
| | | Total | 139 | 64 | |

[a]Phytoplasma detection and identification was based on amplification and sequencing of the 16S rRNA gene.
[b]Data presented for total number of samples of *U. laevis, U. minor* and *U. glabra* trees analysed in the study.
[c]Four samples of *U. minor* were infected: three with '*Ca*. Phytoplasma ulmi' and one with '*Ca*. Phytoplasma asteris'.

represented one sample. After the samples were transported to the laboratory, the leaf main veins were stored at −20 °C until further analysis.

### Plant DNA extraction and amplification of phytoplasma genes

Total nucleic acids were extracted from leaf main veins with the commercial kit OmniPrep for plant (G-Bioscience) according to the manufacturer's instructions, and used as template for amplification of phytoplasma genes. The following phytoplasma genomic loci were amplified using nested PCR: (i) the 16S rRNA gene, (ii) the operon consisting of the *rpl22* and *rps3* genes, (iii) the FD9 genetic locus that contains the 3′-end of the *rplO* gene encoding ribosomal protein L15 and the *secY* gene, and (iv) the *secY-map* genetic locus that contains the 3′-end of the *secY* gene and the *map* gene.

Direct amplification of the 16S rRNA gene was performed with the universal phytoplasma-specific primer pair P1/P7 (Deng & Hiruki, 1991; Schneider *et al.*, 1995) in a reaction mixture containing 1× PCR buffer, 1·5 mM $MgCl_2$, 200 μM each dNTP, 0·2 μM each primer, 0·625 Units GoTaq DNA polymerase (Promega) and 20–50 ng DNA template. Reaction conditions were: denaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min and extension at 68 °C for 2 min; and final extension at 68 °C for 10 min. This was followed by nested PCR with primer pairs P1A/P7A (Lee *et al.*, 2004a) and R16F2n/R2 (Lee *et al.*, 1993; Gundersen & Lee, 1996) using 0·5 μL of the first PCR product as template. Amplification with P1A/P7A was performed as described by Lee *et al.* (2004a). The reaction mixture for amplification with R16F2n/R2 was the same as the mixture used for amplification with P1/P7 and reaction conditions were: denaturation at 94 °C for 1 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min and extension at 72 °C for 3 min; and final primer extension at 72 °C for 10 min.

Amplification of a portion of the ribosomal protein operon was performed using primer pair rp(V)F1/R1 (Lim & Sears, 1992; Lee *et al.*, 1998) for direct PCR, and rp(V)F1A/rp(V)R1A (Lee *et al.*, 2004b) for nested PCR, as described by Martini & Lee (2013), except that the extension temperature was 68 °C. For direct amplification of the FD9 DNA fragment, the FD9f/r primer pair was used according to Daire *et al.* (1997), except that the annealing temperature was 52 °C. This was followed by amplification with FD9f2/r and FD9f3/r2 according to Angelini *et al.* (2001). Amplification of the genetic locus *secY-map* was performed with the FD9f5/MAPr1 primer pair, followed by amplification with the FD9f6/MAPr2 primer pair, as described by Arnaud *et al.* (2007), without the use of DMSO. PCR products were separated on 1% agarose gels in 0·5× TBE buffer, 5 V $cm^{-1}$, prestained with DNA Stain G (Serva Electrophoresis) and visualized on a UV transilluminator.

### DNA sequencing

Sequencing of phytoplasma genes was performed by Macrogen Europe. Amplicons obtained by nested PCR with primer pairs P1A/P7A, rp(V)F1A/rp(V)R1A, FD9f2/r and FD9f6/MAPr2 were purified with GeneElute PCR Clean-up kit (Sigma–Aldrich) and sequenced on both strands with the primers used for amplification and/or intermediate primers. Final sequences of each gene were assembled using DNADYNAMO program and nucleotide sequence data were deposited in GenBank under the accession numbers KU202151–KU202213 and KU216230 for the 16S rRNA gene, KU201965–KU202026 for *rpl22-rps3* genes, KU202027–KU202088 for the *secY* gene and KU202089–KU202150 for *secY-map* genes.

DNA extraction, amplification and sequencing were repeated twice for genes of phytoplasma isolates in which differences (insertions or deletions), compared to previously described genotypes of corresponding phytoplasma species, were detected. If more than one isolate had identical, newly detected change(s), DNA extraction, amplification and sequencing was repeated for only one randomly selected sample. Repeated analyses always confirmed previous results.

### Analysis of nucleotide sequences and phylogenetic analysis

Sequences obtained in this study were compared with those available in GenBank using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST). Representative phytoplasma gene sequences were retrieved from GenBank and multiple sequence alignments were performed using CLUSTALW integrated in MEGA 6 software (Tamura *et al.*, 2013). Nucleotide substitutions, deletions and/or insertions were recorded and the positions of these changes were determined by comparison to the EY1 reference strain (Lee *et al.*, 2004b) for '*Ca*. Phytoplasma ulmi', the OAY reference strain (Lee *et al.*, 2004a) for '*Ca*. Phytoplasma asteris' and the STOL11 reference strain (Quaglino *et al.*, 2013) for '*Ca*. Phytoplasma solani'.

Phylogenetic analysis on the basis of all four sequenced genomic loci was conducted with MEGA 6 software (Tamura *et al.*, 2013) using the maximum parsimony method. The accession numbers of all sequences retrieved from GenBank that were used for phylogenetic tree reconstructions are shown on the resulting phylogenetic trees, next to the sequence labels. The reliability of the phylogenetic analysis was subjected to a bootstrap test with 1000 replicates.

### Statistical analysis

The difference in the prevalence of phytoplasma for investigated elm species was tested by Fisher's exact test using STATISTICA 12 software (Stat Soft, Inc.).

## Results

### Incidence and impact of phytoplasma infections

Phytoplasma infection was confirmed in 64 out of 139 elm samples by 16S rRNA gene amplification and sequencing. In 62 elm samples, '*Ca*. Phytoplasma ulmi' (16SrV-A subgroup) was detected, while '*Ca*. Phytoplasma asteris' (16SrI group) and '*Ca*. Phytoplasma solani' (16SrXII-A subgroup) were each detected in one *U. minor* and one *U. glabra* sample, respectively (Table 1). '*Ca*. Phytoplasma ulmi' infection was proven for both *U. laevis* and *U. minor*, but the prevalence of the infection was significantly higher in *U. laevis*, as shown by Fisher's exact test ($P < 0.001$). Approximately 75% of analysed *U. laevis* trees were infected with '*Ca*. Phytoplasma ulmi'.

One or more symptoms indicating phytoplasma infection, such as leaf yellowing, drying and premature

abscission, were observed on 27 *U. laevis* and three *U. minor* trees infected with '*Ca*. Phytoplasma ulmi'. Development of witches' broom, considered to be a typical symptom of elm yellows disease for *U. minor* (Braun & Sinclair, 1979; Sinclair *et al.*, 2000), was not observed. Leaf yellowing and drying were observed on *U. minor* trees infected with '*Ca*. Phytoplasma asteris' and on *U. glabra* trees infected with '*Ca*. Phytoplasma solani'. However, '*Ca*. Phytoplasma ulmi' was identified in samples from 32 symptomless trees of *U. laevis*.

### Genetic diversity and phylogenetic relationships of '*Ca*. Phytoplasma ulmi'

Analyses of the 16S rRNA gene, *rpl22-rps3*, *secY* and *secY-map* genes revealed high genetic diversity of '*Ca*. Phytoplasma ulmi' in Croatia. The *secY* gene had the highest degree of genetic variability compared to the other sequenced genes. A total of 24 genotypes designated as EY-secY-1 to EY-secY-24 were detected based on *secY* gene sequence analysis. The sequence similarity among these genotypes ranged from 93% to 99.9%. When these genotypes of '*Ca*. Phytoplasma ulmi' were compared to those previously found, the similarity ranged from 92.1% to 99.9%. Two genotypes (EY-16S-1 and EY-16S-2) with 99.9% sequence similarity were detected based on 16S rRNA gene analysis. The similarity in the 16S rRNA gene when compared to previously described '*Ca*. Phytoplasma ulmi' isolates ranged between 99.7% and 100%, because genotype EY-16S-1 was identical to isolates EY1_SRB, EY10_SRB, EY18_SRB and EY20_SRB from Serbia (Jović *et al.*, 2011). Five genotypes (EY-rp-1 to EY-rp-5) with 99.7–99.9% sequence similarity were detected based on *rpl22-rps3* genes, and seven genotypes (EY-map-1 to EY-map-7) with 99.6–99.9% sequence similarity were detected based on *secY-map* genes. When compared to previously described '*Ca*. Phytoplasma ulmi' isolates, similarity in *rpl22-rps3* genes ranged from 99.1% to 100% and similarity in *secY-map* genes ranged from 98.5% to 100%. A 100% sequence identity of *rpl22-rps3* genes was found between Croatian genotype EY-rp-1 and Serbian isolates EY1_SRB, EY10_SRB, EY18_SRB and EY20_SRB, while a 100% sequence identity in *secY-map* genes was found between Croatian genotype EY-map-1 and Serbian isolate EY1_SRB (Jović *et al.*, 2011).

A total of three variable sites were identified in the 16S rRNA genes, 10 in *rpl22-rps3* gene sequences and 56 within *secY* gene sequences when Croatian isolates were compared to the EY1 reference strain. One variable site in the 16S rRNA gene, at the 1100 bp position, was located within an oligonucleotide sequence unique to '*Ca*. Phytoplasma ulmi'. This mutation was present in 16S rRNA sequences of both EY-16S-1 and EY-16S-2 genotypes and it has been previously reported in isolates from the Czech Republic and Serbia (Navrátil *et al.*, 2009; Jović *et al.*, 2011). Nucleotide changes were also found within oligonucleotide sequences unique to '*Ca*. Phytoplasma ulmi' in *rpl22-rps3* and the *secY* genes (Fig. 1). Variability has not been previously reported at the 753 bp position within the signature sequence of *rps3*, nor at several positions within the signature sequence of *secY* (Fig. 1). Isolate E04-D714, which is identical to the EY1 reference strain for the *secY-map* locus (Arnaud *et al.*, 2007), was used as a reference isolate for the *secY-map* genes. In comparison to isolate E04-D714, Croatian *secY-map* genotypes had a total of 10 variable sites, nine within the *map* gene and one within the intergenic sequence. In addition to nucleotide changes, insertions and deletions were also detected in the *secY* gene in 8 and 12 *secY* genotypes, respectively. Only insertions of 3 or 6 nucleotides and deletions of 6, 24, 36 or 39 nucleotides were detected; therefore these mutations did not result in translation frameshifts. Deletions and insertions were present within the range of three out of four *secY* gene signature sequences, and for some genotypes deletions encompassed the entire, or almost the entire, range of signature sequences (Fig. 1d,e,f). A single nucleotide deletion at two different positions within the intergenic region was also detected in *secY-map* genotypes EY-map-3 and EY-map-4.

Phylogenetic analysis based on 16S rRNA gene sequences revealed that within the phylogenetic group composed of isolates of the 16SrV group, isolates of the 16SrV-B subgroup (CLY5, JWB and PY-In) clearly formed a separate cluster, while all other 16SrV subgroups were more closely related with each other, forming a second cluster (Fig. 2). Ribosomal protein genes *rpl22* and *rps3*, the *secY* gene and the *secY-map* genetic locus were more variable compared to the 16S rRNA gene and thus more efficient in delineating phytoplasmas within the 16SrV group. Phylogeny based on these genes clearly indicated a monophyletic origin of all '*Ca*. Phytoplasma ulmi' isolates, but also indicated phylogenetic divergence within this group and existence of more than one phylogenetic lineage of '*Ca*. Phytoplasma ulmi' (Fig. 3). According to Jović *et al.* (2011), two lineages could be resolved based on ribosomal protein gene phylogeny (rpV-EY1 and rpV-EY2), as well as based on *secY* gene phylogeny (SecY-EY1 and SecY-EY2), while three lineages could be resolved based on phylogenetic analysis of *secY-map* genetic locus (Map-EY1, Map-EY2 and Map-EY3). '*Candidatus* Phytoplasma ulmi' isolates detected in Croatia were grouped together with isolates from Serbia within rpV-EY2, SecY-EY2 and Map-EY3 phylogenetic lineages. The isolate EYCZ1 from the Czech Republic (Navrátil *et al.*, 2009) with known sequences of ribosomal proteins and *secY* gene, but not the *map* gene, was not grouped within the phylogenetic lineages of '*Ca*. Phytoplasma ulmi' listed above, but in an additional lineage, indicating further divergence of this '*Ca*. Phytoplasma' species.

Affiliation of all Croatian '*Ca*. Phytoplasma ulmi' isolates to the same phylogenetic lineage based on the *rpl22-rps3* genes, the *secY* gene and the *secY-map* locus indicates their common ancestry. However, rpV-EY2, SecY-EY2 and Map-EY3 phylogenetic lineages do not

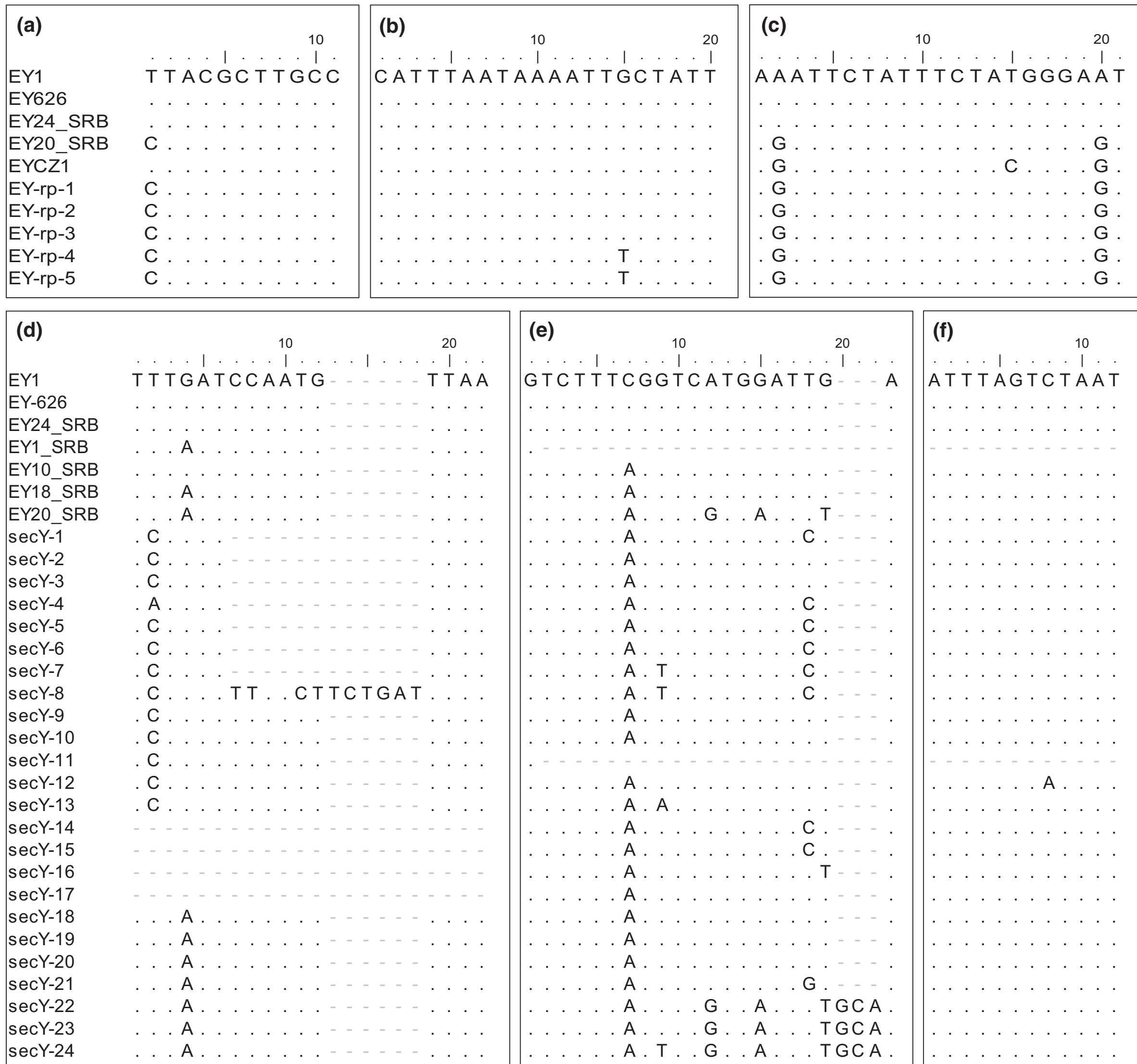

Figure 1 Nucleotide changes of sequences unique to '*Candidatus* Phytoplasma ulmi' in the ribosomal protein operon in position 284–294 within the *rpl22* gene (a), and positions 739–758 (b) and 910–932 (c) within the *rps3* gene; and the *secY* gene in positions 350–365 (d), 595–614 (e) and 616–627 (f).

represent a homogenous group, and further splitting of these lineages was observed (Fig. 3). Within the rpV-EY2 lineage, genotypes EY-rp-4 and EY-rp-5 from the Cetina site were grouped together. Genotypes EY-map-1, EY-map-2, EY-map-3 and EY-map-4 were shown to be more closely related to each other than to the other members of the Map-EY3 phylogenetic lineages. Within the SecY-EY2 lineage, two major clusters were observed: one cluster comprised isolates EY1_SRB and EY20_SRB from Serbia (Jović *et al*., 2011) and Croatian genotypes EY-secY-22, EY-secY-23 and EY-secY-24 found at the Jastrebarsko site, while all other isolates from Croatia and isolates EY10_SRB and EY18_SRB from Serbia were grouped into the second cluster.

## Distribution of particular '*Ca*. Phytoplasma ulmi' genotypes

All isolates from Eastern and Central Croatia had the same 16S rDNA genotype, EY-16S-1, and the majority had the same *rpl22-rps3* genotype, EY-rp-1. The 16S rDNA genotype EY-16S-2 was found only at the Southern littoral site Cetina, and isolates from that site also had unique *rpl22-rps3* genotypes, EY-rp-4 and EY-rp-5. The *secY* genotypes were usually represented by one or more isolates at a single site, except for genotype EY-secY-1, which was found at two sites: Donji Miholjac and Đurđevac. The most frequent *secY-map* genotype was EY-map-1, which was detected at sites Donji Miholjac,

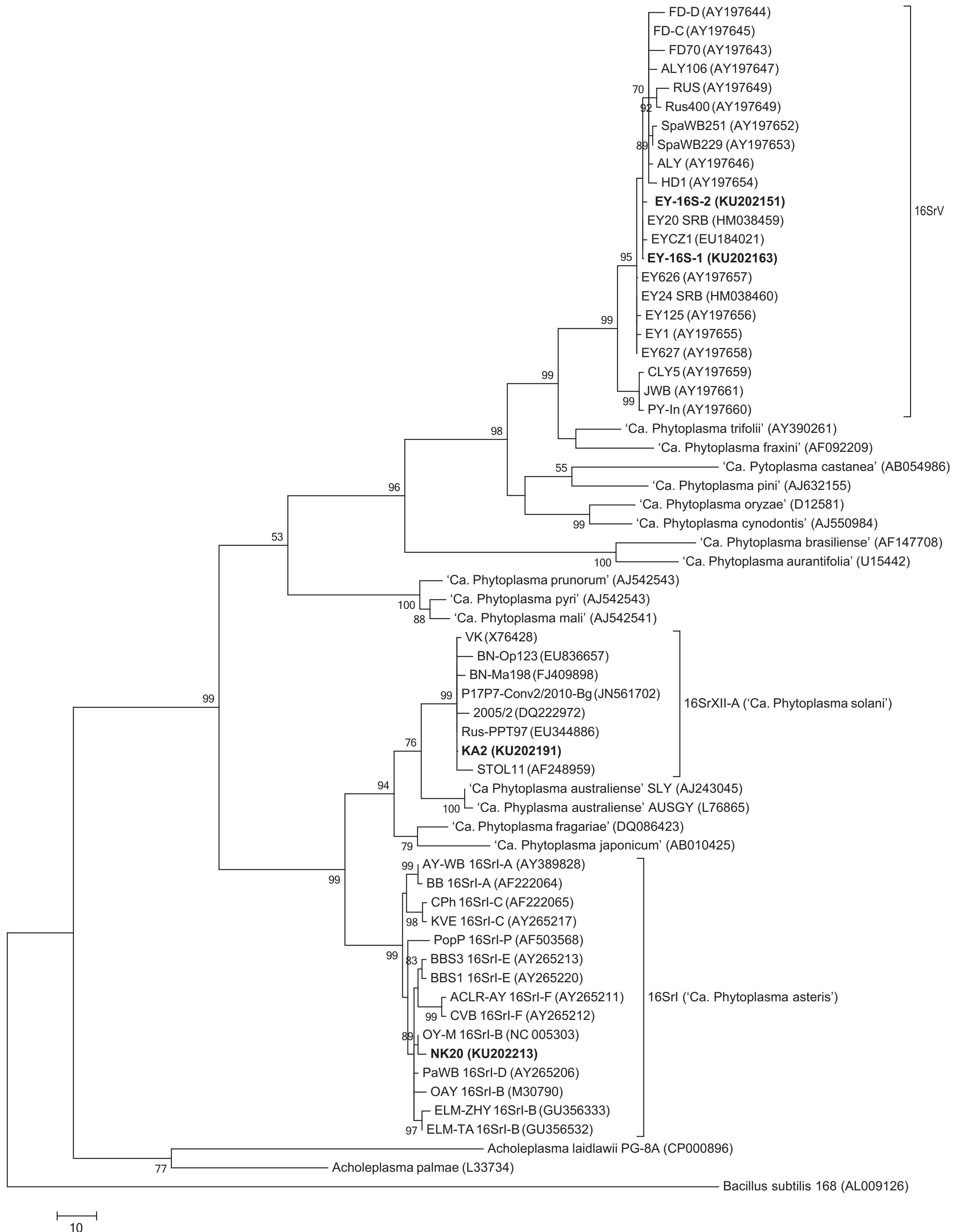


Figure 2 Phylogenetic tree inferred from partial 16S rRNA gene using maximum parsimony method. GenBank (NCBI) accession numbers of all sequences included in the phylogenetic analysis are shown next to the sequence label. Sequences from this study are in bold. Only one representative sequence of each '*Candidatus* Phytoplasma ulmi' genotype was included in the analysis. Sequence of *Bacillus subtilis* is used as the out-group to root the tree. Bootstrap values (>50%) for 1000 replicates are indicated on branches.

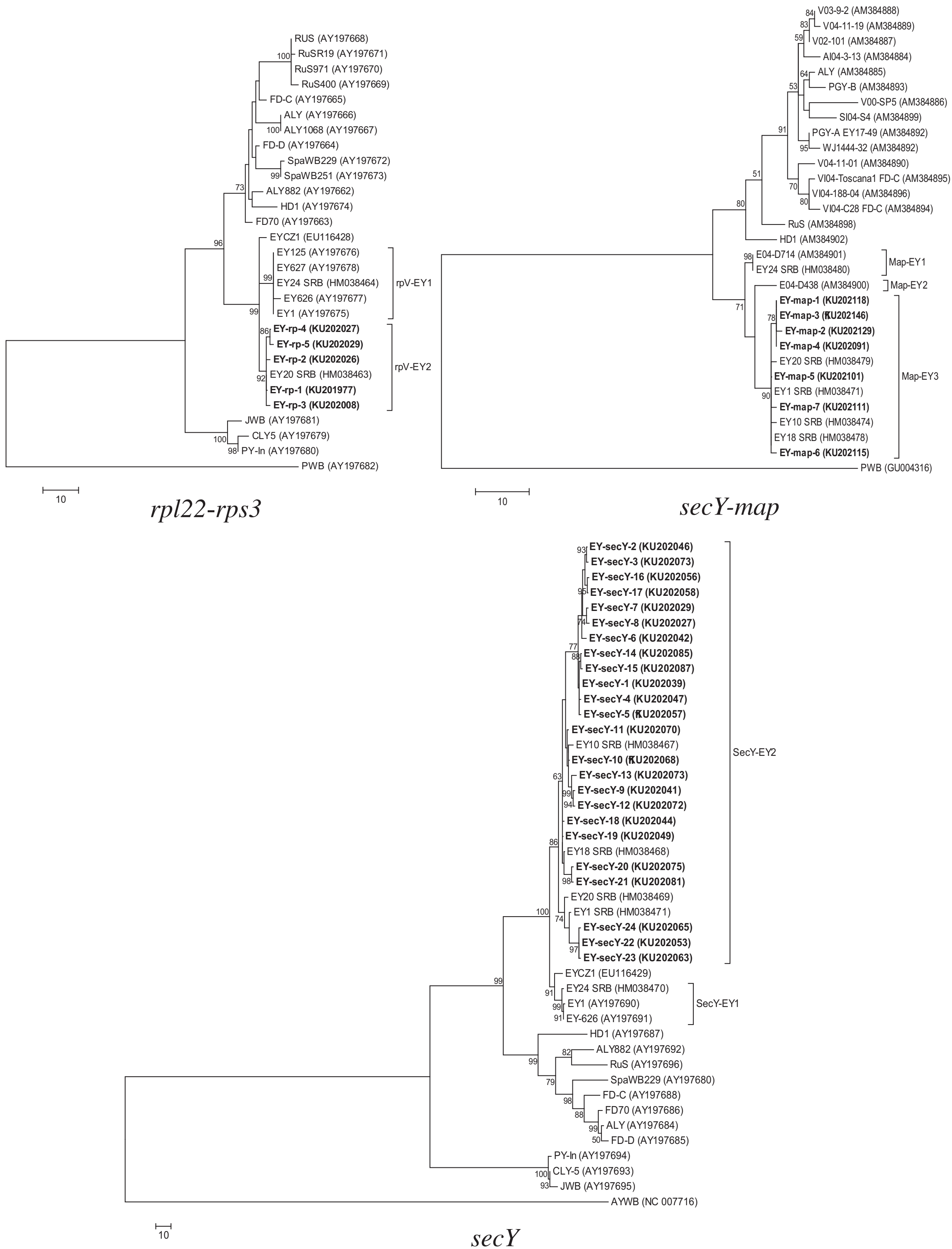


Figure 3 Phylogenetic trees inferred from sequences of *rpl22-rps3*, *secY* and *secY-map* genetic loci using maximum parsimony method. GenBank (NCBI) accession numbers of all sequences included in the phylogenetic analysis are shown next to the sequence label. Only one representative sequence of each '*Candidatus* Phytoplasma ulmi' genotype detected in Croatia was included; these sequences are in bold. Potato witches' broom phytoplasma PWB (16SrVII) is used as the out-group to root *rpl22-rps3* and *secY-map* trees, and aster yellows witches' broom phytoplasma AYWB (16SrI) is used as out-group to root the *secY* tree. Phylogenetic lineages of '*Ca.* Phytoplasma ulmi' identified according *rpl22-rps3*, *secY* and *secY-map* genetic loci by Jović *et al.* (2011) are indicated on the right of the trees. Bootstrap values (>50%) for 1000 replicates are shown on branches.

Đurđevac, Jastrebarsko and Cetina. Genotypes EY-map-3 and EY-map-5 were also present at more than one site, while the rest of the *secY-map* genotypes were each found only at a single site. Combining exact 16S rDNA, *rpl22-rps3*, *secY* and *secY-map* genotypes for each sample gave 31 comprehensive '*Ca*. Phytoplasma ulmi' genotypes. Distribution of '*Ca*. Phytoplasma ulmi' genotypes at the investigated elm sites is shown in Table 2.

### Characterization of other phytoplasmas

'*Candidatus* Phytoplasma solani' isolate KA2 had four nucleotide differences when compared to the STOL11 reference strain, but these differences had no influence on recognition sites for any of 17 restriction enzymes used for *in silico* restriction analysis of phytoplasmas (Lee *et al*., 1998; Wei *et al*., 2007) (data not shown). '*Candidatus* Phytoplasma asteris' isolate NK20 was most similar to phytoplasmas belonging to the 16SrI-B subgroup and was most closely related to isolate OY-M (16SrI-B subgroup; Fig. 2). However, the substitution of adenine by guanine in the 16S rRNA sequence of isolate NK20 at position 898 according to OAY reference strain generated an additional recognition site for *Alu*I endonuclease and consequently, a unique virtual RFLP pattern for the NK20 isolate, compared to all previously

Table 2 Distribution of '*Candidatus* Phytoplasma ulmi' genotypes across Croatia

| Site | | Plant host | No. of isolates | Isolates | Genotype | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 16S rDNA | *rpl22-rps3* | *secY* | *secY-map* | Combined[a] |
| Eastern Croatia | Nova Kapela | *Ulmus laevis* | 1 | NK3 | EY-16S-1 | EY-rp-1 | EY-secY-14 | EY-map-3 | EY-H1 |
| | | | 2 | NK4, NK7 | EY-16S-1 | EY-rp-1 | EY-secY-14 | EY-map-5 | EY-H2 |
| | | | 1 | NK15 | EY-16S-1 | EY-rp-1 | EY-secY-15 | EY-map-5 | EY-H3 |
| | | | 1 | NK16 | EY-16S-1 | EY-rp-2 | EY-secY-14 | EY-map-5 | EY-H4 |
| | Donji Miholjac | *U. laevis* | 3 | DM1, DM10, DM19 | EY-16S-1 | EY-rp-1 | EY-secY-1 | EY-map-5 | EY-H5 |
| | | | 3 | DM2, DM13, DM25 | EY-16S-1 | EY-rp-1 | EY-secY-1 | EY-map-1 | EY-H6 |
| | | | 1 | DM4 | EY-16S-1 | EY-rp-1 | EY-secY-9 | EY-map-1 | EY-H7 |
| | | | 2 | DM8, DM21 | EY-16S-1 | EY-rp-1 | EY-secY-6 | EY-map-1 | EY-H8 |
| | | | 1 | DM11 | EY-16S-1 | EY-rp-1 | EY-secY-18 | EY-map-5 | EY-H9 |
| | | | 1 | DM14 | EY-16S-1 | EY-rp-1 | EY-secY-2 | EY-map-5 | EY-H10 |
| | | | 1 | DM16 | EY-16S-1 | EY-rp-1 | EY-secY-4 | EY-map-5 | EY-H11 |
| | | | 1 | DM20 | EY-16S-1 | EY-rp-1 | EY-secY-19 | EY-map-7 | EY-H12 |
| | | | 1 | DM23 | EY-16S-1 | EY-rp-1 | EY-secY-19 | EY-map-5 | EY-H13 |
| Central Croatia | Đurđevac | *U. laevis* | 1 | KP3 | EY-16S-1 | EY-rp-1 | EY-secY-10 | EY-map-1 | EY-H15 |
| | | | 6 | KP6, KP8, KP19, KP20, KP24, KP30 | EY-16S-1 | EY-rp-1 | EY-secY-11 | EY-map-1 | EY-H16 |
| | | | 1 | KP7 | EY-16S-1 | EY-rp-3 | EY-secY-11 | EY-map-1 | EY-H17 |
| | | | 1 | KP10 | EY-16S-1 | EY-rp-1 | EY-secY-12 | EY-map-1 | EY-H18 |
| | | | 2 | KP11, KP16 | EY-16S-1 | EY-rp-1 | EY-secY-3 | EY-map-5 | EY-H19 |
| | | | 1 | KP13 | EY-16S-1 | EY-rp-1 | EY-secY-20 | EY-map-2 | EY-H20 |
| | | | 1 | KP15 | EY-16S-1 | EY-rp-1 | EY-secY-11 | EY-map-5 | EY-H21 |
| | | | 1 | KP25 | EY-16S-1 | EY-rp-1 | EY-secY-21 | EY-map-2 | EY-H122 |
| | | | 1 | KP12 | EY-16S-1 | EY-rp-1 | EY-secY-1 | EY-map-5 | EY-H5 |
| | | *U. minor* | 1 | KP1 | EY-16S-1 | EY-rp-1 | EY-secY-1 | EY-map-2 | EY-H14 |
| | | | 1 | KP38 | EY-16S-1 | EY-rp-1 | EY-secY-21 | EY-map-2 | EY-H22 |
| | Jastrebarsko | *U. laevis* | 4 | J2, J5, J13, J33 | EY-16S-1 | EY-rp-1 | EY-secY-22 | EY-map-6 | EY-H23 |
| | | | 1 | J4 | EY-16S-1 | EY-rp-1 | EY-secY-13 | EY-map-2 | EY-H24 |
| | | | 2 | J7, J15 | EY-16S-1 | EY-rp-1 | EY-secY-16 | EY-map-1 | EY-H25 |
| | | | 1 | J9 | EY-16S-1 | EY-rp-1 | EY-secY-5 | EY-map-1 | EY-H26 |
| | | | 3 | J10, J17, J24 | EY-16S-1 | EY-rp-1 | EY-secY-17 | EY-map-1 | EY-H27 |
| | | | 1 | J18 | EY-16S-1 | EY-rp-1 | EY-secY-23 | EY-map-6 | EY-H28 |
| | | | 1 | J28 | EY-16S-1 | EY-rp-1 | EY-secY-24 | EY-map-6 | EY-H29 |
| | | *U. minor* | 1 | J16 | EY-16S-1 | EY-rp-1 | EY-secY-13 | EY-map-2 | EY-H24 |
| Southern littoral Croatia | Cetina | *U. laevis* | 11 | C1, C4, C6, C11, C12, C14, C15, C16, C18, C22, C23 | EY-16S-2 | EY-rp-4 | EY-secY-1 | EY-map-8 | EY-H30 |
| | | | 1 | C5 | EY-16S-2 | EY-rp-5 | EY-secY-7 | EY-map-5 | EY-H31 |

[a]The combination of 16S rDNA, *rpl22-rps3, secY* and *secY-map* genotypes.

described 16SrI subgroups (data not shown). Therefore isolate NK20 could represent a new 16SrI subgroup.

## Discussion

The focus of this research was molecular characterization of '*Ca*. Phytoplasma ulmi', the causal agent of elm yellows. In contrast to its first description as a novel phytoplasma taxon (Lee *et al*., 2004b), '*Ca*. Phytoplasma ulmi' was recently shown to be highly diverse in Europe (Jović *et al*., 2011). This is further supported by characterization of '*Ca*. Phytoplasma ulmi' in Croatia using a multilocus sequence analysis approach that included four genomic loci: the 16S rRNA gene, ribosomal protein genes *rpl22* and *rps3*, the *secY* gene and *secY-map* genes. Comparative sequence analysis and comparison with previously described isolates revealed high genetic variability of this phytoplasma species across all investigated elm populations. All Croatian isolates differed from reference strain EY1 and had mutations even in the highly conserved 16S rRNA gene sequence. Diversity of this phytoplasma species was particularly evident from the *secY* gene (FD9 genetic locus) analysis, which was the most variable genetic locus analysed. Aside from nucleotide changes, insertions and/or deletions were recorded within *secY* gene sequences, which is in agreement with earlier studies (Lee *et al*., 2004b, 2010; Jović *et al*., 2011). Furthermore, all isolates had changes in signature sequences of the 16S rRNA gene, ribosomal protein genes *rpl22* and *rps3* and the *secY* gene, unique for '*Ca*. Phytoplasma ulmi'. This is not the first evidence of such variability in elm yellows phytoplasma, and isolates with mutations in oligonucleotide signature sequences, supposedly specific for '*Ca*. Phytoplasma ulmi', were found in the Czech Republic (Navrátil *et al*., 2009) and Serbia (Jović *et al*., 2011). However, many mutations recorded in this study, both within signature sequences and elsewhere, were at positions where variability has not previously been detected, pointing to an even higher diversity of this particular phytoplasma species than previously reported. Altogether, studies of elm yellows phytoplasma in Europe, including the present study, indicate that the formal description of '*Ca*. Phytoplasma ulmi' made by Lee *et al*. (2004b) should be revised and continuously upgraded.

Despite their diversity, all '*Ca*. Phytoplasma ulmi' described so far, including those from this study, are monophyletic in origin. This was clearly indicated by phylogeny inferred from ribosomal protein genes *rpl22* and *rps3*, the *secY* gene and *secY-map* genes. Due to high sequence similarity, phylogeny inferred from the 16S rRNA gene could not resolve phylogenetic relationships among members of the 16SrV group. Phylogeny inferred from *rpl22-rps3*, *secY* and *secY-map* genes also pointed to delineation of '*Ca*. Phytoplasma ulmi' and existence of more than one phylogenetic lineage within this group, which is in agreement with previous studies (Arnaud *et al*., 2007; Jović *et al*., 2011). All phylogenetic analyses indicated that all Croatian '*Ca*. Phytoplasma ulmi' isolates are related to each other. All isolates from this study belonged to the same phylogenetic lineage and were most closely related to isolates from Serbia (Jović *et al*., 2011), which is expected due to geographic proximity.

'*Candidatus* Phytoplasma asteris' and '*Ca*. Phytoplasma solani' infecting two elm trees were probably transmitted by occasional elm-feeding vectors, as previously reported (Lee *et al*., 1995; Credi *et al*., 2006), because both of these phytoplasmas have wide host and vector ranges (Lee *et al*., 2004a; Quaglino *et al*., 2013). However, '*Ca*. Phytoplasma asteris' isolate NK20 differed from other members of the 16SrI group. This isolate was most closely related to the 16SrI-B subgroup, but according to virtual RFLP analysis it may represent a new 16SrI subgroup. This is an interesting contribution to characterization of the aster yellows group (16SrI), one of the most diverse phytoplasma groups associated with diseases of many plant species, including economically important ones (Lee *et al*., 2004a).

The frequent symptomless '*Ca*. Phytoplasma ulmi' infections of *U. laevis* are in accordance with the observation that European elms are less susceptible to phytoplasma infections compared to American elms (Sinclair *et al*., 2000). However, such significant difference in prevalence of '*Ca*. Phytoplasma ulmi' among investigated elm species has not been reported prior to the present study. Infections of *U. minor* with 'Ca. Phytoplasma ulmi' were often detected in previous studies conducted in Europe in Italy, France, Germany, the Czech Republic and Serbia (Mäurer *et al*., 1993; Marcone *et al*., 1997; Boudon-Padieu *et al*., 2004; Navrátil *et al*., 2009; Jović *et al*., 2011) and infected *U. glabra* trees were detected in France and Germany (Mäurer *et al*., 1993; Boudon-Padieu *et al*., 2004; Arnaud *et al*., 2007). Most of these studies were primarily focused on trees with symptoms, although it is known that elm yellows phytoplasma infections of European elms can be symptomless (Carraro, *et al*., 2004). In the present study, more than half of the *U. laevis* trees infected with '*Ca*. Phytoplasma ulmi' did not express any disease symptoms at the time of sampling. This indicates that trees both with and without symptoms should nevertheless be sampled and analysed in order to determine infection rates and distribution of elm yellows phytoplasma in Europe. Due to high prevalence of elm yellows phytoplasma infections in *U. laevis*, it is assumed that there must be efficient vector(s) present across Croatia. *Macropsis mendax* was shown to be a vector of the elm yellows phytoplasma in Italy (Carraro *et al*., 2004) and other species, such are *Hyalesthes luteipes*, *Philenus spumarium* and *Allygus atomarius*, were suggested as potential vectors in Europe (Jović *et al*., 2010; Navrátil *et al*., 2009). Better understanding of host sensitivity and the ecology and behaviour of vector species could provide a possible explanation for observed differences in prevalence of '*Ca*. Phytoplasma ulmi' infections between various elm species.

Altogether, results of this study revealed that Croatia is another European country where '*Ca*. Phytoplasma

ulmi' is widely distributed. The abundance of phylogenetically related but highly variable isolates of '*Ca*. Phytoplasma ulmi', together with their relatively low impact on elms in Croatia, could be used to support the hypothesis that this pathogen may be native to Europe (Jović *et al*., 2011). The relationship between '*Ca*. Phytoplasma ulmi' and *U. laevis* has similarity with another phytoplasmosis of a forest tree in Europe caused by phytoplasmas belonging to the 16SrV group. Phytoplasma belonging to the 16SrV-C group, alder yellows (AldY) phytoplasma, is widespread among European alders (*Alnus* spp.), but infected trees often remain symptomless (Arnaud *et al*., 2007; Holz *et al*., 2016). The hypothesis of a balanced parasitism of AldY phytoplasma in *A. glutinosa*, possibly associated with a long co-evolution of the pathogen and its host, was proposed by Holz *et al*. (2016). This concept may be applied to the elm yellows phytoplasma as well, further supporting the hypothesis of its European origin. AldY phytoplasma belongs to the same phylogenetic subclade as flavescence dorée (FD) phytoplasma and Palatinate grapevine yellows (PGY) phytoplasma that cause economically important disease of grapevine in Europe. Therefore it was assumed that FD and PGY phytoplasmas could have originated from AldY phytoplasma as a consequence of erratic transmission from alder to grapevine (Arnaud *et al*., 2007). Thus, even if the elm yellows disease does not present a direct threat for elms, it can be a starting point for the development of new phytoplasma diseases if transitions to more sensitive hosts occur. The possibility for this is very hard to predict, but it is important to continue investigation of this pathogen in Croatia as well as to extend research to additional elm sites.

## Acknowledgements

This research was supported by grants from the University of Zagreb.

## References

Angelini E, Clair D, Borgo M, Bertaccini A, Boudon-Padieu E, 2001. Flavescence dorée in France and Italy – occurrence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. *Vitis* **40**, 79–86.

Arnaud G, Malembic-Maher S, Salar P *et al*., 2007. Multilocus sequence typing confirms the close genetic interrelatedness of three distinct flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Applied and Environmental Microbiology* **73**, 4001–10.

Boudon-Padieu E, Larrue J, Clair D *et al*., 2004. Detection and prophylaxis of Elm Yellows phytoplasma in France. *Investigación Agraria: Sistemas y Recursos Forestales* **13**, 71–80.

Braun EJ, Sinclair WA, 1979. Phloem necrosis of elms: symptoms and histopathological observations in tolerant hosts. *Phytopathology* **69**, 354–8.

Carraro L, Ferrini F, Ermacora P, Loi N, Martini M, Osler R, 2004. *Macropsis mendax* as a vector of elm yellows phytoplasma of *Ulmus* species. *Plant Pathology* **53**, 90–5.

Credi R, Terlizzi F, Milanesi L *et al*., 2006. Wild host plants of stolbur phytoplasma and its vector, *Hyalesthes obsoletus*, at sites of grapevine Bois noir occurrence in Emilia-Romagna, Italy. In: *Proceedings of the 15th Meeting ICVG 2006*. Stellenbosch, South Africa: South African Society for Enology and Viticulture, 182–3. http://icvg.org/data/extabstr2006part2.pdf. Accessed 4 March 2016.

Daire X, Clair D, Reinert W, Boudon-Padieu E, 1997. Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. *European Journal of Plant Pathology* **103**, 507–14.

Deng S, Hiruki C, 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *Journal of Microbiological Methods* **14**, 53–61.

EFSA, PLH Panel (EFSA Panel on Plant Health), 2014. Scientific opinion on the pest categorisation of elm phloem necrosis mycoplasma. *EFSA Journal*. doi: 10.2903/j.efsa.2014.3773.

Gundersen DE, Lee IM, 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea* **35**, 144–51.

Hogenhout SA, Oshima K, Ammar ED, Kakizawa S, Kingdom HN, Namba S, 2008. Phytoplasmas: bacteria that manipulate plants and insects. *Molecular Plant Pathology* **9**, 403–23.

Holz S, Duduk B, Büttner C, Kube M, 2016. Genetic variability of alder yellows phytoplasma in *Alnus glutinosa* in its natural Spreewald habitat. *Forest Pathology* **46**, 11–21.

IRPCM, 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology* **54**, 1243–55.

Jacobs KA, Lee IM, Griffiths HM, Miller FD Jr, Bottner KD, 2003. A new member of the clover proliferation phytoplasma group (16SrVI) associated with elm yellows in Illinois. *Plant Disease* **87**, 241–6.

Jović J, Cvrković T, Mitrović M, Petrović A, Krnjajić S, Toševski I, 2008. New strain of '*Candidatus* Phytoplasma ulmi' infecting *Ulmus minor* and *U. laevis* in Serbia. *Plant Pathology* **57**, 1174.

Jović J, Cvrković T, Mitrović M, Petrović A, Krstić O, Krnjanjić S, Toševski I, 2010. Genetic variability among '*Candidatus* Phytoplasma ulmi' strains infecting elms in Serbia and survey of potential vectors. In: Bertaccini A, Laviña A, Torres E, eds. Current status and perspectives of phytoplasma disease research and management, Work Groups 1-4 meeting, COST Action FA0807, Sitges, Spain, 18-18. [http://www.costphytoplasma.ipwgnet.org/PDF%20files/WG%20BookwithiISBN.pdf]. Accessed 10 March 2016.

Jović J, Cvrković T, Mitrović M *et al*., 2011. Multigene sequence data and genetic diversity among '*Candidatus* Phytoplasma ulmi' strains infecting *Ulmus* spp. in Serbia. *Plant Pathology* **60**, 356–68.

Lee IM, Hammond RW, Davis RE, Gundersen DE, 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology* **83**, 834–42.

Lee IM, Bertaccini A, Vibio M *et al*., 1995. Detection and characterization of phytoplasmas associated with diseases in *Ulmus* and *Rubus* in northern and central Italy. *Phytopathologia Mediterranea* **34**, 174–83.

Lee IM, Gundersen-Rindal DE, Davis RE, Bartoszyk IM, 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology* **48**, 1153–69.

Lee IM, Gundersen-Rindal DE, Davis RE, Bottner KD, Marcone C, Seemüller E, 2004a. '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. *International Journal of Systematic and Evolutionary Microbiology* **54**, 1037–48.

Lee IM, Martini M, Marcone C, Zhu SF, 2004b. Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of '*Candidatus* Phytoplasma ulmi' for the phytoplasma associated with elm yellows. *International Journal of Systematic and Evolutionary Microbiology* **54**, 337–47.

Lee IM, Bottner-Parker KD, Zhao Y, Davis RE, Harrison NA, 2010. Phylogenetic analysis and delineation of phytoplasmas based on *secY*

gene sequences. *International Journal of Systematic and Evolutionary Microbiology* **60**, 2887–97.

Lim PO, Sears BB, 1992. Evolutionary relationships of a plant-pathogenic mycoplasmalike organism and *Acholeplasma laidlawii* deduced from two ribosomal protein gene sequences. *Journal of Bacteriology* **174**, 2606–11.

Marcone C, Ragozzino A, Seemüller E, 1997. Identification and characterization of the phytoplasma associated with elm yellows in southern Italy and its relatedness to other phytoplasmas of the elm yellows group. *European Journal of Forest Pathology* **27**, 45–54.

Martini M, Lee IM, 2013. PCR and RFLP analyses based on the ribosomal protein operon. In: Dickinson M, Hodgetts J, eds. *Phytoplasma: Methods and Protocols*. Berlin, Germany: Springer Science+Business Media, 173–86.

Mäurer R, Seemüller E, Sinclair WA, 1993. Genetic relatedness of mycoplasmalike organisms affecting elm, alder, and ash in Europe and North America. *Phytopathology* **83**, 971–6.

Navrátil M, Šafářová D, Válová P, Fránová J, Šimková M, 2009. Phytoplasma associated with witches'-broom disease of *Ulmus minor* Mill. in the Czech Republic: electron microscopy and molecular characterization. *Folia Microbiologica* **54**, 37–42.

Quaglino F, Zhao Y, Casati P *et al*., 2013. '*Candidatus* Phytoplasma solani', a novel taxon associated with stolbur-and bois noir-related diseases of plants. *International Journal of Systematic and Evolutionary Microbiology* **63**, 2879–94.

Schneider B, Seemüller E, Smart CD, Kirkpatrick BC, 1995. Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In: Razin S, Tully J, eds. *Molecular and Diagnostic Procedures in Mycoplasmology*, Vol. **1**. New York, USA: Academic Press, 369–80.

Sfalanga A, Martini M, Surico G, Bertaccini A, 2002. Involvement of phytoplasmas in a decline of *Ulmus chenmoui* in Central Italy. *Forest Pathology* **32**, 265–75.

Sinclair WA, Townsend AM, Griffiths HM, Whitlow TH, 2000. Responses of six Eurasian *Ulmus* cultivars to a North American elm yellows phytoplasma. *Plant Disease* **84**, 1266–70.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA 6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–9.

Wei W, Davis RE, Lee M, Zhao Y, 2007. Computer-simulated RFLP analysis of 16S rRNA genes: identification of ten new phytoplasma groups. *International Journal of Systematic and Evolutionary Microbiology* **57**, 1855–67.