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Distribution of phytoplasma diseases in the Lombardy poplar tree population of Zagreb urban area

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Phytoplasmas are uncultivable prokaryotic wall-less pathogens belonging to the class *Mollicutes* that inhabit plant phloem and insects. Their identification and classification is difficult and mainly based on the polymorphism of their 16S rRNA gene sequences. Aster yellows (AY; '*Candidatus* Phytoplasma asteris') phytoplasmas (ribosomal subgroup 16SrI-P) had been previously identified in several *Populus nigra* L. 'Italica' (Lombardy poplar) trees from the urban area of Zagreb. The aim of this research was to examine phytoplasmosis distribution in the poplar tree population of the wider Zagreb urban area. Total nucleic acids were extracted from leaf samples of 30 symptomatic and 4 asymptomatic trees. Phytoplasma 16S rDNA was amplified in direct and nested PCRs by using universal and group-specific primers. The pathogens were classified on the basis of 16S rDNA amplicon RFLP analyses. Phytoplasmas belonging to the 16SrI ribosomal group (AY) were detected in 12 out of 34 trees examined. In addition, a phytoplasma putative gene for aa kinase was analyzed for positive samples. RFLP profiles from 10 samples were referable to the phytoplasma 16SrI-P ribosomal subgroup previously found only in poplars from Zagreb area. In two samples, unique restriction patterns were found, showing the presence of molecular variability within this conserved gene region. In the northern and north-western part of the area the infection was equally distributed, while in the southern part of the city phytoplasmas were not detected in sampled trees. Further research, including a search for potential insect vectors, is needed in order to indentify the ecological and epidemiological implications of these diseases and their impact on the sanitary status of ornamental trees in the city of Zagreb.

Keywords: 16S rDNA, aster yellows, *Candidatus*, Phytoplasma asteris, phytoplasmosis, poplar tree

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Introduction

Phytoplasmas, formerly known as *mycoplasma-like organisms* (MLOs), are wall-less plant pathogens from the class of *Mollicutes* which cause numerous diseases and high losses in plant production worldwide. They have been detected in several hundred economically important plant species (LEE et al. 2000). Vectors responsible for phytoplasma transmission in nature are insects of the order Hemiptera, primarily phloem-feeding leafhoppers (Cicadellidae). Vector specificity varies from high, when phytoplasmas are transmitted by only one or two vectors, to extremely low, when a specific phytoplasma can be transmitted by up to 24, mostly polyphagous, leafhopper species (SEEMÜLLER et al. 2002, LEE et al. 2003). Another important feature of phytoplasma epidemiology is the wide plant host range of some phytoplasmas. In combination with the above mentioned low vector specificity, this trait enables overlapping of plant hosts and vectors, which provides opportunity for different phytoplasma species to interact and exchange genetic information (CHRISTENSEN et al. 2005).

The phytoplasma genome is extremely reduced, lacking genes which have been considered essential for self-dividing organisms (OSHIMA et al. 2004, BAI et al. 2006). This feature is probably the key to our inability to cultivate phytoplasma in *axenic* media. However, this major obstacle to research has not completely discouraged researchers in this field. Major advances were made in the late 1980 and early 1990 with the development of generic and group specific phytoplasma primers based on the 16S rRNA gene. The use of molecular-based tools, such as polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (RFLP) provided sensitive, reliable and relatively rapid diagnosis of diseases associated with these wall-less prokaryotes (DAVIS et al. 1993, LEE et al. 1993).

With the increasing knowledge on the phytoplasma genes and their sequences, the classification of these prokaryotes advanced towards a delineation of their new taxonomic status (IRPCM, 2004). Phytoplasmas were recently assigned to a novel genus '*Candidatus* Phytoplasma'. The designation *Candidatus* refers to organisms that cannot be cultivated in pure cultures *in vitro*. This new classification takes into account the phylogenetic as well as the biological/ecological characteristics of the organisms. So far, about thirty '*Candidatus* Phytoplasma' species have been described. (IRPCM 2004, AROCHA et al. 2005, SCHNEIDER et al. 2005, LEE et al. 2006, VALIUNAS et al. 2006).

Hitherto, phytoplasmas infecting *Populus nigra* L. 'Italica' (Lombardy poplar) trees have been detected by means of molecular biology tools in France (COUSIN 1996), Germany (BERGES et al. 1997) and Croatia (ŠERUGA et al. 2002). Phytoplasmas previously found to infect Lombardy poplar trees in the city of Zagreb (Croatia) have been assigned to a newly described ribosomal subgroup 16SrI-P (ribosomal group 16SrI; aster yellows) and ribosomal protein subgroup rp-O on the basis of exhaustive analyses of several phytoplasma conserved genes. This new phytoplasma could be specific, infecting only Lombardy poplar trees (ŠERUGA et al. 2003). According to the new classification, Lombardy poplar phytoplasma is still denoted as a '*Candidatus* Phytoplasma asteris' member since this taxon (16SrI ribosomal group) currently encompasses many isolates with equivalent status at the species level. This could change in the future because the 16SrI phytoplasma group is the most diverse and may consist of more than one species (FIRRAO et al. 2005).

Witches' broom is a typical symptom associated with phytoplasma infections of Lombardy poplar trees (SHARMA and COUSIN 1986, COUSIN 1996). Besides witches' broom, sev-

eral less specific symptoms regularly appear on affected poplar trees such as undersized leaves, yellowing, sparse foliage, stunting and dieback (BERGES et al. 1997). Nearly all the above mentioned symptoms were observed on the trees growing in the Zagreb urban area. The first report on phytoplasma-associated diseases in the Lombardy poplar tree population of Zagreb was made after analyzing a relatively small number of samples (ŠERUGA et al. 2002). Therefore, the aim of this research was to examine the distribution of Lombardy poplar phytoplasmoses in the city of Zagreb on the basis of a larger group of samples to represent the wider urban area.

Materials and methods

Plant samples

In May 2003, symptomatic and asymptomatic leaf and branch samples from *Populus nigra* L. 'Italica' trees were collected in the Zagreb urban area. Only two sampled trees exhibited typical witches' broom symptoms, but the majority of Lombardy poplars developed leaf yellowing, sparse foliage, dieback and undersized leaves (Tab. 1). Samples from 34 trees were collected from the lower parts of the trunks. Sampling was performed uniformly throughout the city urban area, in order to provide clear data about the disease distribution (Fig. 1). Phloem tissue from leaf midveins was dissected and stored at –80 °C prior to total nucleic acids extraction.

Total nucleic acids extraction

Total nucleic acids (TNA) were extracted from approximately 500 mg of plant phloem tissue by using CTAB buffer, following the procedure already described in ŠERUGA et al. (2003). The TNA concentration was measured by Beckman Spectrophotometer DU-64 (SAMBROOK et al. 1989) and the final concentration for PCR experiments was adjusted to 20 ng/μL.

Amplification of phytoplasma genes

***Amplification of phytoplasma 16S rRNA gene*:** Polymerase chain reaction (PCR) was performed for amplification of phytoplasma 16S rRNA gene by using phytoplasma-universal primer pairs R16F1/R0 (LEE et al. 1995), R16F2n/R2 (GUNDERSEN and LEE 1996) and $16R_{738f}$/$16R_{1232r}$ (GIBB et al. 1995), as well as the primer pair R16(I)F1/R1 (LEE et al. 1994), specific for phytoplasma ribosomal groups 16SrI and 16SrXII. Direct PCR assay was performed with R16F1/R0 primer pair. Obtained products were diluted 1:30 (v/v) with sterile deionized water and used as templates in the nested PCR with primer pair R16F2n/R2. In the second nested PCRs, products obtained from previous amplification were diluted as mentioned above, and used as templates for two separate reactions using either general ($16R_{738f}$/$16R_{1232r}$) or group-specific (R16(I)F1/R1) phytoplasma primers.

***Amplification of phytoplasma putative gene for aa kinase*:** A putative gene for aa kinase was amplified by using the primer pair BB88F1/R1 (GUNDERSEN et al. 1996) specific for phytoplasma ribosomal 16SrI group and by using TNA extracted from positively-tested samples as template in a PCR reaction.

Tab. 1. Presence of phytoplasmosis-like symptoms and the phytoplasma 16S rRNA gene in the collected Lombardy poplar samples from Zagreb.

sample	location	symptoms	Phytoplasma 16S rRNA gene
1	Marulić square	dieback	+
2	Marulić square	irregular branching	–
3	Marulić square	irregular branching, yellowing	+
4	Mažuranić square	yellowing	–
5	Mažuranić square	asymptomatic	–
6	Mažuranić square	witches' broom, yellowing	–
7	Žitnjak	asymptomatic	–
8	»Konzum«, Žitnjak	dieback, yellowing	–
9	Žitnjak	asymptomatic	+
10	»Diona«, Ferenščica	yellowing	+
11	High school, Volovčica	dieback, yellowing	+
12	High school »R. Bošković«, Ferenščica	witches' broom, lesions	+
13	»Konzum«, Žitnjak	dieback, yellowing	–
14	High school, Volovčica	dieback, lesions	–
15	Health centre Kruge	dieback, yellowing	–
16	Health centre Kruge	dieback, yellowing	–
17	Health centre Kruge	dieback, yellowing	–
18	Primary school »Grigor Vitez«, Kruge	yellowing	+
19	Primary school »Vladimir Ruždjak«, Savica	dieback	–
20	Primary school »Vladimir Ruždjak«, Savica	dieback	–
21	Settlement Folnegovićevo	undersized leafs	–
22	Settlement Folnegovićevo	undersized leafs	–
23	Settlement Folnegovićevo	dieback, yellowing	–
24	Lake Bundek	stunting	–
25	Hippodrome	extreme yellowing	–
26	Shopping center Prečko	yellowing	–
27	Shopping center Prečko	yellowing	–
28	Shopping center Prečko	yellowing	+
29	Shopping center Prečko	yellowing, leaf deformation	+
30	Theatre »Trešnja«, Selska road	extreme yellowing	+
31	The Street of Baron Filipović	extreme yellowing	+
32	The Street of Baron Filipović	extreme yellowing, undersized leafs	+
33	Vrapče	yellowing, dieback	–
34	Trešnjevka	asymptomatic	–

Each reaction was performed in a total volume of 25 μL, containing 1.5 μL of reaction buffer, 200 μM of dNTPs, 0.2 μM of each primer and 0.625 U of *Taq*-polymerase (Eppendorf AG, Hamburg, Germany). In every PCR assay, a reaction mix devoid of DNA template was included as a contamination control. All reactions were performed under conditions already described (ŠERUGA et al. 2003). The amplified fragments were analyzed by electrophoresis in 1% agarose gels, stained with ethidium-bromide and visualized on a UV-transilluminator (Sigma T 2202).

RFLP analysis

Products amplified by PCR assays were subjected to the restriction fragment length polymorphism (RFLP) analysis. Two restriction endonucleases were used, *Tru*9I and *Tsp*EI (Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions. Digestion was carried out for 16 hours at 65 °C. Restriction fragments were analyzed by electrophoresis in a 5% polyacrylamide gel, stained with ethidium-bromide and visualized on the UV-transilluminator. RFLP patterns were compared with those of standard strains: STOL (ribosomal subgroup 16SrXII-A, to be described as '*Ca*. P. solani'), HYDB (ribosomal subgroup 16SrI-B, '*Ca*. P. asteris'), P (poplar isolate from ribosomal subgroup 16SrI-P (ŠERUGA et al. 2003). HYDB and STOL standard strains were obtained from Prof. Assunta Bertaccini, University of Bologna (IRPCM, 2004; http://137.204.42.130/person/collection-september_2003.pdf).

Results

Symptoms

Lombardy poplar trees from Zagreb urban area were visually examined for phytoplasmosis-like symptoms during May 2003 (Tab. 1, Fig. 1). In the spring, the symptoms of

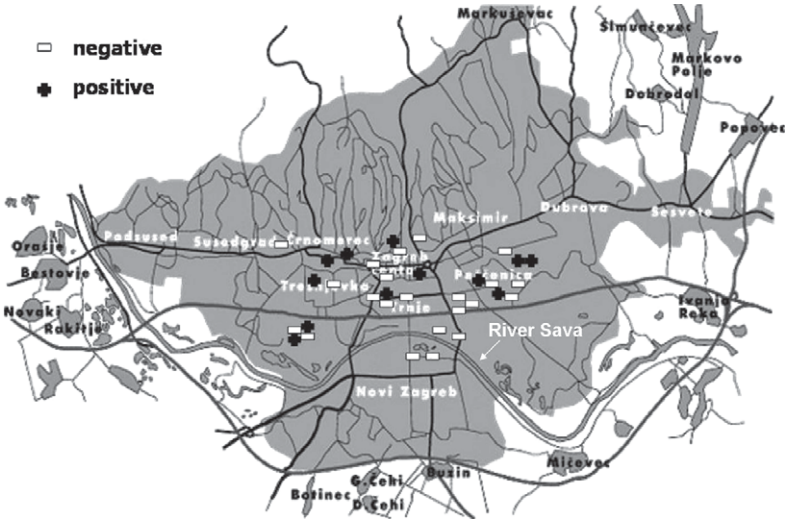

Fig. 1. Distribution of phytoplasma infection in Lombardy poplar tree population of Zagreb urban area. Locations of both positive (infected) and negative samples are indicated.

phytoplasma infection are the most apparent (COUSIN et al. 1999). The majority of the sampled poplars exhibited atypical symptoms of infections such as undersized leaves, yellowing, sparse foliage, stunting and dieback. Only two trees in researched Zagreb urban area showed witches' broom, a symptom that is typical of phytoplasma infection (BERGES et al. 1997). For the purpose of control, four samples, out of 34 in total, were collected from asymptomatic trees and examined for the presence of phytoplasma.

PCR amplification

In the agarose gel electrophoreses performed after direct PCR by using the generic phytoplasma primer pair R16F1/R0 no bands were detected, with the exception of reference strains HYDB and STOL (Fig. 2A). All PCR products were diluted and used as templates in the first nested PCR performed by using the second general primer pair R16F2n/R2. This nested PCR procedure was sensitive enough for obtaining visible amplicons from poplar samples in the agarose gels (Fig. 2 B). The second nested PCRs performed by using two different primer types (primer pairs $16R_{738f}/16R_{1232r}$ and R16(I)F1/R1) on the R16F2n/R2 amplicons resulted in visible bands (Fig. 2C and D). Out of 30 collected symptomatic samples, 11 were positive for the presence of phytoplasma 16S rRNA gene. Regarding four

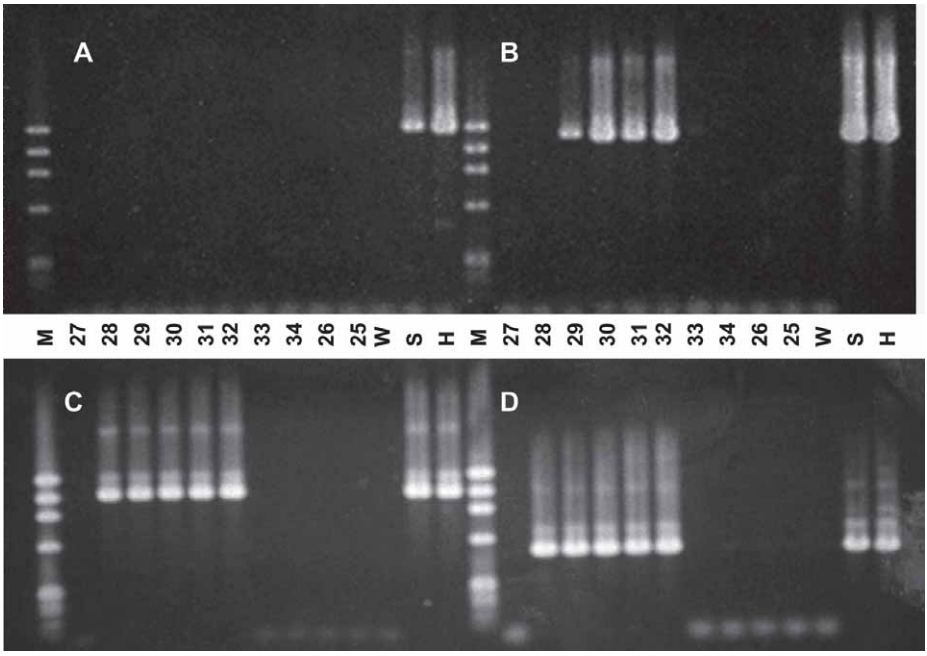

Fig. 2. Agarose gel electrophoreses of phytoplasma 16S rDNA PCR amplification products from Lombardy poplar samples and positive controls. Detected fragments were obtained with primer pairs: R16F1/R0 (A), R16F2n/R2 (B), R16(I)F1/R1 (C) and $16R_{738f}/16R_{1232r}$ (D). *W* – negative (water) control; *H* – reference strain of ribosomal subgroup 16SrI-B; *S* – reference strain of ribosomal subgroup 16SrXII-A; *26–34* – samples, numbered as given in Table 1; *M* – marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281 and 271.

asymptomatic samples, three of them tested negative for the presence of phytoplasma DNA, while one of them, despite the absence of infection symptoms, tested positive. In the case of 12 positivly-tested samples, another direct PCR was performed using the primer pair BB88F1/R1 (GUNDERSEN et al. 1996) specific for phytoplasma ribosomal 16SrI group. All PCR reactions provided visible bands in the agarose gel electrophoreses (not shown).

RFLP analyses

Amplified phytoplasma fragments obtained from Lombardy poplar tree samples, together with the amplified fragments of reference strains HYDB, STOL and P, were subjected to the RFLP analysis with *Tru*9I (Fig. 3) and *Tsp*9I (not shown).

All restriction profiles of phytoplasma 16S rDNA amplified from 12 poplar tree extracts corresponded to the restriction profile of the ribosomal subgroup 16SrI-B reference strain HYDB (Fig. 3), as well as the profiles of the phytoplasma previously isolated from poplars in Zagreb urban area (ŠERUGA et al. 2002).

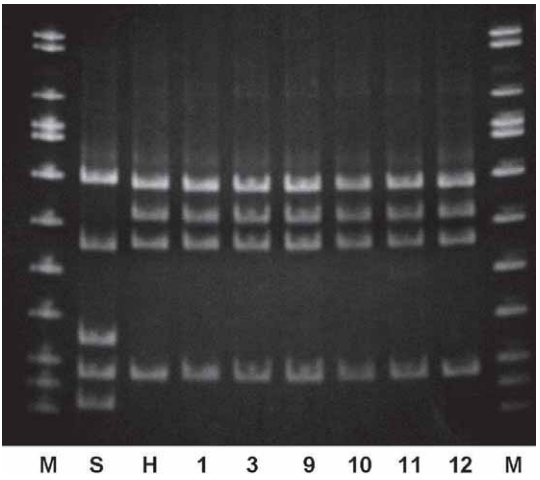

Fig. 3. Polyacrylamide gel showing the RFLP analysis of phytoplasma 16S rDNA fragments obtained with general phytoplasma primer pair R16F2n/R2. Amplified 16S rDNA was digested with *Tru*9I. *H* – reference strain of ribosomal subgroup 16SrI-B; *S* – reference strain of ribosomal subgroup 16SrXII-A; *1, 3, 9–12*- samples, numbered as given in Table 1; *M* – marker, pUCBM21 digested with *Hpa*II and with *Bra*I + *Hind*III. Fragment sizes in base pairs from top to bottom: 1114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110.

Restriction profiles obtained from 10 amplicons of phytoplasma gene for putative aa kinase were identical to the restriction profile of the ribosomal subgroup 16SrI-P (ŠERUGA et al. 2003), whilst profiles of two samples did not correspond entirely to either of the standard strains used. Molecular variability was observed in the analysis of both enzymes, *Tru*9I and *Tsp*EI (Fig. 4).

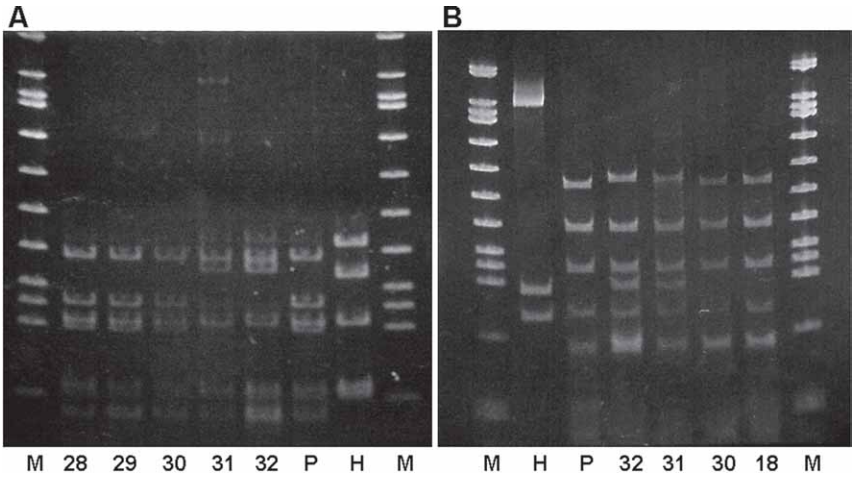

Fig. 4. Polyacrylamide gel showing the RFLP analysis of phytoplasma DNA fragments (putative phytoplasma gene for aa kinase) obtained with specific primer pair BB88F1/R1. Amplified fragments were digested with *Tru9I* (A) and *Tsp9I* (B). *H* – reference strain of ribosomal subgroup 16SrI-B; *P* – poplar phytoplasma isolate belonging to ribosomal subgroup 16SrI-P; *18, 28 – 32* – samples, numbered as given in Table 1; *M* – marker same as in Fig. 3.

Discussion

Populus nigra L. 'Italica' is a very common ornamental tree growing in parks, along roads and in other horticultural areas of the city of Zagreb. In 2002, there was a report on the presence of phytoplasma infection in Lombardy poplar tree population of Zagreb urban area, covering only a small area of the city (ŠERUGA et al. 2002). Comprehensive analyses also including phytoplasma conserved genes other than 16S rRNA enabled classification of the detected pathogen into a newly described ribosomal subgroup 16SrI-P and ribosomal protein subgroup rp-O (ŠERUGA et al. 2003). In the spring 2003, a bigger and more representative sampling was performed throughout the wider Zagreb urban area (Fig. 1).

Out of 30 collected symptomatic samples, 11 were positive for the presence of phytoplasma 16S rRNA gene. In almost two-thirds of the samples collected from trees exhibiting nonspecific phytoplasmosis symptoms, no phytoplasma DNA was detected (Tab. 1). There could be several reasons for such a result. Phytoplasmas could be present in a very low titer in those trees thus making it impossible to detect the infection, which is not unusual for woody plants (MARCONE et al. 1996a, b). Furthermore, disease development and the distribution of phytoplasmas in the woody hosts can vary in the sampled tree portion depending on numerous factors (tree size, age, general sanitary and metabolic status of the plant host). In addition, environmental conditions can influence all the parameters mentioned above (COUSIN et al. 1999). There is also a possibility that nonspecific symptoms could have been caused by other pathogens such as viruses, fungi or bacteria. Conversely, in one asymptomatic sample, the presence of phytoplasma was detected. This was probably the case of relatively recent, still latent infection, where symptoms had not appeared by the time of sampling (BERGES et al. 1997).

Phytoplasma-infected trees were equally distributed throughout the northern and north--western part of the city. In the southern part of the city, situated along the Sava River, phytoplasma infection was not detected in the scope of this research (Fig. 1). This distribution could indicate the transmission of the phytoplasmas from a primary focus in the north or north-west part of the city. Phytoplasmosis development is dependent on, among other environmental conditions, the irrigation level of the poplar growth area. Its incidence is rare in well-irrigated areas, which corresponds to our findings in southern part of the city, along the River Sava (COUSIN et al. 1999).

In the course of this research, specific vectors responsible for the disease transmission were not found. However, the detection of vectors was reported in areas around Paris where two leafhopper species, *Rhytidodus decimusquartus* and *Tremulicerus vitreus*, were identified as transmitters of phytoplasma infection in the *Populus nigra* L. 'Italica' population. Since the specificity of the different leafhopper species to different species of poplar is well known (COUSIN et al. 1999), *R. decimusquartus* and *T. vitreus* could also be potential vectors in the case of phytoplasmosis of Zagreb poplars.

RFLP analyses of 16S rDNA revealed that all detected phytoplasmas belonged to the ribosomal group 16SrI ('*Candidatus* Phytoplasma asteris'). Subjecting another phytoplasma conserved gene (putative gene for aa kinase) to RFLP analysis enabled further classification of phytoplasmas as members of 16SrI-P subgroup previously described only in Croatian poplars. Samples from two poplars showed unique restriction patterns. To elucidate whether those unique patterns represent a new subgroup or mixed profile of ribosomal protein gene regions, analysis of other conserved phytoplasma genes (*tuf* gene, ribosomal protein genes etc.) followed by sequencing is necessary.

The data on ornamental tree phytoplasma infection is not abundant, especially in comparison with crops and other plants important for human nutrition or even with herbaceous ornamentals that have bigger economic importance (LEE et al. 2000). In addition to *Populus nigra* L. 'Italica', several other *Populus* species and landscape trees were found to be infected with phytoplasmas: *P. alba* L. (Cousin 1996), *P. tremula* L. (SEEMULLER and LEDERER 1988), *Fraxinus velutina* (BRICKER and STUTZ 2004), *Ulmus minor* (MARCONE et al. 1997), *Eucalyptus spp.* MARCONE et al. 1996a), *Magnolia spp.* (KAMINSKA et al. 2001). Some of these trees are also common in Zagreb. It would be interesting to investigate the incidence of phytoplasmoses in these trees, as well as their importance, if any, in the Lombardy poplar-phytoplasma-vector pathosystems.

No effective and standard treatment of phytoplasma infected trees exists. There were several attempts at antibiotic applications in order to free infected plants from phytoplasmas. Diseased *Magnolia liliiflora* plants, infected by aster yellow phytoplasma, were treated with three different antibiotics. The repeated treatments of plants resulted in temporary remission of symptoms, but did not cure the disease (KAMINSKA and SLIWA 2003). Tetracycline treatment of *Euphorbia pulcherrima* was partially successful, since only one plant exposed to the highest concentration of the antibiotic escaped phytoplasma infection (BRADEL et al. 2000). Applying antibiotics to poplar trees *in vivo* is not manageable at present, due to the lack of standardized procedure and high costs of the treatment. Other methods for controlling the infection could be detection and eradication of vectors together with the removing of infected trees. These measures would be effective only if more details on the Lombardy poplar phytoplasmosis molecular epidemiology are revealed. Hence, subse-

quent research into phytoplasmoses in the Lombardy poplar tree population of the Zagreb urban area should focus on identifying the potential insect vector and detecting phytoplasma diversity in the tree population. Identifying the preferential routes of disease transmission and ecological factors that could affect pathogenesis, thus having impact on the sanitary status of these trees, should also be considered.

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References

AROCHA, Y., LOPEZ, M., PINOL, B., FERNANDEZ, M., PICORNELL, B., ALMEIDA, R., PALENZUELA, I., WILSON, M. R., JONES, P., 2005: »*Candidatus* Phytoplasma graminis«, and »*Candidatus* Phytoplasma caricae«, two novel phytoplasmas associated with diseases of sugarcane, weeds and papaya in Cuba. Int. J. Syst. Evol. Microbiol. 55, 2451–2463.

BAI, X., ZHANG, J., EWING, A., MILLER, S. A., JANCSO, RADEK, A., SHEVCHENKO, D. V., TSUKERMAN, K., WALUNAS, T., LAPIDUS, A., CAMPBELL, J. W., HOGENHOUT, S. A., 2006: Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. J. Bacteriol. 188, 3682–3696.

BERGES, R., COUSIN, M. T., ROUX, J., MAURER, R., SEEMÜLLER, E., 1997: Detection of phytoplasma infections in declining *Populus nigra* L. 'Italica' trees and molecular differentiation of the aster yellows phytoplasmas identified in various *Populus* species. Eur. J. For Pathol. 27, 33–43.

BRADEL, B. G., PREIL, W., JESKE, H., 2000: Remission of the free-branching pattern of *Euphorbia pulcherrima* by tetracycline treatment. J. Phytopathol. 148, 587–590.

BRICKER, J. S., STUTZ, J. C., 2004: Phytoplasma associated with ash decline. J. Arboric. 30, 193–199.

CHRISTENSEN, N. M., AXELSEN, K. B., NICOLAISEN, M., SCHULZ, A., 2005: Phytoplasmas and their interactions with hosts. Trends Plant Sci. 10/11, 526–535.

COUSIN, M. T., 1996: Witches' broom: a phytoplasma disease of poplar. In: MARAMOROSCH, K., RAYCHAUDHURI, S. P. (eds), Forest trees and palm diseases and control, 245–262. Oxford and IBH Publishing Company, New Delhi.

COUSIN, M. T., BERGES, R., ROUX, J., MOREAU, J. P., HIRUKI, C., SEEMULLER, E., 1999: *Populus nigra* L. 'Italica' decline in France. Variability of the phytoplasma responsible for the disease in Europe. Results and perspective. Acta Hort. 496, 77–86.

DAVIS, R. E., DALLY, E. L., BERTACCINI, A., CREDI, R., OSLER, R., SAVINO, V., CARRARO, L., DI TERLIZZI, B., LEE, I. M., BARBA, M., 1993: Restriction fragment length polymorphism analyses and dot blot hybridization distinguish mycoplasmalike organisms associated with Flavescence dorée and Southern European grapevine yellows disease in Italy. Phytopathology 83, 772–776.

FIRRAO, G., GIBB, K., STRETEN, C., 2005: Short taxonomic guide to the genus '*Candidatus* phytoplasma'. J. Plant Pathol. 87, 249–263.

GIBB, K. S., PADOVAN, A. C., MOGEN, B. A., 1995: Studies on sweet potato little-leaf phytoplasmas detected in sweet potato and other plant species growing in Northern Australia. Phytopathology 85, 169–174.

GUNDERSEN, D. E., LEE, I. M., 1996: Ultrasensitive detection of phytoplasmas by nested--PCR assays using two universal primer pairs. Phytopath. Medit. 35, 144–151.

IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group 2004: '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. Int. J. Syst. Evol. Microbiol. 54, 1243–1255.

KAMINSKA, M., SLIWA, H., RUDZINSKA-LANGWALD, A., 2001: The association of phytoplasma with stunting, leaf necrosis and Witches' Broom symptoms in *Magnolia* plants. J. Phytopathol. 149, 719–724.

KAMINSKA, M., SLIWA, H., 2003: Effect of antibiotics on the symptoms of stunting disease of *Magnolia liliiflora* plants. Phytopathology 151, 59–63.

LEE, I. M., DAVIS, R. E., SINCLAIR, W. A., DEWITT, N. D., CONTI, M., 1993: Genetic relatedness of mycoplasmalike organisms detected in *Ulmus* spp. in USA and Italy by means of DNA probes and polymerase chain reactions. Phytopathology 83, 829–833.

LEE, I. M., GUNDERSEN, D. E., HAMMOND, R. W., DAVIS, R. E., 1994: Use of mycoplasmalike organism (MLO) group-specific oligonucleotid primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology 84, 559–566.

LEE, I. M., BERTACCINI, A., VIBIO, M., GUNDERSEN, D. E., 1995: Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology 85, 728–735.

LEE, I. M., DAVIS, R. E., GUNDERSEN-RINDAL, D. E., 2000: Phytoplasma: Phytopathogenic mollicutes. Annu. Rev. Microbiol**.** 54, 221–255.

LEE, I. M., MARTINI, M., BOTTNER, K. D., DANE, R. A., BLACK, M. C., TROXCLAIR, N., 2003: Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas**.** Phytopathology 93, 1368–1377.

LEE, I. M., BOTTNER, K. D., SECOR, G., RIVERA-VARAS, V., 2006: '*Candidatus* Phytoplasma americanum' a phytoplasma associated with a potato purple top wilt disease complex. Int. J. Syst. Evol. Microbiol. 56, 1593–1597.

MARCONE, C., RAGOZZINO, A., SEEMULLER, E., 1996a: Detection of an elm yellows-related phytoplasma in eucalyptus trees affected by little-leaf disease in Italy. Plant Dis. 80, 669–673.

MARCONE, C., RAGOZZINO, A., SEEMMULLER, E., 1996b: Association of phytoplasmas with the decline of European hazel in southern Italy. Plant Pathol. 45, 857–863.

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. Forest Pathol. 27, 45–54.

OSHIMA, K., KAKIZAWA, S., NISHIGAWA, H., JUNG, H-Y., WEI, W., SUZUKI, S., ARASHIDA, R., NAKATA, D., MIYATA, S-I., UGAKI, M., NAMBA, S., 2004: Reductive evolution suggested from the complete genome sequence of a plant-pathogenic phytoplasma. Nat. Genet. 36, 27–29.

SAMBROOK, J., FRITSCH, E. F., MANIATIS, T., 1989: Molecular cloning: a laboratory manual. CSH Laboratory Press, Cold Spring Harbor.

SCHNEIDER, B., TORRES, E., MARTIN, M. P., SCHRODER, M., BEHNKE, H. D., SEEMÜLLER, E., 2005: '*Candidatus* Phytoplasma pini', a novel taxon from *Pinus silvestris* and *Pinus halepensis*. Int. J. Syst. Evol. Microbiol. 55, 303–307.

SEEMULLER, E., LEDERER, W., 1988: MLO-associated decline of *Alnus glutinosa, Populus tremula* and *Crataegus monogyna*. J. Phytopathol. 121, 33–39.

SEEMULLER, E., GARNIER, M., SCHNEIDER B., 2002: Mycoplasmas of plant and insects. In: RAZIN, S., HERRMANN R. (eds), Molecular biology and pathogenicity of mycoplasmas, 91–116, Kluwer Academic / Plenum Publisher, New York.

SHARMA, A. K., COUSIN, M. T., 1986: Mycoplasmalike organisms (MLOs) associated with the witches broom disease of poplar. J. Phytopathol. 117, 349–356.

ŠERUGA, M., ŠKORIĆ, D., BOTTI, S., PALTRINIERI, S., JURETIĆ, N., BERTACCINI, A., 2002: Molecular identification of a phytoplasma naturally infecting *Populus nigra* L. 'Italica' trees in Croatia. Plant Protect. Sci. 38, 28–30.

ŠERUGA, M., ŠKORIĆ, D., BOTTI, S., PALTRINIERI, S., JURETIĆ, N., BERTACCINI, A. F., 2003: Molecular characterization of a phytoplasma from the aster yellows (16SrI) group naturally infecting *Populus nigra* L. 'Italica' trees in Croatia. Forest Pathol. 33, 113–125.

VALIUNAS, D., STANIULIS, J., DAVIS, R. E., 2006: '*Candidatus* Phytoplasma fragariae', a novel phytoplasma taxon discovered in yellows diseased strawberry, *Fragaria* x *ananassa*. Int. J. Syst. Evol. Microbiol. 56, 277–281.