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MOLECULAR CHARACTERIZATION OF PHYTOPLASMAS DETECTED IN AGRONOMICALLY RELEVANT CROPS IN SERBIA

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INDEX

SUMMARY

Detection and molecular characterization of phytoplasmas infecting different plant species, selected among those most agronomically relevant in Serbia were carried out. Correlation between molecular polymorphisms of relevant phytoplasma strains and their geographical distribution and, when possible, comparison with homologous genes from phytoplasma strains detected in the same crops in other geographical areas worldwide was also achieved. Molecular diversity was studied on genes coding ribosomal proteins S3, tuf gene, helicases, aminoacid kinases, amp and imp besides phytoplasma 16S ribosomal gene used for classification.

Plant species studied were carrot in Serbia infected with aster yellows phytoplasmas, corn in Serbia and in Colombia respectively infected with stolbur and aster yellows phytoplasmas, grapevine in Serbia and in Italy infected by Flavescence dorée and Bois noir, apple in Serbia, Italy and Hungary infected with apple proliferation.

Selected samples infected by phytoplasmas belonging to diverse ribosomal groups and associated with different plant disease and were further studied coupling with PCR/RFLP technology the cloning and sequencing followed by phylogenetic analyses. The allows detecting genetic variability not always related do geographical strain distribution but possibly related to ability of phytoplasmas to rapidly evolve and modify genome in short time and therefore to be able to induce severe epidemic within cyclic periods.

INTRODUCTION

Numerous yellows-type diseases of plants were believed to be caused by viruses considering their infective spreading, symptomatology, and transmission by insects (Kunkel, 1926; 1931; 1955: McCoy *et al*., 1989; Maramorosch, 2008). The first demonstration that the etiological agents of these diseases could be wall-less prokaryotes rather than viruses caught the field of plant pathology by surprise (Doi *et al.*, 1967). This discovery of a new group of plant pathogens related to bacteria led to find of pleomorphic, wall-less prokaryotes in the phloem of many plant species affected by yellows-type diseases.

The term mycoplasma-like organisms (MLOs) was first used to name these micro-organisms due to their morphological and ultrastructural similarity to mycoplasmas. Both pathogenic groups (MLOs and mycoplasmas) are prokaryotes belonging to the *Mollicutes* class (cell wall-less prokaryotes). However, in contrast to mycoplasmas, which cause an array of disorders in animals and humans, the phytopathogenic MLOs resisted all attempts to culture them *in vitro* in cell free media (Lee and Davis, 1986). Following the application of molecular technologies the enigmatic status of MLOs amongst the prokaryotes was resolved and led to the new trivial name of "phytoplasma", and eventually to the designation of a new taxon named '*Candidatus* phytoplasma' (IRPCM, 2004).

Plants infected by phytoplasma exhibit an array of symptoms that suggests profound disturbances in the normal balance of growth regulators. Symptoms include virescence/phyllody (development of green leaf like structures instead of flowers), sterility of flowers, proliferation of axillary (side) buds resulting in a witches' broom behaviour, abnormal internodes elongation, generalized stunting (Bertaccini, 2007). These micro-organisms are transmitted in a persistent manner by insects belonging to the families Cicadellidae, Cixidae, Psyllidae, Delphacidae and Derbidae (Weintraub and Beanland, 2006).

Recent molecular data on phytoplasmas have provided considerable insights into their diversity and genetic interrelationships that are the basis for several comprehensive studies on phytoplasma phylogeny and taxonomy (Hogenhout *et al*., 2008). Some investigations, particularly sequence analysis of 16S rDNA, have shown that phytoplasmas constitute a coherent, genus-level taxon. In the monophyletic phytoplasma clade, groups and subgroups were delineated, many of which are now considered as species under the provisional status '*Candidatus*' for incompletely described prokaryotes, according to Murray and Stackebrandt

(1995). Several provisional species have been described to date and rules for future putative species delineation have been defined (IRPCM, 2004).

The first comprehensive phytoplasma classification scheme was based on restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S rRNA (Lee *et al.*, 1998a, 2000), providing a reliable means for the differentiation of a broad array of phytoplasmas. This system has classified phytoplasmas in 19 groups and more than 40 subgroups and has become the most comprehensive and widely accepted phytoplasma classification system (Lee *et al.*, 2004a; b; 2006b; Arocha *et al.*, 2005; Al-Saady *et al.*, 2008).

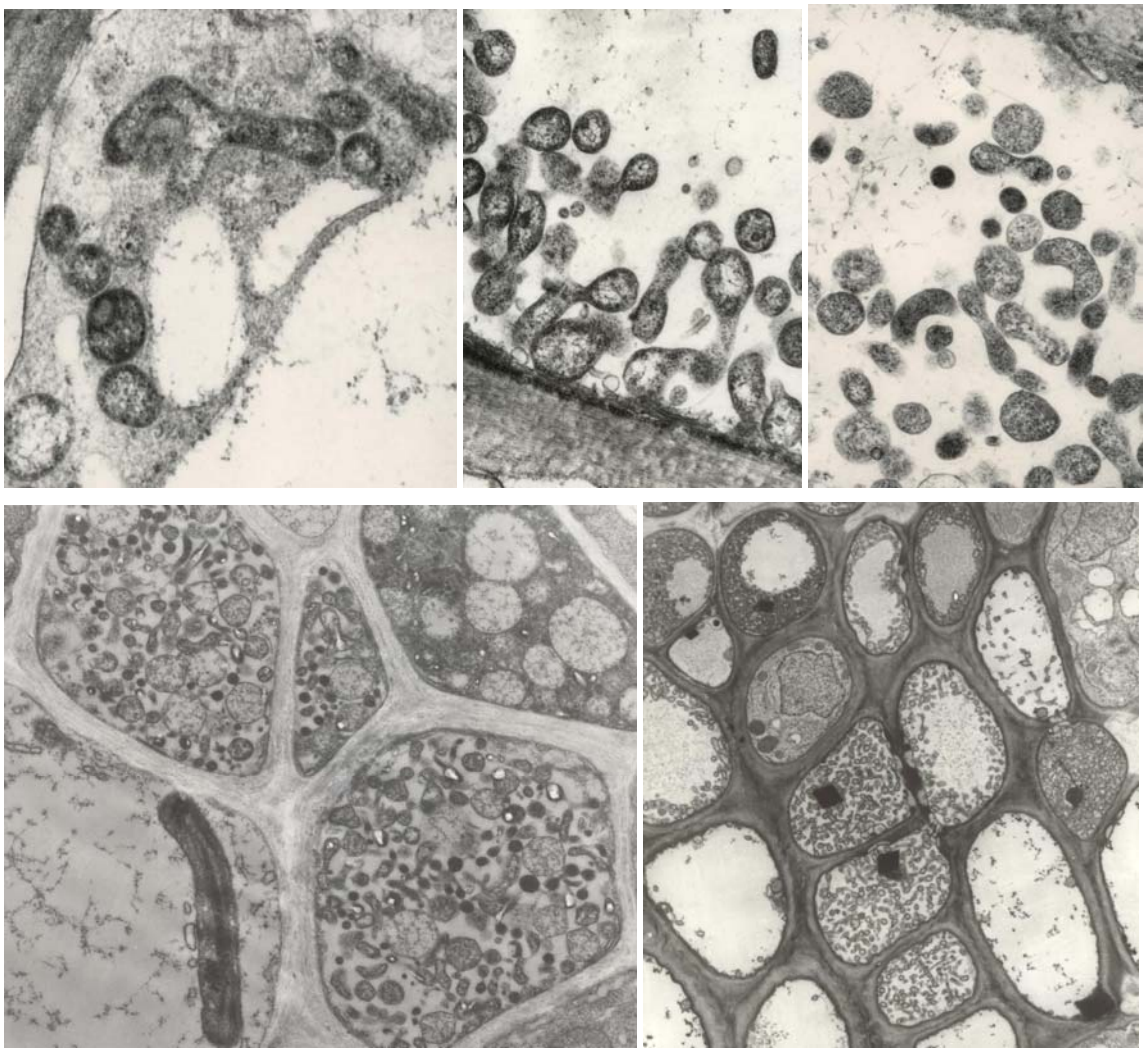
Sensitive and accurate detection of these micro-organisms is a prerequisite for the management of phytoplasma-associated diseases. Following their discovery, phytoplasmas have been difficult to detect due to their low concentration especially in woody hosts and their erratic distribution in the sieve tubes of the infected plants (Berges *et al.*, 2000). The establishment of electron microscopy (EM) based techniques represents an alternative approach to the traditional indexing procedure for phytoplasmas based on graft transmission of the pathogen to healthy indicator plants. EM observation (Bertaccini and Marani, 1982; Cousin *et al.*, 1986) and less frequently scanning EM (Haggis and Sinha, 1978) were the only diagnostic techniques until staining with DNA-specific dyes such as DAPI was developed (Seemüller, 1976). Lately, protocols for the production of enriched phytoplasma-specific antigens have been developed, thus introducing serological-based detection techniques for the study of these pathogens in plants or insect vectors (Hobbs *et al.*, 1987). Phytoplasma detection is now routinely done by different nucleic acid techniques based on polymerase chain reaction (PCR) (Schaff *et al*, 1992; Baric and Dalla-Via; 2004; Green *et al.*, 1999; Zhang *et al.*, 1998). The procedures developed in the last 20 years are now used routinely and are adequate for detecting phytoplasma infection in plant propagation material and identifying insect vectors, thus helping in preventing the spread of the diseases and their economical impact.

Diseases associated with phytoplasma presence occur worldwide in many crops, although individual phytoplasmas may be limited in their host range or distribution. There are more than 300 distinct plant diseases attributed to phytoplasmas, affecting hundreds of plant genera (Hoshi *et al.*, 2007). Many of the economically important diseases are those of woody plants, including coconut lethal yellowing, peach X-disease, grapevine yellows, and apple proliferation.

What are Phytoplasmas?

Phytoplasma, formerly known as 'Mycoplasma-like organisms' or MLOs, are specialized bacteria that are obligate parasites of plant phloem tissue, and of several insect species. Phytoplasmas have diverged from gram-positive bacteria, and belong to the '*Candidatus* Phytoplasma' genus within the Class *Mollicutes* (IRPCM, 2004). They can't be cultured *in vitro* in cell-free media. They are pleomorphic (Fig. 1) since they lack of a cell wall, with a diameter less than 1 micrometer, and very small genomes (680-1,600 kb).

Fig. 1. Electron micrographs of sieve tubes cross sections showing the polymorphism in shape and in dimensions of phytoplasmas infecting plants.



The genomes of phytoplasmas became greatly reduced in size when compared with those of their ancestors (walled bacteria in the *Bacillus*/*Clostridium* group). Phytoplasmas thus lack several biosynthetic pathways for the synthesis of compounds necessary for their survival,

and they must obtain those substances from plants and insects in which they are parasites (Bai *et al.*, 2006).

Phytoplasma diseases and their economical importance

Phytoplasmas are associated with plant diseases in several hundred plant species including many important food, vegetable, and fruit crops; ornamental plants; and timber and shade trees (Ahrens *et al.*, 1993; Andersen *et al.*, 1998; Davis *et al.*, 1998; Errampalli *et al.*, 1991; Zreik *et al.*, 1995). The list of diseases caused by phytoplasmas continues to grow. Many newly emerging diseases have been identified in the last years or know diseases were associated with different phytoplasmas according with diverse geographic distribution such as for the citrus Huanglongbing disease that was associated with aster yellows-related phytoplasmas in China (16SrI) (Chen *et al*., 2008) and with pigeon pea witches' broom-related phytoplasmas (16SrIX) in Brazil (Teixeira *et al*., 2009) that are only the last on the list confirming the widespread occurrence of similar symptoms associated with diverse phytoplasmas obliging to a molecular identification in spite identical symptoms in order to be able to efficiently reduce the disease impact on the different ecosystems.

Many of the cultivated plants are affected by phytoplasma infection not only in countries where agriculture is still not advanced, but also in the so called more advanced countries where these pathogen are severely affecting both herbaceous and woody plants (Bertaccini, 2007). Phytoplasmal infections are the primary limiting factors for production of many important crops all over the world. For example, the aster yellows phytoplasma contributes to the major economic loss of many vegetable crops (e.g. lettuce, carrot, and celery) and ornamental plants (e.g. gladiolus, hydrangea, China aster, and purple coneflower) in North America and parts of Europe. Peach yellows and the X-disease contribute during the 90ties to the loss of peach and cherry respectively in the United States. In several regions of Middle East citrus species are affected by phytoplasma diseases such as lime witches' broom, that is almost eliminating traditional lime production in the Sultanate of Oman and in Iran.

Rice yellow dwarf severely affects rice crops in several regions of Southeast Asia; potato witches' broom and maize bushy stunt contribute to the losses of yield of potato and corn, respectively in Central and South America. Sweet potato witches' broom and related diseases are responsible for the loss of sweet potato crops in Asia and Australia; cassava witches' broom to the loss of cassava crops in South America; grapevine yellows severely affects grapevine production in Europe and Australia. Pear decline, apple proliferation, European stone fruit yellows and other fruit declines reduce both production and quality of fresh fruits

in Europe; legume diseases such as peanut witches' broom, sesame and soybean phyllody cause the loss of these crops in Asia.

On the other hand also forests are severely damaged by phytoplasma associated diseases such as paulownia witches' broom, coconut lethal yellowing, and mulberry dwarf that reduce the presence of these woody species in different continents. Elm yellows or witches' broom is a disease that almost eliminated historical as well as new elm plantation in Europe and in North America; in particular plant surviving the severe epidemic of Dutch elm disease were killed by successive phytoplasma infections (Sinclair *et al.*, 1996; Bertaccini, 2007). Because of these diseases, the movement of many of the affected plant species should be internationally restricted by quarantine regulations (Lee *et al.*, 2000; Bertaccini, 2007).

Symptoms of phytoplasma diseases

A common symptom caused by phytoplasma infection is phyllody, the production of leaf like structures in place of flowers. Evidence suggests that the phytoplasma deregulates a gene involved in flower formation (Pracros *et al.*, 2006). Other symptoms, such as the yellowing of leaves, are thought to be caused by the phytoplasma presence in the phloem affecting its function, and changing the transport of carbohydrates. Photosynthesis, especially photosystem II, is also inhibited in many phytoplasma infected plants. These infected plants often show yellowing which is caused by the breakdown of chlorophyll and carotenoids, whose biosynthesis is also inhibited (Bertamini and Nedunchezhian, 2001). Induced expression of sucrose synthase and alcohol dehydrogenase I genes in phytoplasma-infected grapevine plants grown in the field was also recently demonstrated (Hren *et al*., 2009) Phytoplasma infected plants may also suffer from virescence - the development of green flowers due to the loss of pigment in the petal cells (Lee *et al.*, 2000). Sometimes sterility of the flowers is also described. Many phytoplasma infected plants gain a bushy or witches' broom appearance due to changes in normal growth patterns caused by the infection. Most plants show apical dominance but phytoplasma infection can cause the proliferation of axillary (side) shoots (Lee *et al.*, 2000) and a decrease in size of the internodes (Fig. 2).

Fig. 2. Symptoms of phytoplasmas in different plant species: (upper row) left, Forsithia in Cambridge botanical garden infected with 16SrIII phytoplasmas (Duduk *et al*., unpublished); right, periwinkle showing flower virescence from Colombia infected with 16SrIX-C phytoplasmas (Duduk *et al.*, 2008a). Low row: left, *Sophora japonica* in China infected by '*Ca*. P. japonicum' and stolbur phytoplasmas (Duduk *et al*., 2009b); right, lime witches' broom ('*Ca*. P. aurantifolia') on lime tree in Oman.

Phytoplasmas may cause many other aspecific symptoms that are induced just because of the stress in the plant. Symptoms of phytoplasma infections are in some case useful in the commercial production. Phytoplasma infection produces more axillary shoots that enable to production of poinsettia plants that have more than one flower and reduced size that allow its growth in pots (Bertaccini *et al*., 1996; Lee *et al.*, 1997a).

Phytoplasma transmission and some aspects of their epidemiology

Phytoplasmas are mainly spread by insects of the families Cicadellidae (leafhoppers), Fulgoridae (planthoppers), and Psyllidae, which feed on the phloem tissues of infected plants acquiring the phytoplasmas and transmitting them to the next plant they feed on. For this reason the host range of phytoplasmas is strongly dependent upon its insect vector. Phytoplasmas contain a major antigenic protein that makes up the majority of their cell surface proteins and this has been shown to interact with microfilament complexes of intestinal muscles of insect and is believed to be important for transmission and infection (Suzuki *et al.*, 2006; Hoshi *et al.*, 2007). Phytoplasmas may overwinter in insect vectors or in

perennial plants: they can have various affects on their insect hosts; examples of both reduced and increased fitness were described (Christensen *et al.*, 2005).

Phytoplasmas are found in most of the major organs of an infected insect host once they are established. They enter the insect's body through the stylet and then move through the intestine and been absorbed into the haemolymph. From here they proceeded to colonize the salivary glands, a process that can take up to some weeks. The time between phytoplasmas being taken up by the insect and the phytoplasmas reaching an infectious titer in the salivary gland (possibility to be transmitted) is called the latency period. Some phytoplasma transmissions were reported in insects as transovarially such as for the combinations *Scaphoideus titanus*/aster yellows (Danielli *et al*., 1996; Alma *et al.*, 1997); *Hishimonoides sellatiformis*/mulberry dwarf (Kawakita *et al*., 2000), *Matsumuratettix hiroglyphicus* (Matsumura)/sugarcane white leaf (Hanboonsong *et al.*, 2002), and *Cacopsylla melanoneura* (Tedeschi *et al.*, 2006).

Phytoplasmas and some plant-infecting viruses may be transmitted from infected to healthy plant through dodder (*Cuscuta* sp.). Experimental transmission of a phytoplasma from infected to healthy plant of the same or different species through dodder can be accomplished in the laboratory or greenhouse.

Recently the possibility of phytoplasma transmission by seed was also under investigation. After first suspect related to the epidemiological spreading to coconut lethal yellowing (Cordova *et al.*, 2003) other studies on Oman alfalfa (*Medicago sativa* L.) cultivations severely affected by phytoplasmas verify the possibility of phytoplasma transmission by seed. Lime (*Citrus aurantiaca*) and tomato (*Licopersicum esculentum*) seeds respectively from Oman, and Italy allowed to germinate under sterile conditions were tested at several growth stages and provided positive results from some of the tested samples indicating the presence of phytoplasmas belonging to ribosomal groups 16SrI, 16SrXII and 16SrII (Khan *et al*., 2002a; Botti and Bertaccini, 2006a).

Phytoplasmas can also be spread via vegetative propagation such as the grafting of a piece of infected plant onto a healthy plant, cutting, micropropagation or other ways to propagate plant germplasm avoiding sexual reproduction are also propagating the phytoplasmas.

Phytoplasmas are able to move within plants through the phloem from source to sink and they are able to pass through sieve tube elements (Christensen *et al.*, 2004). Several studies showed uneven phytoplasma distribution in the host plant (Seemüller *et al.*, 1984), and a seasonal fluctuation of the pathogen population in woody hosts. Generally, levels were low in roots (sink organ) and moderate in stems. The highest titer is found in source organs (mature leaves) (sometimes ≈40 times higher titer than in roots). In sink leaves, phytoplasma concentration is low or below detection. Highest concentration of phytoplasmas in source regions indicates that phytoplasmas multiply fastest here. Variation in the amount of

phytoplasma DNA between individual plants propagated from one infected parent plant has been also reported (Christensen *et al.*, 2004).

For deciduous woody plants it has been proposed that phytoplasmas disappear from aerial parts of trees during the winter and survive in the root system to re-colonize the stem and branches in spring (Seemüller *et al.*, 1984, Guthrie *et al.*, 1998). Waterworth and Mock (1999) detect phytoplasmas in dormant fruit tree scion-wood collected during the winter season. The presence of phytoplasmas in aerial parts of pear cultivars and *Prunus* species in the winter has been reported by Errea *et al.* (2002) and Jarausch *et al.* (1999b), respectively.

Phytoplasma detection and identification

Before development of molecular techniques the detection of phytoplasma diseases was difficult due to the fact that they could not be cultured. Thus diagnostic techniques such as observation of symptoms, insect or dodder/graft transmission to host plant, together with electron microscopy observation of ultra-thin sections of the phloem tissue were employed.

Serological diagnostic techniques for the detection of phytoplasma began to emerge in the 1980's with ELISA based methods. In the early 1990's PCR coupled with RFLP analysis allowed the accurate identification of different strains and species of phytoplasma (Namba *et al*., 1993; Lee *et al*., 1993a; Schneider *et al*., 1993).

The disappearance of symptoms in some cases after antibiotic (i.e. tetracycline) treatment provided additional evidence to support the diagnosis of prokaryotic micro-organisms as agents of several plant diseases (Doi *et al.*, 1967; Lee and Davis, 1992). Phytoplasma strains were differentiated and identified by their biological properties, such as the similarity in symptoms induced in infected plants, plant hosts and insect vector ranges (Chiykowski, 1991; Errampalli *et al.*, 1991; Lee and Davis, 1992). Determination of biological properties was laborious and time-consuming, and often the results were inconsistent. In many cases, identities of insect vectors remained unknown, further complicating identification based on biological criteria. The presence of phytoplasmas in sieve cells was detected by electron microscopy of ultra-thin sections of infected tissues or indirectly by light microscopy of stained-sieve cells by Diene's stain or DAPI stain (Lee and Davis, 1992).

Polyclonal and monoclonal antisera have been tested for the detection phytoplasmas (Lee *et al.*, 1993b; Chen *et al*., 1993; 1994), some are still commercially available for economically important phytoplasma-associated diseases such as "Flavescence dorée" and apple proliferation. Serological tools have also been used with success to detect different phytoplasmas in leafhopper vectors or potential vectors, by immunofluorescence (Lherminier *et al.*, 1990), immunosorbent electron microscopy (Sinha, 1979; Sinha and Benhamou, 1983),

dot blot or ELISA (Boudon-Padieu *et al.*, 1989). In other approaches, tissue blotting with direct or indirect antigen detection has been used for specific phytoplasma detection (Lin and Chen, 1985; 1996). In more recent years, antibodies have been prepared to partial sequences of the major immunodominant proteins of some phytoplasmas (Berg *et al.*, 1999; Blomquist *et al.*, 2001; Hong *et al.*, 2001; Mergenthaler *et al.*, 2001) expressed as fusion proteins in *Escherichia coli* by cloning immunodominant membrane protein genes of phytoplasmas and obtaining their *in planta* expression (Kakizawa *et al*., 2009).

Molecular detection

In the 1990's, following the first cloning of phytoplasma DNA (Kirkpatrick *et al*., 1987), nucleic acid-based probes (randomly cloned DNA or its complementary RNA) were widely applied in different assays to detect and differentiate phytoplasmas in plants and vectors (Lee and Davis, 1988; Bertaccini *et al.*, 1990a; Bonnet *et al.*, 1990; Harrison *et al.*, 1992). In the same years probes based on cloned phytoplasma-specific chromosomal and extrachromosomal DNAs provides the first evidences of genetic differences in the phytoplasma DNA among strains derived from different plant hosts or from different geographical locations (Lee *et al.*, 1992; Bertaccini *et al*., 1990b; 1993). Moreover genomic sequence-specific oligonucleotides were developed for diagnostic purposes (Schaff *et al*., 1992; Firrao *et al.*, 1993). PCR assays using primers based on cloned DNA fragments (non-ribosomal DNAs), specific to a given phytoplasma, provide sensitive as well as specific means for phytoplasma detection. In contrast, PCR assays using generic or broad-spectrum primers designed based on conserved sequences (e.g. 16S rRNA, ribosomal protein, *tuf*, 16S-23S spacer) allow detection of a wide array of phytoplasmas associated with plants and insects (Bertaccini *et al.*, 1992a; Gundersen and Lee, 1996; Gundersen *et al.*, 1996; Schneider *et al.*, 1997). PCR assays using universal primers are useful for preliminary identification of phytoplasma diseases. Several universal and many phytoplasma group specific primers have been designed for routine detection of phytoplasmas on 16S ribosomal gene (Deng and Hiruki, 1991; Ahrens and Seemüller, 1992; Namba *et al.*, 1993; Davis and Lee, 1993; Lee *et al.*, 1993a, 1998a; b; Lorenz *et al.*, 1995; Schneider *et al.*, 1997).

The success of the PCR approach for phytoplasma detection in field collected samples is largely dependent on obtaining total nucleic acid preparations of good quality and enriched in phytoplasma DNA, but this has always been a hard task (Firrao *et al.*, 2007). The amount of phytoplasma DNA is lower than 1% of total DNA extracted from tissue (Bertaccini, 2007). Different protocols for total DNA extraction have been reported for the detection of these plant pathogens. The main goal of each protocol was to concentrate phytoplasma DNA while reducing enzyme-inhibitory plant polyphenolic and polysaccharide molecules. This is generally attained by including a phytoplasma enrichment step. However nested-PCR assay,

designed to increase both sensitivity and specificity, is indispensable for the amplification of phytoplasmas from samples in which unusually low titers, or inhibitors are present that may interfere the PCR efficacy (Gundersen *et al.*, 1994). Nested-PCR is performed by preliminary amplification using a universal primers pair followed by second amplification using a second universal primer pair. By using a universal primer pair followed by PCR using a group-specific primer pair, nested-PCR is capable of detection of dual or multiple phytoplasmas present in the infected tissues in case of mixed infection (Lee *et al.*, 1994).

Immuno-capture PCR assay, in which the phytoplasma of interest is first selectively captured by specific antibody adsorbed on microtiter plates, and then the phytoplasma DNA is released and amplified using specific or universal primers, can be an alternative method to increase detection sensitivity (Rajan and Clark, 1995; Heinrich *et al.*, 2001). This method is aimed at avoiding the lengthy extraction procedures to prepare target DNAs.

The design of primers based on various conserved sequences such as 16S rRNA gene, ribosomal protein gene operon, *tuf* and *SecY* genes was the major breakthrough in detection, identification, and classification of phytoplasmas (Gundersen *et al.*, 1996; Schneider *et al.*, 1997; Marcone *et al*., 2000; Wei *et al*., 2004a; Martini *et al*., 2002; 2007). Differentiation of putative phytoplasmas now is routinely carried out on 16S rRNA gene that must be accomplished through Restriction Fragment Length Polymorphism (RFLP) analysis of PCR amplified DNA sequences using a number of endonuclease restriction enzymes (Lee *et al.*, 1998a; b). Because the RFLP patterns characteristics of each phytoplasmas are conserved, unknown phytoplasmas can be identified by comparing the patterns of the unknown with the available RFLP patterns for known phytoplasmas without co-analyses of all reference representative phytoplasmas (Lee, 1998a; b; Wei *et al*., 2007; 2008a; Cai *et al*., 2008).

The continuous effort to improve of the diagnostic procedures aims quicker, more economic and robust methods. Sensitivity is not an issue *per se*, as the current nested PCR protocols are extremely sensitive, but the achievement of high levels of sensitivity without the risk of false positive results that can be associated with nested PCR is highly desirable. The recent introduction of diagnostic assays based on real time PCR fulfils these requirements: due to the high sensitivity and direct reading of the results which reduce the risk of amplicon contamination and the need for a gel-based post PCR analysis, real time PCR is candidate for replacing standard PCR in routine testing (Baric and Dalla-Via, 2004; Firrao *et al.*, 2007).

Phytoplasma classification

To date, no phytoplasma culture has been established in a cell-free medium; thus, differentiation and classification of phytoplasmas by means of the biophysical and

biochemical-based classic phenotypic criteria that are routinely used for cultivable micro-organisms is not possible. Reference phytoplasma strain collection maintained in periwinkle is however available for research and classification purposes (Bertaccini *et al*., 1992b).

In the 1980's and early 1990's, the employment of serological (Lee *et al.*, 1993b) and nucleic acid-based (Lee and Davis, 1988; Lee *et al.*, 1992) assay techniques revealed new insights into the diversity and genetic interrelationships of phytoplasmas. These studies showed that several phytoplasma groups could be clearly distinguishable for their genomic DNA sequences. In order to achieve a general and reliable system of phytoplasma detection and identification molecular tools such as PCR/RFLP and nested-PCR on the conserved (16SrDNA) ribosomal phytoplasma region were developed and applied for general classification (Lee *et al.*, 1998a; Seemüller *et al.*, 1998a).

RFLP analyses of 16S rDNA nested PCR products from 34 representative phytoplasma strains with 17 restriction enzymes was used by Lee *et al.* in 1998 to differentiate various phytoplasmas by their distinct RFLP patterns. Based on similarity coefficients derived from RFLP analyses, the 34 phytoplasma strains were differentiated into 14 major groups (termed 16Sr groups) and 32 sub-groups. By including additional groups and sub-groups from which RFLP analyses of available 16S rDNA sequence data, Lee *et al.* (1998a) proposed a total of 14 groups and 41 sub-groups (Table 1). The phytoplasma 16Sr groups has been shown to be consistent with the phylogenetic groups (clades) defined by phylogenetic analysis of near-full-length 16S rRNA gene sequences, indicating that the RFLP-based groups are phylogenetically valid. The approach using RFLP analyses of PCR amplified 16S rDNA provides a simple, reliable and rapid means for differentiation and identification of known phytoplasmas.

For finer differentiation of phytoplasmas, additional genetic markers such as ribosomal protein (rp) genes, *secY*, *tuf*, and the 16S-23S rRNA intergenic spacer region sequences have been used as supplementary tools (Lee *et al.*, 1994; 2004a; b; 2006b; Martini *et al.*, 2002; 2007; Schneider *et al.*, 1997; Smart *et al.*, 1996). Finer subgroup delineation could be achieved by combining RFLP analyses of 16S rRNA and rp gene sequences: the subgroups recognized by these methods were consistent with the subclusters identified by analysis of phytoplasma genomes through dot and Southern hybridizations using a number of cloned phytoplasma DNA probes (Lee *et al*., 1992; 1998a; Gundersen *et al*., 1996; Martini *et al*., 2007).

Finally based on extensive RFLP or phylogenetic analysis of 16S rRNA gene sequences from a wide array of phytoplasma strains, 19 RFLP groups and more than 20 distinct phylogenetic groups have been identified to date (Montano *et al*., 2001; Arocha *et al.*, 2005; Lee *et al.*, 2006a; Al Saady *et al*., 2008). A consensus for naming novel phytoplasmas was reached and recommended by the IRPCM Phytoplasma/Spiroplasma Working Team-

Phytoplasma Taxonomy Group (IRPCM, 2004) that "a '*Candidatus* (*Ca.*) Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence (>1200 bp)", and "a strain can be recognized as a novel '*Ca.* Phytoplasma' species if its 16S rRNA gene sequence has <97.5% similarity to that of any previously described '*Ca.* Phytoplasma' species". So far, 32 members of '*Ca.* Phytoplasma' have been proposed (Arocha *et al.*, 2005; 2007; IRPCM, 2004; Lee *et al.*, 2006b; Schneider *et al.*, 2005; Valiunas *et al.*, 2006; Al-Saady *et al*., 2008). Because of the highly conserved nature of the 16S rRNA gene, many biologically or ecologically distinct phytoplasma strains, which may warrant designation of a new taxon but may fail to meet the requirement of sharing <97.5% sequence similarity with existing '*Ca.* Phytoplasma', cannot be readily differentiated and classified. In this case, additional unique biological properties such as antibody specificity, host range and vector transmission specificity as well as other molecular criteria (gene) need to be included for speciation (Seemüller and Schneider, 2004).

Table 1. Classification of phytoplasmas based on RFLP analyses of 16S rRNA gene (based on Lee *et al.*, 1998a).

16Sr group	Phytoplasma strain	GenBank Acc. no.	Reference
16SrI: Aster yellows			
I-A	Aster yellows witches'-broom (AYWB) *rrnA; rrnB*	NC_007716	Bai *et al*., 2006
I-A	Tomato big bud (BB)	L33760	Lee *et al.*, 1992
I-B	Onion yellows mild strain (OY-M) *rrnA; rrnB*	NC_005303	Oshima *et al*., 2004
I-B	'*Ca.* Phytoplasma asteris'	M30790	Lee *et al*., 2004a
I-C	Clover phyllody (CPh)	AF222065	Lee *et al*., 2004a
I-D	Paulownia witches' broom (PaWB)	AY265206	Lee *et al*., 2004a
I-E	Blueberry stunt (BBS3)	AY265213	Lee *et al*., 2004a
I-F	Aster yellows from apricot - Spain (ACLR-AY)	X68338 AY265211	Kison, 1992 (GenBank submission) Lee *et al*., 2004a
I-I	Strawberry witches' broom (STRAWB1)	U96614	Jomantiene *et al*., 1998a; b
I-K	Strawberry witches' broom STRAWB2	U96616	Jomantiene *et al*., 1998a; b
I-L	Aster yellows (AV2192)	AY180957	Lee *et al*., 2003
I-M	Aster yellows (AVUT)	AY265209	Lee *et al*., 2004a
I-N	Aster yellows (IoWB)	AY265205	Lee *et al*., 2004a
I-O	Soybean purple stem (SPS)	AF268405	Lee *et al*., 2002
I-P	Aster yellows from *Populus*	AF503568	Šeruga *et al*., 2003
I-Q	Cherry little leaf (ChLL)		Valiunas *et al*., 2005
I-R	Strawberry phylloid fruit (StrawbPhF)	AY102275	Jomantiene *et al*., 2002a
16SrII: Peanut WB			
II-A	Peanut witches' broom (PnWB)	L33765	Gundersen *et al*., 1994
II-B	'*Ca.* P. aurantifolia' (witches' broom of lime, WBDL)	U15442	Zreik *et al*., 1995
II-C	Faba bean phyllody (FBP)	X83432	Schneider *et al.*, 1995
II-D	Sweet potato little leaf (SPLL)	AJ289193	Gibb *et al.*, 1995
II-E	*Pichris echioides* phyllody (PEY)	Y16393	Seeműller *et al*., 1998a

II-F	Cotton phyllody (CoP)	EF186827	Martini *et al*., 2007
16SrIII: X-disease			
III-A	Western X-disease (WX)	AF533231	Liefting and Kirkpatrick, 2003
III-B	Clover yellow edge (CYE)	L33766 8	Gundersen *et al.*, 1994
III-C	Pecan bunch (PB)	EF186807	Martini *et al.*, 2007
III-D	Goldenrod yellows (GR1)	EF186810	Martini *et al.*, 2007
III-E	Spiraea stunt (SP1)	AF190228	Jomantiene and Davis, 1999 (GenBank submission)
III-F	Milkweed yellows (MW1)	AF510724	Davis and Dally, 2002 (GenBank submission)
III-G	Walnut witches' broom (WWB)	AF190226 AF190227	Jomantiene and Davis, 1999 (GenBank submission)
III-H	Poinsettia branch-inducing (PoiBI)	AF190223	Jomantiene and Davis, 1999 (GenBank submission)
III-I	Virginia grapevine yellows (VGYIII)	AF060875	Davis *et al*., 1998
III-J	Chayote witches' broom (ChWBIII)	AF147706 AF1477067	Montano *et al*., 2000
III-K	Strawberry leafy fruit	AF274876	Jomantiene and Davis, 2000 (GenBank submission)
III-L	Cassava frog skin disease	EU346761	Alvarez *et al.*, 2009
III-P	Dandelion virescence	AF370119 AF370120	Jomantiene and Davis, 2001 (GenBank submission)
III-Q	Black raspberry witches' broom (BRWB7)	AF302841	Davis *et al*., 2001
III-T	Sweet and sour cherry (ChD)	FJ231728	Valiunas *et al*., 2009
III-U	Cirsium white leaf (CWL)		
16SrIV: Coconut lethal yellows			
IV-A	Coconut lethal yellowing (LYJ-C8)	AF498307	Harrison *et al*., 2002
IV-B	Yucatan coconut lethal decline (LDY)	U18753	Harrison *et al.*, 1994
IV-C	Tanzanian coconut lethal decline (LDT)	X80117	Harrison *et al.*, 1994
16SrV: Elm yellows			
V-A	'*Ca.* P. ulmi' (EY)	AY197655	Lee *et al*., 2004b
V-B	'*Ca.* P. ziziphi' (Jujube witches' broom, JWB-G1)	AB052876	Jung *et al*., 2003a
V-C	Alder yellows (ALY882)	AY197642	Lee *et al*., 2004b
V-C	Flavescence dorée (FD-C)	X76560	Daire *et al.*, 1992
V-D	Flavescence dorée (FD-D)	AJ548787	Torres *et al*., 2005
V-E	Rubus stunt (RuS)	Y16395	Seemüller *et al.*, 1998a
16SrVI: Clover proliferation			
VI-A	'*Ca.* P. trifolii' (CP)	AY390261	Hiruki and Wang, 2004
VI-B	*Fragaria multicipita*	AF036354 AF268895	Jomantiene *et al*., 1998a
VI-C	Illinois Elm Yellows (ILEY)	AF409069 AF409070	Jacobs *et al*., 2003
16SrVII: Ash yellows			
VII-A	'*Ca.* P. fraxini' (AshY)	AF092209	Griffiths *et al*., 1999
VII-B	Erigeron witches' broom	AY034608	Barros *et al*., 2002
16SrVIII: Loofah witches' broom			
VIII-A	Loofah witches' broom	AF086621	Ho *et al.*, 2001
16SrIX: Pigeon pea witches' broom			
IX-A	Pigeon pea witches'-broom	AF248957	Davis and Dally, 2000 (GenBank submission)

IX-B	'*Ca.* P. phoenicium'	AF515636 AF515637	Verdin *et al*., 2002
IX-C	Naxos periwinkle virescence		Heinrich *et al*., 2001
16SrX: Apple proliferation			
X-A	'*Ca.* P. mali'	AJ542541	Seemüller and Schneider, 2004
X-B	'*Ca.* P. prunorum'	AJ542544	Seemüller and Schneider, 2004
X-C	'*Ca.* P. pyri'	AJ542543	Seemüller and Schneider, 2004
X-D	'*Ca.* P. spartii'	X92869	Marcone *et al*., 2004a
X-E	Black alder witches'-broom BAWB (Buckthorn witches'-broom BWB)	X76431	Seemüller *et al.*, 1994
16SrXI: Rice yellow dwarf			
XI-A	'*Ca.* P. oryzae'	AB052873	Jung *et al*., 2003b
XI-B	Sugarcane white leaf SCWL	X76432	Lee *et al.*, 1997b
XI-C	Leafhopper-borne BVK	X76429	Seemüller *et al.*, 1994
16SrXII: Stolbur			
XII-A	Stolbur STOL (*Capsicum annum*)	X76427	Seemüller *et al.*, 1994
XII-B	'*Ca.* P. australiense' (Australian grapevine yellows, AUSGY)	L76865	Davis *et al*., 1997
16SrXIII: Mexican periwinkle virescence			
XIII-A	Mexican periwinkle virescence MPV	AF248960	Dally *et al*., 2000 (GenBank submission)
XIII-B	Strawberry green petal (Florida)	U96616	Jomantiene *et al.*, 1998b
16SrXIV: Bermudagrass white leaf			
XIV-A	'*Ca.* P. cynodontis'	AJ550984	Marcone *et al*., 2004b
16SrXV: Hibiscus witches'-broom			
XV-A	'*Ca*. P. brasiliense'	AF147708	Montano *et al.*, 2001
16SrXVI: Sugarcane yellow leaf syndrome			
XVI-A	'*Ca*. P. graminis'	AY725228	Arocha *et al*., 2005
16SrXVII: Papaya bunchy top			
XVII-A	'*Ca*. P. caricae'	AY725234	Arocha *et al*., 2005
16SrXVIII: American potato purple top wilt			
XVIII-A	'*Ca*. P. americanum'	DQ174122	Lee *et al.*, 2006
16SXIX: Cassia witches'-broom			
16SXIX-A	'*Ca*. P. omanense'	EF666051	Al-Saady *et al*., 2008

Over the last few years, numerous and diverse phytoplasmas have been discovered at an increasingly rapid pace in emerging diseases worldwide. These developments have raised expectations that the number of 16S rRNA RFLP groups (16Sr groups) and subgroups could rise considerably, warranting expansion of the existing phytoplasma classification scheme. A new computer-simulated RFLP (*in silico* restriction) analysis method resulted in the identification of putative new phytoplasma groups, significantly expanding existing 16S RNA gene based phytoplasma classification scheme (Wei *et al.*, 2007), but the reality of this system is still under discussion.

More '*Candidatus* Phytoplasma' species were published without reference to 16S grouping to reach 26 today. These phytoplasmas are listed in table below.

'*Candidatus* Phytoplasma'	**GenBank Acc. no.**	**Associated diseases**	**Country**	**Literature**
'*Ca.* P. japonicum'	AB010425	Hydrangea phyllody	Japan	Sawayanagi *et al*., 1999
'*Ca.* P. castaneae'	AB054986	Chestnut witches' broom	Korea	Jung *et al*., 2002
'*Ca.* P. pini'	AJ310849	Pinus decline	Germany	Schneider *et al*., 2005
'*Ca.* P. rhamni'	AJ583009	Rhmnus witches' broom	Italy	Marcone *et al*., 2004a
'*Ca.* P. allocasuarinae	AY135523 AY135524	Allocasuarina yellows	Australia	Marcone *et al*., 2004a
'*Ca.* P. fragariae'	DQ086423	Strawberry yellows	Lithuania	Valiunas *et al*., 2006
'*Ca.* P. lycopersici'	EF199549	'Brote grande' tomato	Peru	Arocha *et al*., 2007

To these officially published it should be added seven that were proposed (IRPCM, 2004) but not yet officially published: '*Ca*. P. pruni', '*Ca*. P. vitis', '*Ca*. P. solani', '*Ca*. P. palmae', '*Ca*. P. luffae', '*Ca*. P. cocostanzianae', '*Ca.* P. cocosnigeriae'. These '*Candidatus*' names proposed at the X International Congress of the International Organization of Mycoplasmology, 1994, held in Bordeaux, France, but not yet formally described, are reported here as incidental citations which do not constitute prior citations, according to rule 28b of the bacteriological code (Lapage *et al*., 1992).

Phytoplasma genes and genomics properties

Similar to other members of the *Mollicutes,* phytoplasmas contain one circular double-stranded chromosomal DNA (Lim and Sears, 1989; 1991a; b; Neimark and Kirkpatrick, 1993); they also have the smallest genome among bacteria, and phylogenetic studies propose that the common ancestor for phytoplasmas is *Acholeplasma laidlawii* in which the triplet coding for tryptophan (trp) is UGG, while in the other prokaryotes, enclosing mycoplasmas and spiroplasmas, trp is coded by UGA; phytoplasmas use UGA as a stop codon. In the other hand, phytoplasmas are genetically distinguishable from mycoplasmas for the presence of a spacer region (about 300 bp) between 16S and 23S ribosomal regions, which codes isoleucine tRNA ($tRNA^{Ile}$) and part of the sequences for alanine tRNA ($tRNA^{Ala}$).

Phytoplasmas have a genome with a low content G+C (sometimes as little as 23% which is thought to be the threshold for a viable genome), a feature common to all members of the class *Mollicutes*. Despite their very small genomes, many predicted genes are present in multiple copies; they contain 2 rRNA operons, and heterogeneity of the two operons is demonstrated in some phytoplasmas (Liefting *et al.*, 1996; Lee *et al.*, 1993a; Schneider and

Seemüller, 1994). Phytoplasma genome contains large numbers of transposon genes and insertion sequences in which is described the presence of portions unique to phytoplasmas and responsible for genome variability, to adjust phytoplasma to survive in diverse environments of plants and insects, as well as leading to the marked heterogeneity of phytoplasma genome sizes. These sequences were named as variable mosaic (SVM) (Jomantiene and Davis, 2006; Jomantiene *et al*., 2007) and as potential mobile units, (PMUs) (Bai *et al*., 2006). They also contain a unique family of repetitive extragenic palindromes (REPs) called PhREPS whose role is unknown though it is theorized that the PhREPS (capable of forming the stem loop structures) may play a role in transcription termination or genome stability (Jomantiene and Davis, 2006; Jomantiene *et al*., 2007) and were described as being at the roots of phytoplasma evolution (Wei *et al*., 2008b).

Some phytoplasmas contain extrachromosomal DNA (EC-DNA) such as plasmids (Rekab *et al*., 1999; Nishigawa *et al.*, 2002a; b). Short circular extrachromosomal DNAs (1.7-7.4 kb) or plasmids were found in all members of the aster yellows group (16SrI) and stolbur group (16SrXII) and in some members of the X-disease (16SrIII) and clover proliferation (16SrVI) groups. Large plasmids may also be present in some phytoplasmas. Some small plasmids may be of viral origin. Two extrachromosomal DNAs have been sequenced and shown to share significant sequence similarity with genes in geminiviruses, a type of DNA plant virus (Kuboyama *et al.*, 1998).

Genes encoded in EC-DNAs, such as plasmids, are known to play important roles in the pathogenicity and virulence of many plant pathogenic bacteria. The isolation and characterization of EC-DNAs in some phytoplasmas have been described (Denes and Sinha, 1991; Kuske *et al*., 1991; Schneider *et al*., 1992; Goodwin *et al*., 1994; Nakashima and Hayashi, 1997; Kuboyama *et al*., 1998; Oshima *et al*., 2001a; Nishigawa *et al*., 2001; 2002b; Liefting *et al*., 2004; 2006). '*Candidatus* Phytoplasma asteris', onion yellows strain (OY), has shown to have diverse lines: a mildly pathogenic line (OY-M) insect transmitted contains two types of plasmids (EcOYM and pOYM), each of which possesses a gene encoding the putative transmembrane protein, ORF3. Likewise, a non-insect-transmissible line (OY-NIM) has the corresponding plasmids (EcOYNIM and pOYNIM), but pOYNIM lacks orf3. It was recently shown that, in OY-M, orf3 was transcribed from two putative promoters while, on EcOYNIM, one of the promoter sequences was mutated and the other deleted. It was also demonstrated that ORF3 was not expressed in the OY-NIM-infected plants by immuno-hystochemical analysis. Moreover, ORF3 protein seemed more specifically expressed in OY-M-infected insects rather than in plants. These results allow speculating that ORF3 might play a role in the interactions of OY with its insect host (Ishii *et al*., 2009).

Differences in the chromosome size among phytoplasma species were reported; '*Ca.* P. cynodontis' and a tomato strain of the stolbur phytoplasma that belong to different 16Sr

groups have the chromosomal size of 530 and 1,350 kb, respectively; or the rape virescence phytoplasma and the hydrangea phyllody from the same subgroup differ greatly in their genome size by 1,130 kb vs. 660 kb (Marcone *et al.*, 1999). These differences are usually due to the occurrence of gene duplication and redundancy. In the genome of the onion yellows (OY) phytoplasma (Oshima *et al.*, 2004), it was estimated that 18% of the total genes are multiple redundant copies of only five genes: *uvrD* (ATP-dependent DNA helicase , 3,117 nucleotides, 7 copies), *hflB* (ATP-dependent Zn protease, 1,551 nucleotides, 17 copies) , *tmk* (thymidylate kinase, 624 nucleotides, 6 copies), *dam* (DNA methylase, 660 nucleotides, 4 copies) , and *ssb* (single-stranded DNA-binding protein, 345 nucleotides, 15 copies), all of which are generally single copies (if they exist at all) in the other *Mollicutes* whose genome has so far been sequenced. In addition, 5 genes encoding elements of transporter systems have multiple copies, presumably not all functional. Multiple copies of insertion sequence-like elements are also present in the genome of the OY and other phytoplasma strains (Lee *et al.*, 2005) and functional gene for thymidylate kinase (Miyata *et al*., 2003).

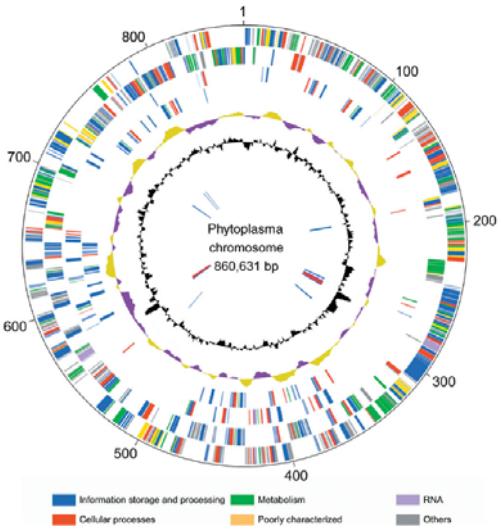
Phytoplasmas lack many genes for standard metabolic functions (reduction of biosynthetic genes) and have no functioning homologous recombination pathways, but do have a *sec* transport pathway (a major route of protein translocation across various cell membranes) (Bai *et al.*, 2006). The phytoplasma genome encodes even fewer metabolic functions than do mycoplasma genomes (Razin, 2007).

The analysis of the genome sequence of the OY phytoplasma (Fig. 3a), shed some light on the nutritional requirements of these micro-organims. The genome of the OY phytoplasma is about 861 kb and contains 754 ORFs, corresponding to 73% coding capacity. Like other *Mollicutes*, the OY phytoplasma lacks genes for the biosynthesis of the amino acids and fatty acids, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (production of ATP). Unlike the mycoplasmas, phytoplasma genome also lacks genes for the phosphotransferase system and for metabolizing UDP-galactose to glucose 1-phosphate, suggesting that phytoplasmas possess a unique sugar intake and metabolic system (Oshima *et al.*, 2004). In addition, (unlike mycoplasmas) phytoplasmas lack the pentose phosphate cycle (normally used to synthesize NADPH and also supplies the ribose 5-phosphte necessary to synthesize nucleotides), arginine dehydrolase pathway, and more unexpectedly, ATP-synthase subunits, which are thought to be essential for life (Fig. 3b). It suggests that phytoplasma imports host ATP by an unknown mechanism. Alternatively, ATP synthesis in phytoplasma might be strongly dependent on the glycolysis. Lack of these important functions in phytoplasmas may be the result of reductive evolution as a consequence of life as an intracellular parasite in a nutrient-rich environment (Oshima *et al.*, 2004).

Phytoplasmas lack also the PTS system (phosphotransferase system) for import of sugars essential for glycolysis. Most bacteria use this system as an energy-efficient way of

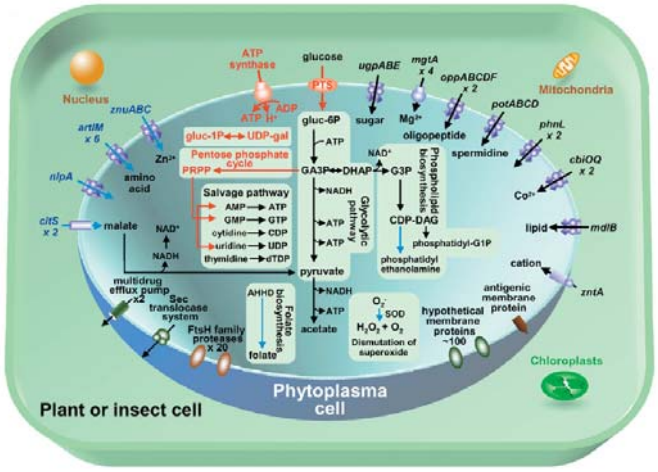
simultaneously importing and phosphorylating sugars such as sucrose, glucose and fructose. However, phytoplasmas have a maltose ABC transport system. The maltose binding protein may have affinity to maltose, trehalose, sucrose and palatinose, and therefore import them to phytoplasma cells. Trehalose is a major sugar of the insect haemolymph. However, enzymes for converting these sugars to glucose-6-phosphate (available for glycolysis) were not found in phytoplasmas (Christensen *et al.*, 2005; Razin, 2007). The possibility that the phytoplasmas take up phosphorylated hexoses from the host (Bai *et al.*, 2006) and other possibilities remain to be investigated further.

Fig. 3. Schematic representation of chromosome and metabolic pathways of OY phytoplasma. **(a)** Circular representation of the phytoplasma chromosome. The first (outer) and second circles show predicted protein-coding regions, respectively, on the plus and minus strands classified by function using the colour code at the bottom of the figure. The third and fourth circles show the multiple redundant genes, respectively, on the plus and minus strands, respectively. The fifth circle shows the GC-skew value ((G – C)/(G + C)). The sixth circle shows the G + C content (higher values outward). The seventh and eighth circles show tRNAs (blue) and rRNAs (red) on the plus and minus strands, respectively. The scale in kb is indicated (numbers on the outside of the chromosome). **(b)** An overview of phytoplasma metabolism and transport. Shown are transporters and the main elements of metabolic pathways, deduced from the set of genes with predicted functions. Red arrows: pathways or transporters found in the *Mycoplasma genitalium* genome but not in the phytoplasma genome. Blue arrows: pathways or transporters found in the phytoplasma genome but not in the *M. genitalium* genome (from Oshima *et al.*, 2004).



a

b



Although metabolic genes are scarce, the phytoplasma genome contain many genes encoding transporter systems, such as malate, metal-ion and amino-acid transporters, some of which have multiple copies, suggesting aggressive import of many metabolites from host cell, that it greatly disturbs the metabolic balance and causing disease symptoms. Also, the phytoplasma genome encodes folate synthesis genes, which may allow phytoplasmas to adapt to the very different plant and insect environments (Oshima *et al.*, 2004).

Interaction with hosts

At the present, exploration of the interactions of phytopathogenic *Mollicutes* with their plant and insect hosts is a fascinating and very active field of research. Several lines of investigation are important in the interactions of phytopathogenic *Mollicutes* with both plants and insects.

Phytoplasma membrane proteins

Serological studies recognized one or two abundant immuno-dominant proteins (highly antigenic membrane proteins at the surface of phytoplasmas) with trans-membrane domains. Major surface proteins may play roles in *Mollicutes* recognition, adherence to plant or insect host cells (a prerequisite for colonization and infection), pathogenicity and triggering of host resistance responses (Christensen *et al.*, 2005). The occurrence of major surface epitopes unique to each phytoplasma species suggests that these proteins may be key participants in specific interactions with host cells. Previous studies have shown that immunodominant membrane protein is a major portion of the total cellular membrane proteins in most phytoplasmas (Shen and Lin, 1993).

Genes encoding immunodominant membrane proteins were isolated from several phytoplasma groups. They are classified into three distinct types: (1) immunodominant membrane protein (Imp): sweet potato witches' broom (SPWB) (Yu *et al*., 1998), apple proliferation (AP) (Berg *et al*., 1999), European stone fruit yellows (ESFY) (Morton *et al*., 2003), pear decline (PD) (Morton *et al*., 2003), and peach yellow leaf roll (PYLR) phytoplasmas (Morton *et al*., 2003); (2) immunodominant membrane protein A (IdpA): western X-disease (WX) phytoplasma (Blomquist *et al*., 2001); and (3) antigenic membrane protein (Amp): aster yellows (AY) (Barbara *et al*., 2001, 2002), clover phyllody (CPh) (Barbara *et al*., 2002), and onion yellows (OY) phytoplasmas (Kakizawa *et al*., 2004). They are not orthologues of each other (Barbara *et al*., 2002; Kakizawa *et al*., 2006); nonhomologous proteins play the role of 'immunodominant', the major portion of the total cellular membrane proteins, in diverse phytoplasmas. A high expression level of Amp protein was confirmed in AY, CPh, and OY phytoplasmas (Kakizawa *et al*., 2004; Barbara *et al*., 2002), and Amp protein was shown to be exported via the Sec protein secretion system, accompanied by the cleavage of its N-terminal signal sequence (Kakizawa *et al*., 2004).

Cloning of amp genes from several strains in the AY-group phytoplasma showed that Amp proteins were under positive selection and positively selected amino acids were encoded in the central hydrophilic domain of the Amp (Kakizawa *et al*., 2006a). Recently, it was reported that the Amp of OY phytoplasma forms a complex with an insect microfilament. In addition, the formation of Amp–microfilament complexes was correlated with the phytoplasma transmitting capability of leafhoppers, suggesting that the interaction between Amp and insect microfilament complexes plays a major role in determining the transmissibility of phytoplasmas (Suzuki *et al*., 2006).

Analysis of other types of immunodominant membrane proteins was also reported. Morton *et al*. (2003) isolated genes encoding Imps from several phytoplasma strains and found that the sequence identities of imp in several phytoplasmas were not correlated with that of 16S rRNA gene; this result suggests that the variability of immunodominant membrane proteins could reflect some factors other than evolutionary time. However, as the information about other types of immunodominant membrane protein is limited, analyses of several types of immunodominant membrane protein would shed light on the biological diversity and evolution of phytoplasmas (Kakizawa *et al*., 2009).

Although the gene encoding immunodominant membrane protein of WX phytoplasma is idpA, Liefting and Kirkpatrick (2003) reported that a gene homologous to imp was also encoded in the WX genomic fragment. However, it is not known if imp was expressed and found as a protein on the phytoplasma surface membrane. This observation leads to a hypothesis that imp may be a common ancestor of phytoplasmal immunodominant membrane proteins. The sequence identity of imp was found quite low among different groups, and a

BLAST search did not detect similarity between imp genes from different groups. However, the gene organizations around imp were well conserved in most phytoplasmas, and all Imps had a transmembrane region in their Nterminus. Therefore, despite dissimilarities in sequence identity, imp genes studied till now would be orthologous because of the similar gene organization and conserved transmembrane structure (Kakizawa *et al*., 2009).

In contrast, the orthologue of idpA, which is the immunodominant membrane protein of WX, in the complete genomic sequences of OY-M (Oshima *et al*., 2004), AY-WB (Bai *et al.*, 2006), '*Ca*. Phytoplasma australiense' (Tran-Nguyen *et al*., 2008), or '*Ca*. P. mali' (Kube *et al*., 2008); in addition, the orthologue of amp, in the complete genomic sequence of '*Ca*. P. mali' was not found. It was suggested that the ancestral type of immunodominant membrane protein had been Imp, and subsequently expression levels of Amp or IdpA in the AYgroup or WX-group, respectively, were increased (Kakizawa *et al*., 2009). Further sequence analyses of immunodominant membrane protein genes from many phytoplasma strains would contribute to a better understanding of the biological and evolutionary roles of immunodominant membrane protein.

It was suggested that Imp has an important role in host–phytoplasma interactions, just like many positively selected proteins that have previously been reported (Hughes and Nei, 1988; Nielsen and Yang, 1998; Bishop *et al.*, 2000; Jiggins *et al*., 2002; Urwin *et al*., 2002; Andrews and Gojobori, 2004). The accumulation of Amp was calculated as about 10-fold higher than that of Imp. This result was consistent with the 'immunodominant' property of Amp in AY-group phytoplasmas.

However, detection of Imp in phytoplasma infected plants is also possible, and therefore the protein amount of Imp must also be high. Results from blot analysis of Imp from OY-W suggested that its signal sequence would be uncleaved and Imp would be retained in the phytoplasma cell membrane. This result agrees with the previous reports that Imp is an immunodominant membrane protein in several phytoplasmas including AP and SPWB (Yu *et al*., 1998; Berg *et al.*, 1999). Thus, for the prediction of phytoplasma secretory proteins, it might be important to consider the amino acid length between transmembrane regions and cleavage motifs.

Results from cloning imp from several groups of phytoplasmas suggest that Imp has some important roles in phytoplasma. It was previously reported that Amp forms a complex with microfilaments of insect vectors and is important for the transmissibility of phytoplasmas (Suzuki *et al*., 2006). Elucidation of the function of Imp is important for understanding the biology of phytoplasmas. In addition, it is interesting to ascertain whether Imp has the same function in different groups of phytoplasma.

As Imp is present in all phytoplasmas, isolating imp from many other groups of phytoplasmas should be possible. Further cloning, sequencing, and comparison of imp from

many phytoplasmas will extend our knowledge of phytoplasma evolution. In addition, as Imp is an immunodominant membrane protein in AP and SPWB phytoplasmas, and expression of Imp in OY-W was shown clearly, antibodies against Imp could be useful for detection of phytoplasmas in general. Cloning of Imp and subsequent production of antibodies against Imp would provide a good tool to detect phytoplasmas (Kakizawa *et al*., 2009) as it was recently shown for amp (Arashida *et al*., 2008).

Phytoplasma titer and multiplication

The phytoplasma titer varies greatly from plant to plant. Recently, different competitive PCR procedures have made it possible to estimate the phytoplasma titer in different host plants; periwinkle was confirmed as a high-concentration species irrespective of the phytoplasma's taxonomic affiliation, while other species should be considered medium-or even low-titer hosts (Berges *et al.*, 2000).

Plant species with different susceptibility to phytoplasma infection have been reported such as apple (Kartte and Seemüller, 1991; Bisognin *et al*., 2008), pear (Seemüller *et al.*, 1998b) apricot (Audergon *et al.*, 1989). Using different approaches, a reduction/suppression of phytoplasma multiplication has been observed when susceptible cultivars were grafted on resistant or tolerant rootstocks (Kartte and Seemüller, 1991; Pastore *et al.*, 1998). More recently, the disappearance of phytoplasmas from the canopy but not from the roots of recovered apple proliferation-infected trees has been reported (Musetti *et al.*, 2005). The results suggested that some components of the oxidant-scavenging system in recovered leaves are not very active, leading to an overproduction of H_2O_2 and, possibly, to a membrane lipid peroxidation. The production of H_2O_2 was reported as involved in counteracting pathogen virulence (Musetti *et al.*, 2005).

Host susceptibility is not the only factor relevant in triggering pathogen concentration. Different strains of the same phytoplasma may reach different concentrations in the infected host as reported for ash yellows (Sinclair and Griffiths, 2000). In a co-inoculation experiment, Sinclair and Griffiths showed that the aggressive strain rDNA restriction profile of Ash yellows was detected by PCR sooner and more frequently than a milder one. In the other hand, phytoplasma concentration in woody hosts may also differ according to the season as reported for pear decline (Errea *et al.*, 2002; Garcia-Chapa *et al.*, 2003), European stone fruit yellows (Jarausch *et al.*, 1999b) and "bois noir" (Škorić *et al.*, 1998).

Phytoplasma translocation in plants

Phytoplasmas are transferred by insects to plant sieve element, from which they spread systemically in the plant using the sieve tube system, but never settle in the meristems

(Christensen *et al.*, 2004). Phytoplasmas are pleomorphic and sufficiently small to pass freely through sieve pores, so they might be swept along with the assimilate flow from leaves to sugar consuming plant organs like systemic viruses. Accordingly, phytoplasmas have been found in sink tissues such as immature leaves and roots, whereas source leaves remained uninfected (Siddique *et al.*, 1998). By contrast, a high titer of phytoplasma in source leaves and a low colonization in sink tissue were reported by other researchers (Christensen *et al.*, 2004). Studies focusing on the translocation of phytoplasmas after localized inoculation (Wei *et al.*, 2004b) or the re-colonization of trees (Garcia-Chapa *et al.*, 2003) provide evidence that translocation of phytoplasmas cannot be explained only by assimilate flow. Active movement by the phytoplasmas seems highly unlikely considering lack of any genes coding for cytoskeleton elements or flagella (Christensen *et al.*, 2005).

Pathogenicity and virulence in phytoplasmas

Despite their economic importance and unique biological features, phytoplasmas remain the most poorly characterized plant pathogens, primarily because efforts at *in vitro* culture, gene delivery and mutagenesis have been unsuccessful. It is agriculturally important to identify the factors involved in their pathogenicity to discover effective measures to control phytoplasma diseases.

Energy metabolism is certainly a key topic for understanding phytoplasma biology and pathogenesis. Effect of altered levels of oxygen and carbon dioxide on phytoplasma abundance in *Oenothera* leaftip cultures has been reported (Sears *et al.*, 1997). Lepka *et al.* (1999) and Maust *et al.* (2003) have reported the occurrence of changes in the concentration of carbohydrates in phloem, root and leaves of phytoplasma infected plants, as compared to the healthy control. Because of the large array of symptoms of phytoplasma diseases, it can be speculated that nutrient depletion may not be restricted to sugars, but might include other compounds depending on the pathogen involved. This is confirmed by the discovery that reduction in the concentration of photosynthetic pigments (Bertamini *et al.*, 2002) and total soluble proteins (Bertamini *et al.*, 2002; Musetti *et al.*, 2000; 2005), as well as alterations in hormone balance (Maramorosch, 1957; Ulrychowá, 1975; Plavšić *et al*., 1978; Pecho and Vizarova, 1990; Hegele and Bangerth, 1997; Das and Mitra, 1998; Pertot *et al.*, 1998; Maust *et al.*, 2003; Jagoueix-Eveillard *et al.*, 2001), amino-acid transport (Lepka *et al.*, 1999) and presence of folate and endopetidase genes homologues in phytoplasma genome (Davis *et al.*, 2003b) were described as potentially affect infection of host plants by different phytoplasmas.

Impaired photosynthesis, accumulation of carbohydrates in mature leaves and decreased starch content in sink tissues such as roots, often described for phytoplasma infection, seem to be secondary effects and can easily be related to inhibition of phloem transport. Accumulation of carbohydrates in source leaves is generally believed to result in a feedback inhibition of

photosynthesis causing chlorosis (Lepka *et al.*, 1999; Maust *et al.*, 2003; Bertamini *et al.*, 2002). A decisive reduction in phloem translocation can be expected when phytoplasmas multiply to such a level that the lumen of sieve elements is clogged. However, reduced translocation has also been observed in plants with a low titer of phytoplasmas, suggesting a more indirect influence of the parasite on the host metabolism and phloem function (Siddique *et al.*, 1998; Guthrie *et al.*, 2001).

Very little is known about the phytoplasma virulence. Phytoplasma possesses none of the known virulence genes (such as *hrp*) found in other phytopathogenic bacteria (Oshima *et al.*, 2004). Because phytoplasmas lack most of the common metabolic pathways, it has been speculated that they must assimilate a wide range of materials from the host cells, probably with detrimental effects on the hosts. In onion yellows (OY) phytoplasma two types of stains were described: mild line (OY-M, causing mild proliferation and yellowing) and severe line (OY-W, causing yellowing, stunting, proliferation and withes' broom). Oshima *et al.* (2001b) described that the OY-W chromosome (1000 kbp) is larger than the OY-M chromosome (860 kbp). Further analysis showed that five glycolytic genes are duplicated in severely pathogenic strain. This implies that it is advantageous for the phytoplasma to retain these genes in its lifestyle and also, it may influence its glycolytic activity. Higher titer of phytoplasma population of OY-W than that of OY-M was previously reported (Oshima *et al.*, 2001b). Taking these results into account, the higher consumption of carbon source (because of duplication of glycolytic genes) may affect the growth rate of phytoplasma and may also directly or indirectly cause more severe symptoms (Oshima *et al.*, 2007). Very recently it was shown that a single virulence factor, "tengu-su" inducer (TENGU), induces witches' broom and dwarfism when expressed in transgenic *Nicotiana benthamiana* and *Arabidopsis thaliana* plants. Although the localization of phytoplasma was restricted to the phloem, TENGU protein was detected in apical buds by immunohistochemical analysis, suggesting that TENGU was transported from the phloem to other cells. Microarray analyses showed that auxin-responsive genes were significantly down-regulated in the "tengu"-transgenic plants compared with GUS-transgenic control plants. These results suggest that TENGU inhibits auxin-related pathways, thereby affecting plant development (Hoshi *et al*., 2009).

Putative genes involved in phytoplasma-host interactions

Several recently developed molecular techniques have prompted new lines of research in phytoplasmology. Differential display and arbitrarily primed PCR, which facilitate detection of differential gene expression, are being applied in several laboratories to investigate the biology of phytopathogenic *Mollicutes* in different environments, such as the plant and insect hosts.

Phytoplasma infection can lead to production of defense proteins, increase of phenolic compounds and hydrogen peroxide overproduction in host plants (Junqueira *et al.*, 2004; Musetti *et al.*, 2000; 2005). Using the messenger RNA differential display method, Smart *et al* (1996) have recognized *Arabidopsis* genes regulated following infection with the aster yellows phytoplasma. Also, Jagoueix-Eveillard *et al.* (2001) have isolated several up- and down-regulated genes from periwinkle plants infected either by *Spiroplasma citri*, the stolbur phytoplasma or '*Candidatus* Phytoplasma aurantifolia'. Eight of them had homologies with genes coding proteins involved in photosynthesis, sugar transport, response to stress, or pathways of phytosterol synthesis. They showed that a gene coding a pathogen-induced protein, namely a wall associated kinase was activated by *S. citri* infection. In *Arabidopsis* spp., this gene also is induced by salicylic acid and is involved in the plant's defence response. In *A. thaliana*, mutation of the transketolase gene affects the expression of several photosynthetic genes and pigment production. So, inhibition of the transketolase gene might therefore be responsible for the repression of the genes involved in photosynthesis. The presence of specific genes for translocation was also reported in several phytoplasmas (Kakizawa *et al*., 2001).

A homologue of an amino acid transporter was found to be down-regulated after infection of *Prunus armeniaca* with '*Candidatus* Phytoplasma prunorum', supporting the effect of phytoplasma on amino acid transport. Besides, three genes were found to be up-regulated in host plant infected with this phytoplasma: a gene coding a heat-shock protein (HSP70), a gene encoding a metallothionein and a homologue of the expressed sequence tag 673 clone of *P. armeniaca*. Metallothioneins are proteins that have potent metal binding and redox properties, and are produced in response to heavy metal stress. This can also induce the production of HSP70, as does growth under extreme temperatures (Carginale *et al.*, 2004).

Phytoplasmas were also detected in floral tissue. Infection of tomato plants by the stolbur phytoplasma induces typical flower abnormalities as sepal hypertrophy, virescence, phyllody, and big buds. The data showed that flower malformations of stolbur phytoplasma-infected tomatoes are associated with early deregulations in the expression of key flower development genes (including *LeDEF*, *LeWUS* and *TAG1*). As phytoplasmas never settle in the meristems, they probably change floral development by long distance signals, such as impairing translocation of sugars in phloem, or hypermethylation of plant genome through activation of plant defense mechanism (Pracros *et al.*, 2006). In plants and animals, the cytosine methylation process is known to regulate gene expression in an epigenetic manner. Studies have demonstrated that genes can be accidentally down-regulated when the number of methylated cytosines is more abundant as compared with the normal methylation status of the gene.

Perspective towards control of phytoplasma diseases

Control of epidemic outbreak of phytoplasma diseases can be carried out theoretically either by controlling the vector or by eliminating the pathogen from the infected plants by meristem tip culture, antibiotics or other chemicals (Bertaccini, 2007).

At the present, insect vector control using pesticides is the tool of choice for limiting outbreaks of phytoplasma diseases. Apart from environmental considerations, the efficacy of this approach is far from satisfying, and phytoplasma diseases continue to be epidemic in several areas of the world, despite the large use of insecticide treatments (Firrao *et al.*, 2007). In the other hand, removal of sources of inoculum is efficient in the case of mollicute diseases spread by monophagous vector feeding on the affected plant (i.e. flavescence dorée/*Scaphoideus titanus* and pear decline/*Cacopsylla pyri*). It is difficult to achieve when wild reservoir plants are sources of contaminations for polyphagous leafhoppers such as stolbur/*Hyalesthes obsoletus*/bindweed/nettle, and when reservoirs are unknown. Similarly, it is easier to control monophagous insects reproducing on the affected crop than insect making their cycle on wild plants.

Also, the cure of infected plants by antibiotics or by stimulating the production of specific antibodies is impossible, because in one hand the use of antibiotics is very expensive, less allowed or prohibited in several countries, and even not always efficient for long-time and on the other hand the production of transgenic plants just producing antibodies against these pathogens is very far from achievement (Le Gall *et al.*, 1998; Chen and Chen, 1998; Ishiie *et al*., 1967; Bertaccini, 2007).

Recent research is shading new light on several aspects of the phytoplasma biology and host relationships. Interference with the phytoplasma colonization of the insect body or with their nutrient uptake in the plant phloem is primary targets for plant protection without the use of toxic compounds. Identification of barriers to phytoplasma colonization of the insect body is a prerequisite to the development of strategies to reduce the infectivity of vector populations. Alternatively, phytoplasma nutrient uptake from the plant phloem may be targeted to reduce pathogen multiplication and symptom expression in the host. Hopefully these approaches will lead to the protection of plants without the use of toxic compounds (Firrao *et al.*, 2007). Therefore, a real way to control phytoplasma infection is to prevent the outbreaks by producing clean material or by finding phytoplasma resistant varieties (Dai *et al.*, 1997; Carraro *et al.*, 1998; Kison and Seemüller, 2001; Jarausch *et al.*, 1999a; 2007; Seemüller *et al.*, 2007).

Knowledge about the mechanisms of plant host resistance to phytoplasmas is also little, but the paucity of effective disease management strategies for these diseases lends a high priority to these questions. Efforts continue to identify germplasm encoding natural resistance to *Mollicutes*, and to incorporate such genes *via* selection and breeding programs into various

crops and trees including fruit trees (Thomas and Hassan,1992; Ponnuswami and Irulappan, 1993; Thomas and Mink, 1998; Seemüller *et al.*, 1992; 1998b; 1998c; Sinclair *et al.*, 1997a; b). It may involve resistance to either the pathogen itself or to the insect vector. Plant defense related proteins, known to be active in host responses to invasion by other types of pathogens, might occur in responses to mollicute infection; confirmation of this hypothesis would require demonstration that the compound is in the right place at the right time, and is present in effective concentrations (Garnier *et al.*, 2001).

Chapter 1

Phytoplasmas associated with carrot yellows in Serbia

1.1. Introduction

Phytoplasma diseases are known to affect vegetables in the major production areas worldwide (Lee *et al*., 2003, 2004a); among them carrot is quite an important crop in Serbia, covering 15,000 ha, with marketable production of about 120,000 t (Thorogood *et al*., 2003). During field surveys in large carrot fields at Begeč, Bačka region, carrot plants with symptoms referable to phytoplasma presence were observed. Presence of symptoms was sporadic but single plants were severely affected. Symptoms included leaf reddening, purpling and yellowing, formation of chlorotic adventitious shoots, and reduction of size and quality of taproots (Fig. 1.1).

Carrot diseases with similar symptoms were reported in several vegetable growing areas worldwide. '*Candidatus* Phytoplasma asteris' (aster yellows, AY) (Lee *et al*., 2004a), beet leafhopper-transmitted virescence agent (BLTVA) and *Spiroplasma citri* (SC) are mollicutes reported to infect carrot (*Daucus carota* L.), causing indistinguishable symptoms on infected plants (Lee *et al*., 2006a). All these pathogens are prokaryotes with phloematic habitats that can be detected both in wild and cultivated plants, and are transmitted by leafhoppers that may have occasional or permanent trophic relationship with their hosts. The symptoms observed in plants in Serbia resemble those already described in carrot in North America and in Israel (Orenstein *et al*., 1999; Lee *et al*., 2003; 2006a) which had been associated with the phytopathogenic mollicutes described above. The identification of phytoplasmas associated with this carrot disease together with investigations about the genetic variability of identified aster yellows phytoplasmas were carried out.

The current aster yellows phytoplasma classification, as the molecular phytoplasma classification in general, relies on PCR amplification of 16SrDNA followed by RFLP analysis and/or sequencing (IRPCM, 2004). However this approach does not always provide a clear

molecular distinction and, since the aster yellows group encompass phytoplasmas infecting numerous different plant species and insect vectors (Lee *et al*., 2004a), it is not completely helpful for epidemiological studies towards possible control of these diseases. Therefore, additional genes such as the *tuf* gene coding the elongation factor Ef-Tu, the putative aa kinase gene and ribosomal recycling factor gene, and a phytoplasma DNA helicase gene were studied to molecularly characterize the aster yellows phytoplasma strains detected in carrot samples. Moreover *groES*, *groEL*, *amp* and *nadE* genes were also sequenced and compared with an aster yellows strains detected in corn in Colombia (Duduk *et al*., 2008b) and with other phytoplasma strains for whose these sequences were available in GenBank.

Figure 1.1. A) Symptomatic carrot (left) showing leaf reddening, formation of chlorotic adventitious shoots and reduction in taproot size and quality, and asymptomatic carrot (right). B) Severely infected carrot plant showing strong reduction of taproot and witches' broom vegetation.

1.2. Material and Methods

Carrot samples

Samples of carrot showing the symptoms described above were collected during September-October 2006 and 2007 in the South Bačka region of Serbia samples from 55 symptomatic and eight asymptomatic plants. The surveyed area covered approximately 40 ha.

Total nucleic acids were extracted from 1 g of carrot leaf and root tissue following the protocol described by Angelini *et al*. (2001), dissolved in TE buffer, maintained at -20°C and employed for polymerase chain reaction (PCR) diluted 1:100 in sterile distilled.

Phytoplasma reference strains

The phytoplasma strains *Chrysanthemum* yellows (CHRY, ribosomal subgroup 16SrI-A), European aster yellows (EAY, ribosomal subgroup 16SrI-B), *Catharanthus* virescence (CVB, ribosomal subgroup 16SrI-F), carrot yellows (CA, ribosomal group 16SrI-C), primula yellows (PRIVA, ribosomal subgroup 16rI-L), clover phyllody from France (KVF, ribosomal subgroup 16SrI-C), and stolbur from pepper from Serbia (STOL C, ribosomal subgroup 16SrXII-A), maintained in collection in periwinkle [*Catharanthus roseus* (G.) Don.] (Bertaccini, 2003), were employed as reference strains in restriction fragment length polymorphism (RFLP) analyses. Two corn samples infected with maize bushy stunt phytoplasma (MBSC1 and MBSC2, ribosomal subgroup 16SrI-B) collected in Palmira (Colombia) were also used as reference strains.

PCR amplification and sequence analyses

16S ribosomal DNA. Direct PCR assays with the universal phytoplasma primer pair P1/P7 (Deng & Hiruki, 1991; Schneider *et al*., 1995) or with primer pair R16(I)F1/R1 (Lee *et al*., 1994) specific to the ribosomal groups I, II (Tolu *et al.*, 2006) and XII were carried out for phytoplasma detection in carrot samples.

Each 25 µl PCR reaction mix contained 20 ng template DNA, 2.5 µl 10X PCR buffer, 0.8 U *Taq* polymerase (Polymed, Italy), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.4 µM of each primer. Samples lacking DNA were employed as negative controls. Thirty-five PCR cycles were performed under the following conditions: 1 min (2 min for the first cycle) for denaturation step at 94°C, 2 min for annealing at 50°C, and 3 min (10 min for the last cycle) for primer extension at 72°C. Six µl of PCR products were separated in 1% agarose gel, stained with ethidium bromide and visualized with UV transilluminator.

Identification of detected phytoplasmas was done using RFLP analyses with *Tsp509*I (New England Biolabs, Beverly, MA, USA), *Taq*I, *Hha*I and *Tru*I (Fermentas, Vilnius, Lithuania) restriction enzymes. RFLP products were separated in a 5% polyacrylamide gel, stained with ethidium bromide and visualized under UV transilluminator.

The P1/P7 amplified products of three carrot samples 2006/1, 2006/5 and 2006/9 were purified using Qiagen PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in both directions with two forward primers P1 and R16F2 (Lee *et al*., 1995), and one reverse primer P7, using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). A database search of homologous sequences was performed by Blast analyses at the National Center for Biotechnology (NCBI) website (http://ncbi.nlm.nih.gov/BLAST).

Ribosomal protein genes. A further molecular characterization was performed by PCR using rpF1C/rp(I)R1A primer pair (Martini *et al*., 2007) that amplifies part of the ribosomal operon which includes the 3' end of the *s19* gene and the complete *l22* and *s3* genes (Lim and Sears, 1992).

Table 1.1. Phytoplasma 16S rDNA and ribosomal protein sequences retrieved from GenBank, including carrot samples 2006/1, 2006/5 and 2006/9 from Serbia.

Phytoplasma strain			Accession number		
	16Sr group	rp group	16S rDNA	rp genes	Reference
2006/1	I-A	I-A	EU215424	EU215428	Duduk *et al*., 2009a
2006/5	n.d.	n.d.	EU215425	EU215429	Duduk *et al*., 2009a
2006/9	I-B	I-B	EU215426	EU215430	Duduk *et al*., 2009a
Br273	I-B	I-B	EU215427	EU215431	Duduk *et al*., 2009a
Btsv2CarD1	I-A	I-A	AY180926.1	AY183690	Lee *et al*., 2003
Btsv2CarD3	I-B	I-B	AY180945.1	AY183710	Lee *et al*., 2003
CabD3	I-B	I-B	AY180947.1	AY183717	Lee *et al*., 2003
OnionD2	I-A	I-A	AY180931.1	AY183699	Lee *et al*., 2003
ParsD1	I-B	I-B	AY180954.1	AY183719	Lee *et al*., 2003
ParsD3	I-A	I-A	AY180940.1	AY183700	Lee *et al*., 2003
PLD1	I-A	I-A	AY180941.1	AY183702	Lee *et al*., 2003
RgwdD1	I-A	I-A	AY180930.1	AY184704	Lee *et al*., 2003
Btsv2M.f.12	I-B	I-B	AY180951.1	AY183711	Lee *et al*., 2003
BtsvS.i.4	I-A	I-A	AY180938.1	AY183695	Lee *et al*., 2003
Btsv2C.a.13	I-A	I-A	AY180925.1	AY183688	Lee *et al*., 2003
Btsv2C.a.17	I-B	I-B	AY180944.1	AY183716	Lee *et al*., 2003
AV2192	I-L	I-B	AY180957.1	AY183708	Lee *et al*., 2003
BB	I-A	I-A	AY180955.1	AY183686	Lee *et al*., 2003
CHRY	I-A	I-A	AY180956.1	AY183696	Lee *et al*., 2003
MIAY	I-B	I-B	M30790.1	M74770	Lee *et al*., 2003
CVB	I-F	I-N	AY265212.1	AY264865	Martini *et al*., 2007
MBS	I-B	I-L	AY265208.1	AY264858.1	Lee *et al*., 2004a
PaWB	I-D	I-D	AY265206.1	AY264857.1	Lee *et al*., 2004a
IoWB	I-N	I-F	AY265205.1	AY264859.1	Lee *et al*., 2004a
BBS3	I-E	I-E	AY265213.1	AY264863.1	Lee *et al*., 2004a
STRAWB2	I-K	I-J	U96616	U96617.1	Lee *et al*., 2004a
CPh	I-C	I-C	AF222065	AY264862.1	Lee *et al*., 2004a
GD1	I-A	I-M	DQ112021	AY264864.1	Lee *et al*., 2006b
A. ladlawii	n. a.	n. a.	M23932	M74771	

n.a.= group not available, n.d.= group not defined

The PCR reaction mix and negative control were as described above. Thirty-eight PCR cycles were performed under the following conditions: 1 min (2 min for the first cycle) for denaturation step at 94°C, 2 min for annealing at 50°C, and 3 min (10 min for the last cycle) for primer extension at 72°C. PCR product separation, RFLP analyses using *Tru*I, *Tsp509*I, *Alu*I and *Dde*I (Fermentas, Vilnius, Lithuania), and visualization of PCR and RFLP products were performed as described above.

The rpF1C/rp(I)R1A amplified products of carrot samples 2006/1, 2006/5 and 2006/9 were purified, sequenced with primers rpF1C and rp(I)R1A, and deposited in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) as described above. These sequences were aligned with those of 24 representative '*Ca.* P. asteris' accessions available in GenBank (Table 1.1) using CLUSTALX program (Thompson *et al*., 1997) and BioEdit (Hall, 1999). A phylogenetic tree was constructed using MEGA version 4 (Tamura *et al*., 2007) with *Acholeplasma laidlawii*, as the out-group to root the tree. A database search of homologous sequences was also performed as described above.

Other chromosomal DNA fragments. Molecular analyses were carried out using the *tuf* gene coding the elongation factor Ef-Tu and the putative aa kinase gene and ribosomal recycling factor gene (Botti and Bertaccini, 2003) amplified using TufAYf/r (Schneider *et al*., 1997) and BB88F1/R1 (Gundersen *et al*., 1996) primer pairs, respectively. The PCR reaction mix and negative control were as described above. For both primer pairs 35 PCR cycles were performed under the following conditions: 30 sec for denaturation step at 95°C, 30 sec for annealing at 55°C, and 1 min for primer extension at 72°C. PCR products were visualized as described above. RFLP was done using *Tsp*509I and *Tru*I restriction enzymes on BB88F1/R1 and only *Tru*I restriction enzyme on *tuf* gene amplicons. The G35p/m primer pair was used with parameters described by Davis *et al*. (1992) to amplify a phytoplasma DNA helicase gene (Duduk and Bertaccini, 2006). RFLP analyses were carried out using *Tru*I and *Alu*I restriction enzymes. Visualization of RFLP products was performed as described above.

Further molecular analyses were performed at the Plant Pathology Department of the University of Tokyo on other two fragments of chromosomal DNA of carrot sample 2005/5 comparing sequences obtained with those of two MBS samples from Colombia and of 14 other phytoplasmas available in genBank (Table 1.2).

The molecular characterization was performed by PCR amplification using the ES-1/Nad-2 primer pair (Kakizawa *et al*., 2006a) that amplifies complete *groES*, *groEL*, *amp* and part of *nadE* genes and the Rnc-1/PssA-1 primer pair (Kakizawa *et al*., 2009) that amplifies complete *rnc*, *dnaD*, *imp*, *pyrG*, *psd* and *pssA* genes. The two analyzed fragments are respectively 3.6 and 5.0 kb long.

The PCR conditions were as described previously (Jung *et al*., 2002), except that LA-*Taq* (Takara) was used, the annealing temperature was 48°C, for 5 minutes. The ES-1/Nad-2 amplified products of carrot sample 2006/5 and MBSC1 from Colombia were purified using ExoSAP-IT (Amersham Bioscience) and sequenced with primers ES-1, EL-1, EL-2, EL-3, Amp-N1, Amp-3, Amp-C1, Nad-1 and Nad-2 (see list a below, Kakizawa *et al*., 2006a). The ES-1/Nad-2 sequences (3.6 kb) were aligned with those of 14 '*Ca.* P. asteris' strains available in GenBank (Table 1.2) using CLUSTALX program (Thompson *et al*., 1997) and BioEdit (Hall, 1999). A Neighbor-Joining phylogenetic tree was constructed using MEGA version 4 (Tamura *et al.*, 2007).

List a:

ES-1: 5'-TTGAGCTCGCGGCCGCATGAAACAAAAAACAATTATC-3'
EL-1: 5'-AGAATTCCATATGAGTAAAAAAATACTTTATGGCAA-3'
EL-2: 5'-AAATTAGTACTGTGCAAGAAA-3'
EL-3: 5'-TATCAAGAATTAAAAGATACT-3'
Amp-N1: 5'-AAGAATTCCATATGCAAAATCAAAAAACTCA-3'
Amp-3: 5'-CTGTTAAAGCTGTAGATGGTA-3'
Amp-C1: 5'-AAGAGCTCGAGTTATTTATTGTTTTTGTTTTTTTTAAC-3'
Nad-1: 5'-TACTCGAGGCGGCCGCCATTTTGCATACTATAAGCAT-3'
Nad-2: 5'-TTTGCCTGATTGAAACCATCT-3'

The Rnc-1/PssA-1 amplified products of carrot sample 2006/5 and MBSC1 from Colombia (5.0 kb) were amplified and purified as described above. Sequencing was carried out with primers Rnc-1, Imp-N1, Imp-N2, Imp-C1, Impw1, Impw2, Impw3, Impw4, Impw5, Impw6, Impw7 and PssA-1 (see list b below, Kakizawa *et al*., 2009. The *Imp* gene sequences were aligned with those of 14 '*Ca.* P. asteris' accessions available in GenBank (Table 1.2) and a Neighbor-Joining phylogenetic tree was constructed as described above.

List b:

Rnc-1: 5'-GGCATTATGACTAAAAAAAGAGCTCAAGC-3'
Imp-N1: 5'-AAGAATTCCATATGAAATTTTTAGCAACCAAACG-3'
Imp-N2: 5'-TTGAATTCCATATGAATCCTGTTAACTGGCTTTCTCC-3'
Imp-C1: 5'-TGAGCTCGAGTTTGGTTAAATTATTAACTTTTTC-3'
Impw1: 5'-TTACAAGAAATAGTTCAGTCAG-3'
Impw2: 5'-AAGTTTGGAATTAACATTGCC-3'
Impw3: 5'-AATCAACTAGCAATGAATACG-3'
Impw4: 5'-GRCAATTACAAAGTGAGCGAT-3'
Impw5: 5'-GTAAATGTAGCACATTGCGGG-3'
Impw6: 5'-TCATCCTTTGCAAACGTTTTC-3'
Impw7: 5'-GTTAAAAGTAACATGCAGGCAAAATGAAACC-3'
PssA-1: 5'- ATATCAGGCGTTTGTGATATGTTTGATGG-3'

Table 1.2 Phytoplasma amp and imp genes sequences used, retrieved from GenBank, including carrot samples 2006/5 from Serbia and MBSC1 from Colombia.

Phytoplasma strain	16Sr group	rp group	Accession number imp	amp	Reference
Carrot 2006/5	n.d.	n.d.	-	-	Unpublished
MBSC1	I-B	I-L	-	-	Unpublished
OY-NIM	I-B	n.d.	AB469008	AB124808	Kakizawa *et al*., 2006a; 2009
OY-W	I-B	n.d.	AB469007	AB124806	Kakizawa *et al*., 2006a; 2009
OY-M	I-B	n.d.	AP006628	AB124807	Oshima *et al*., 2004; Kakizawa *et al*., 2006a
PaWB	I-D	I-D	AB469010	AB124810	Kakizawa *et al*., 2006a; 2009
MD	I	n.d.	AB469009	AB124809	Kakizawa *et al*., 2006a; 2009
AYWB	I-A	n.d.	CP000061		Bai *et al*., 2006
PvWB	n.d.	n.d.	AB469011	AB242237	Kakizawa *et al*., 2006a; 2009
IPY	n.d.	n.d.		AB242234	Kakizawa *et al*., 2006a
AYBG	n.d.	n.d.		AB124811	Kakizawa *et al*., 2006a
LeY	n.d.	n.d.		AB242233	Kakizawa *et al*., 2006a
TY	n.d.	n.d.		AB242232	Kakizawa *et al*., 2006a
MarY	n.d.	n.d.		AB242235	Kakizawa *et al*., 2006a
ED	n.d.	n.d.		AB242231	Kakizawa *et al*., 2006a
SWB	n.d.	n.d.		AB242236	Kakizawa *et al*., 2006a
PPT	n.d.	n.d.		AB167357	Kakizawa *et al*., 2006a
WX	III-A	III-B	AF533231		Liefting and Kirkpatrick, 2003;
'*Ca*. P. mali'	X-A	X-B	AJ011678		Berg *et al*., 1999;
'*Ca*. P. pyri'	X-C	n.d.	AF400588		Morton *et al*., 2003
PYLR	X-C	n.d.	AF400589		Morton *et al*., 2003
'*Ca*. P. prunorum'	X-B	n.d.	AF400587		Morton *et al*., 2003;
PWBK	n.d.	n.d.	AB469013		Kakizawa *et al*., 2009
RYD	XI-A	n.d.	AB469012		Kakizawa *et al*., 2009
TWB	III	n.d.	AB469014		Kakizawa *et al*., 2009
'*Ca*. P. australiense'	XII-B	n.d.	AM422018		Tran-Nguyen *et al*., 2008

n.d.= not defined

Cloning / sequencing of a phytoplasma strain from carrot

The P1/P7 amplicon of carrot sample 2006/5 was purified using Qiagen PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to manufacturers instructions, quantified by GenQuant reading (Pharmacia) and electophoresis on agarose gel. Cloning was then carried out by the InsT/Aclone PCR Product Cloning Kit (Fermentas, MBI, Vilnius, Lithuania) employing Stratagene competent cells *Escherichia coli* (strain DH5α) ready to use.

Recombinant selection was carried out on LB medium Petri dishes containing 50 µg/ml ampicillin, 40 µl IPTG 0,1 M in distilled water and equal volume of N,N-dimetilformammide of X-Gal 20mg/ml. White recombinant colonies were collected with sterile tips and suspended in 0.5 ml Eppendorf tubes containing 10 µl sterile, filtered, distilled water. After 5 minutes incubation at 95°C PCR amplification with universal primers M13f/r was carried out. These

primers anneal on *LacZ* gene of the recombinant plasmid downstream and upstream of the putative insert. Below the sequences of primers employed for the screening of recombinant colonies:

M13f: 5'-CAG GAA ACA GCT ATG AC-3'
M13r: 5'-GTA AAA CGA CGG CCA GT-3'

Cycle for PCR amplification encloses: 5 minutes at 95°C, followed by 30 cycles of 1 minutes at 94°C, 30 seconds at 55°C and 2 minutes at 72°C with a final extension of 10 minutes at 72°C. The electrophoresis of amplicon aliquots carried out in agarose gel 1% in TA buffer followed by ethidium bromide straining and observation at UV light under transilluminator allow to identify colonies containing inserts of the expected size.

Further nested PCR amplification on M13f/r amplicons diluted 1:30 in sterile distilled water with P1/P7 primers followed by RFLP analyses with *Taq*I restriction enzyme allow to confirm the cloning results and select clones for sequencing. The two selected colonies showing different expected RFLP profiles were again amplified with M13f/r primers and cleaned amplicons sequenced in both directions with the same primers using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK).

Distance matrix

2006/5 and 2006/9 carrot phytoplasma 16SrDNA sequences were compared with other 16SrI-B phytoplasma sequences and some other 16SrI strains which clustered in 16SrI-B clade. The number of base substitutions per site from analysis between sequences is shown in Table 1.2. All the results are based on the pairwise analysis of 14 sequences. Analyses were conducted using the Maximum Composite Likelihood method in MEGA4 (Tamura *et al*., 2004; 2007). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 1,542 positions in the final dataset.

1.3. Results

PCR amplification and sequence analyses

16S ribosomal DNA carrots. PCR reactions with P1/P7 and R16(I)F1/R1 primer pairs resulted in amplification of the expected fragment length of about 1,800 and 1,100 bp respectively from all of the symptomatic carrot samples tested. No amplification was obtained from the asymptomatic samples tested.

The restriction profiles obtained with amplicons P1/P7 using *Hha*I restriction enzyme (Fig. 1.2, and data not shown), as well as with R16(I)F1/R1 amplicons using *Hha*I and *Tru*I restriction enzymes allowed different groups of profiles to be distinguished; one of them with both amplicons was indistinguishable from the reference strain CHRY, which belongs to ribosomal subgroup 16SrI-A, while the other profile was indistinguishable from the reference strain EAY, belonging to ribosomal subgroup 16SrI-B.

However, the restriction profiles obtained with amplicons P1/P7 using *Tsp509*I restriction enzyme (Fig. 1.2) allowed three different groups of profiles to be distinguished (Table 1.3). One of the profiles was consistent with the two described above, while those of samples 2006/5 and 2006/6 were not identical to any employed reference strain. The restriction profiles of all samples obtained with amplicons P1/P7 but one, using *Taq*I restriction enzyme (Fig. 1.2) showed no polymorphism, while the profiles of samples 2006/5 and 2006/6 were different from the others and from all phytoplasma controls employed. The restriction profiles obtained with the same amplicons from all carrot samples but one using *Tru*I restriction enzyme showed no polymorphism, the different sample showed restriction profile referable to the reference strain STOL, belonging to 16SrXII-A ribosomal subgroup (Fig. 1.3).

Figure 1.2. Polyacrylamide gel 5% showing the *Hha*I, *Tsp509*I and *Taq*I RFLP patterns of phytoplasma 16SrDNA plus spacer region amplified with P1/P7 primer pair from carrot samples and from phytoplasma reference strains in periwinkle. Sample abbreviations: 2006/1-2006/15, carrot samples; CA, carrot yellows (16SrI-C); CHRY, *Chrysanthemum* yellows (16SrI-A); CVB, *Catharanthus* virescence (16SrI-F); KVF, clover phyllody from France (16SrI-C); EAY, European aster yellows (16SrI-B); PRIVA, primula yellows (16SrI-L); STOL C, stolbur from pepper from Serbia (16SrXII-A); phiX174, marker phiX174 *Hae*III digested: fragment sizes in base pairs from top to bottom: 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72; pBR322, marker pBR322 *Hae*III digested; fragment sizes in base pairs from top to bottom: 587; 540; 502; 458; 434; 267; 234; 213; 192; 184; 124; 123; 104; 89; 80; 64; 57 and 51.

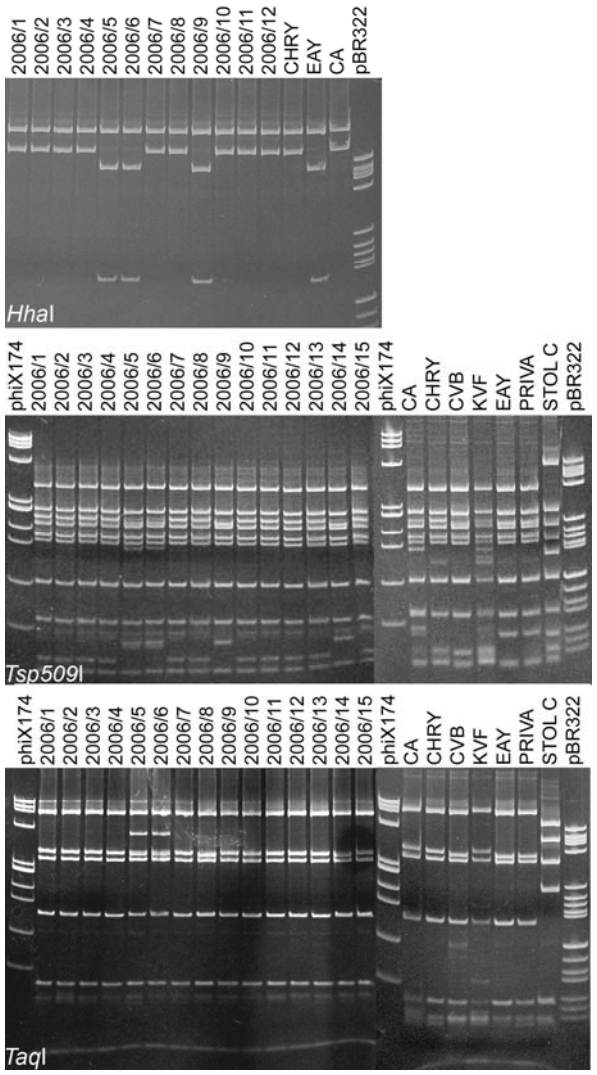
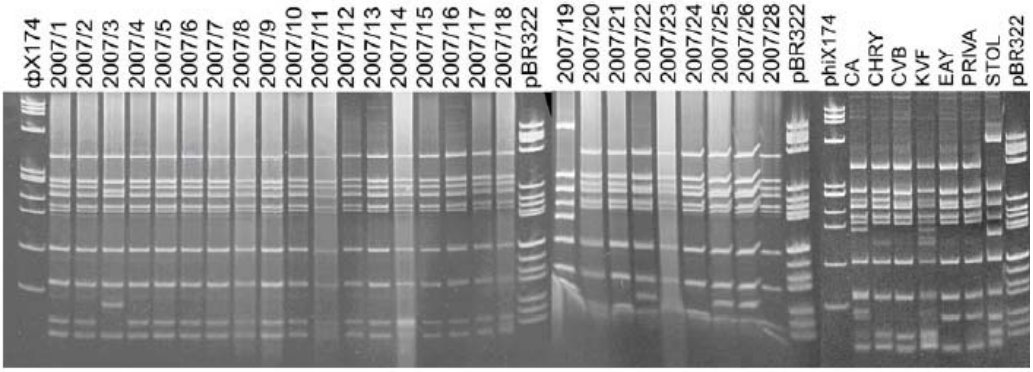


Figure 1.3. RFLP picture of polycrylamide 5% gel of P1/P7 amplicons from carrot samples digested with *Tsp509*I. 2007/1-2007/28: carrot samples; abbreviations of phytoplasma control strains are as in Fig. 1.2. ϕX174: marker phiX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72; pBR322: marker pBR322 *Hae*III digested; fragment sizes in base pairs from top to bottom: 587; 540; 502; 458; 434; 267; 234; 213; 192; 184; 124; 123; 104; 89; 80; 64; 57 and 51.



Sequencing of 16SrDNA plus spacer region of selected carrot samples. The sequences obtained from carrot samples 2006/1 and 2006/9 (1,736 bp and 1,720 bp respectively) were deposited in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) under accession numbers EU215424 and EU215426. The DNA sequence chromatogram of sample 2006/5 clearly indicated ambiguous bases, and this sequence was therefore employed for cloning and, after sequencing of the cloned amplicons, employed for phylogenetic analyses (see below).

The 16Sr DNA plus spacer sequence of sample 2006/1 showed the highest identity value of 99% with aster yellows phytoplasma strains from Lithuania and Ohio (USA) (AF510323.1, CP000061.1, respectively), while the same region of sample 2006/9 showed the highest identity value of 99% with aster yellows phytoplasma strains from Hawaii (USA), Lithuania and Japan (AY665676.1, AY102274.1, and AP006628.1, respectively).

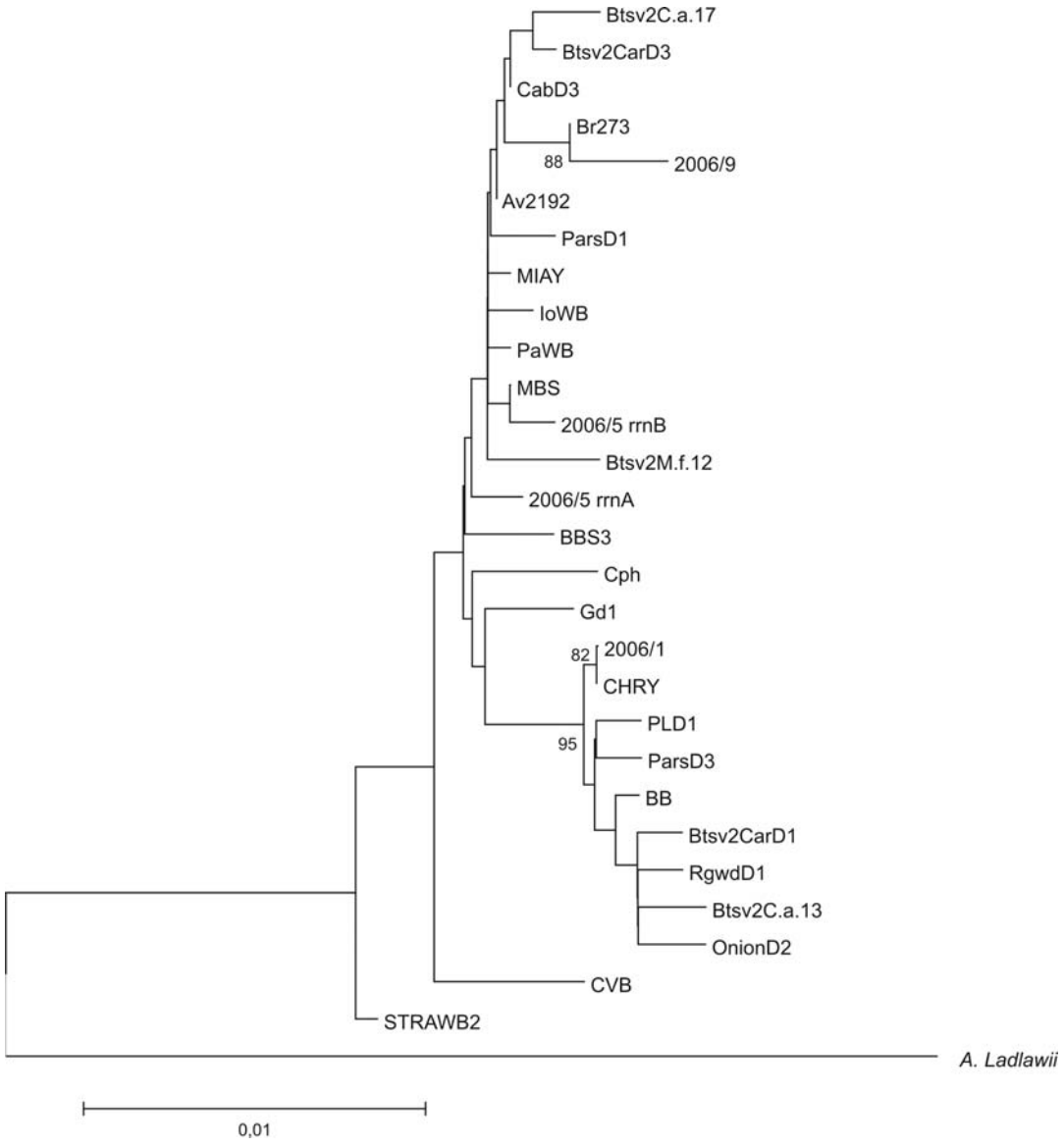
A similar comparison of the 16S rRNA gene alone of samples 2006/1 and 2006/9 with 24 strains of aster yellows phytoplasmas (Table 1.1) confirmed that the phytoplasma detected in carrot sample 2006/1 was most closely related to aster yellows phytoplasma reference strains belonging to the 16SrI-A ribosomal subgroup, while the phytoplasma detected in carrot sample 2006/9 was closely related to phytoplasmas of the 16SrI-B ribosomal subgroup as defined by Lee *et al*. (1998a; b) (data not shown).

Cloning / sequencing of 2006/5 strain from carrot. The sequences obtained from carrot sample 2006/5 (named rrnA and rrnB of 1,829 bp and 1,828 bp, respectively) were deposited in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) under accession No. EU215425 and GQ175789 respectively.

Phylogenetic comparison of the 16S rRNA gene alone of samples 2006/1, 2006/5 and 2006/9 with 24 representative strains of phytoplasmas from aster yellows ribosomal group indicated that the phytoplasma detected in carrot samples 2006/1 and 2006/9 can be enclosed respectively in the 16SrI-A and 16SrI-B ribosomal subgroups as defined by Lee *et al*. (1998a; b).

The two operons separated with the cloning in sample 2006/5 resulted to have two quite differentiable sequences. The sequence 2006/5rrnB was found to be very closely related to maize bushy stunt phytoplasma from the GenBank (AY265208), which belong to 16SrI-B ribosomal subgroup, while the sequence 2006/5rrnA obtained from same sample clustered separately from 16SrI-B group phytoplasmas relatively far from both 16SrI-B and 16SrI-A subgroup phytoplasmas (Lee *et al*., 2004a) (Fig. 1.5).

Figure 1.5. Phylogenetic tree constructed using neighbor joining algorithm of 16SrDNA sequences from carrot samples from Serbia, one broccoli sample from Serbia, 11 samples from Lee *et al*., 2003 and 12 reference strains, employing *Acholeplasma laidlawii* as the outgroup. Phytoplasma strains are described in Table 1.1.



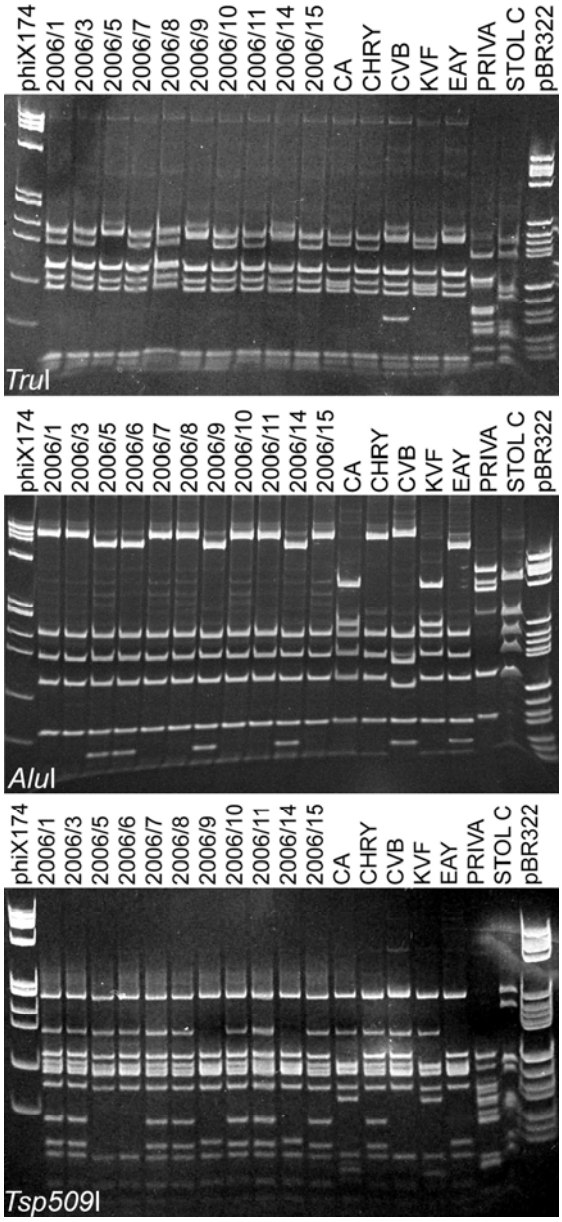
Among these sequences described (Table 1.1) twelve, all belonging to phytoplasmas enclosed in 16SrI-B subgroup clade, were selected for further evaluation to estimate the evolutionary divergence (Table 1.3). Calculation of the number of base substitutions per site values of these 16S rDNA sequences plus those of the two operons of sample 2005/5 showed the average number of 0.003 and varied from 0 to 0.009. The value of comparison between the two sequences obtained after cloning from sample 2006/5 was 0.004 (Table 1.3).

Table 1.3. Estimates of evolutionary divergence among sequences clustered in 16SrI-B subgroup clade.

	1	2	3	4	5	6	7	8	9	10	11	12	13
Br273													
MBSC1	0.001												
MIAY	0.001	0.001											
AV2192	0	0.001	0.001										
Btsv2C.a.17	0.003	0.004	0.004	0.003									
Btsv2M.f.12	0.003	0.004	0.004	0.003	0.007								
IoWB	0.001	0.002	0.002	0.001	0.005	0.005							
ParsD1	0.002	0.003	0.003	0.002	0.005	0.005	0.003						
2006/9	0.001	0.006	0.006	0.005	0.007	0.009	0.007	0.006					
2006/5 rrnA	0.002	0.003	0.003	0.002	0.005	0.005	0.003	0.004	0.007				
2006/5 rrnB	0.002	0.001	0.003	0.002	0.005	0.005	0.003	0.004	0.007	0.004			
Btsv2CarD3	0.001	0.003	0.003	0.001	0.003	0.005	0.003	0.004	0.006	0.004	0.004		
PaWB	0.001	0.001	0.001	0.001	0.004	0.004	0.002	0.003	0.006	0.003	0.003	0.003	
CabD3	0	0.001	0.001	0	0.003	0.004	0.002	0.003	0.005	0.003	0.003	0.001	0.001

Ribosomal protein genes. The PCR assays with rpF1C/rp(I)R1A primer pair resulted in amplification of the expected fragment length of about 1,200 bp from all symptomatic carrot samples tested. RFLP analyses with *Tru*I and *Alu*I restriction enzymes (Fig. 1.6) produced two different restriction profiles, one of which was indistinguishable from that of reference strain CHRY (16SrI-A), while the other was indistinguishable from that of reference strain EAY (16SrI-B). RFLP analyses of the same amplicons with *Dde*I restriction enzyme showed no polymorphism (Table 1.4). The RFLP analyses with *Tsp509*I restriction enzyme produced three different restriction profiles, one of which was indistinguishable from that of reference strain CHRY, the second was indistinguishable from that of reference strain EAY, while the third did not match with any profile of 16SrI reference phytoplasmas employed (Fig. 1.6, and Table 1.4).

Figure 1.6. Polyacrylamide gel 5% showing the *Tru*I, *Alu*I and *Tsp509*I RFLP patterns of phytoplasma *l22* and *s3* genes amplified with rpF1C/rp(I)R1A primer pair from carrot samples and from phytoplasma reference strains in periwinkle. Sample abbreviations: 2006/1-2006/15, carrot samples; see Fig. 2 for abbreviations of reference strains; phiX174, marker phiX174 *Hae*III digested: fragment sizes in base pairs from top to bottom: 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72; pBR322, marker pBR322 *Hae*III digested; fragment sizes in base pairs from top to bottom: 587; 540; 502; 458; 434; 267; 234; 213; 192; 184; 124; 123; 104; 89; 80; 64; 57 and 51.



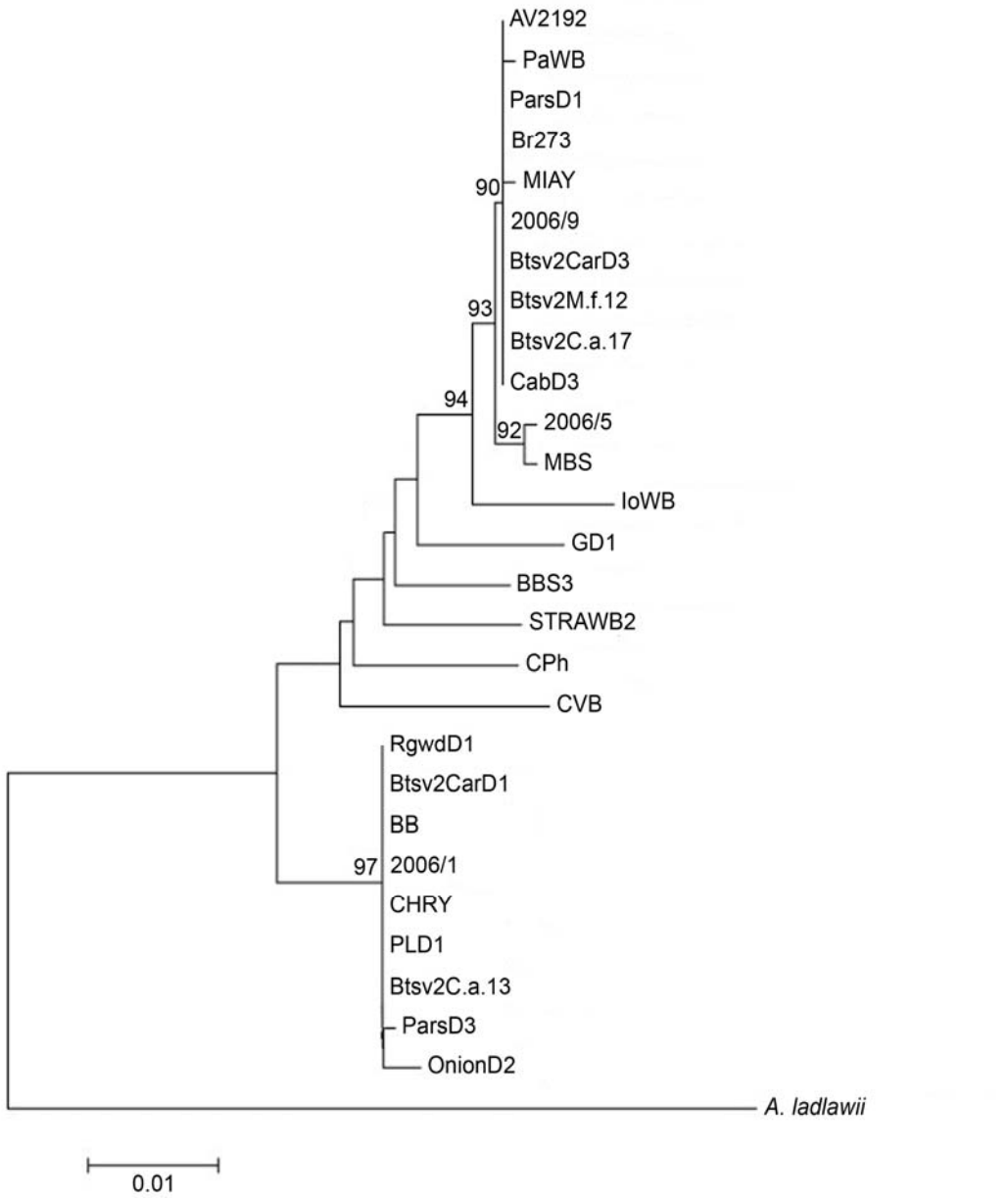
The sequences obtained from samples 2006/1, 2006/5 and 2006/9 (1,137 bp, 1,123 bp, and 1,132 bp respectively) were deposited in the NCBI, under accession numbers EU215428, EU215429 and EU215430.

The 3'end of the *s19* gene and the complete *l22* and *s3* genes of sample 2006/1 was

identical with aster yellows phytoplasma strains from *Chrysanthemum* (AY264869.1) and *Plantago coronopus* from Germany (AY264867.1), and with false ragweed (AY183705.1, AY183704.1), prickly lettuce (AY183702.1, AY183697.1), carrot (AY183694.1, AY183692.1, AY183691.1), *Ceratagallia abrupta* (AY183693.1, AY183689.1, AY183688.1) and tomato from Texas (AY183686.1) and lettuce (CP000061.1).

On the other hand the sequence of the same amplicon from sample 2006/5 showed the highest identity value of 99% with maize bushy stunt phytoplasma strain MBS from Mexico (AY264858), while the same region of sample 2006/9 was identical with Texas aster yellows phytoplasma strains from cabbage (AY183717.1), *Ceratagallia abrupta* (AY183716.1), *Scaphytopius irroratus* (AY183715.1, AY183712.1), *Macrosteles fascifrons* (AY183711.1) and carrot (AY183710.1).

Figure 1.7. Phylogenetic tree constructed using neighbor joining algorithm of ribosomal protein operon sequences from carrot samples from Serbia, one broccoli sample from Serbia, 11 samples from Lee *et al*., 2003 and 12 reference strains, employing *Acholeplasma laidlawii* as the outgroup. Phytoplasma strains are described in Table 1.1.



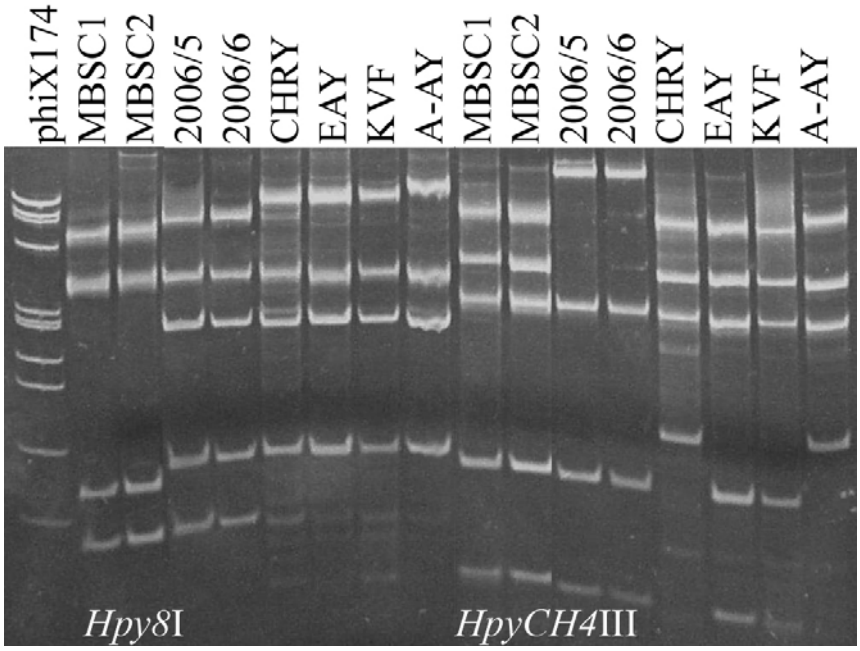
Phylogenetic comparison of the *l22* and *s3* genes of samples 2006/1, 2006/5 and 2006/9 with 24 representative strains of phytoplasmas from aster yellows ribosomal group indicated that the phytoplasmas detected in carrot samples 2006/1 and 2006/9 can be enclosed respectively in the rpI-A and rpI-B ribosomal protein subgroups as defined by Lee *et al*. (1998a; b), while sample 2006/5 was found to be very closely related to maize bushy stunt phytoplasma, which belong to rpI-L ribosomal protein subgroup (Lee *et al*., 2004a) (Fig. 1.7).

Aligned putative restriction site maps for *Hpy8*I and *HpyCH4*III restriction enzymes of

carrot samples 2006/1, 2006/5 and 2006/9, and of phytoplasma strains MBS revealed characteristic restriction profiles differentiating among these strains (data not shown).

Real RFLP analyses with both *Hpy8*I and *HpyCH4*III restriction enzymes (Fig. 1.8) confirmed the virtual results differentiating carrot samples from MBSC1 and MBSC2 samples from Colombia and also collectively from all the reference phytoplasma strains employed.

Figure 1.8 Polyacrylamide gel 5% showing the *Hpy8*I and *HpyCH4*III RFLP patterns of phytoplasma *l22* and *s3* genes amplified with rpF1C/rp(I)R1A primer pair from carrot samples and from phytoplasma reference strains in corn and periwinkle. Sample abbreviations: MBSC1 and MBSC2, Maize bushy stunt from Colombia; 2006/5 and 2006/6, carrot samples; see Fig. 2 for abbreviations of reference strains; phiX174, marker phiX174 *Hae*III digested: fragment sizes in base pairs from top to bottom: 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72; pBR322, marker pBR322 *Hae*III digested; fragment sizes in base pairs from top to bottom: 587; 540; 502; 458; 434; 267; 234; 213; 192; 184; 124; 123; 104; 89; 80; 64; 57 and 51.



Other chromosomal DNA fragments. PCR with the primers TufAYf/r and BB88F1/R1 resulted in amplification of the expected fragment length of about 1,200 bp and 740 bp, respectively from all symptomatic carrot samples tested. RFLP analyses with *Tru*I restriction enzyme on TufAYf/r amplicons and with *Tsp509*I restriction enzyme on both amplicons provided two different groups of profiles (Table 1.4). One group of profiles with both amplicons was indistinguishable from strain CHRY, while the other group was indistinguishable from strain EAY. RFLP analyses with *Tru*I restriction enzyme on

BB88F1/R1 amplicon produced three different restriction profiles, one of which was indistinguishable from the profile of reference strain CHRY, the second was indistinguishable from reference strain EAY, while the third did not match any reference phytoplasma profile except with profiles of MBSC1 and MBSC2 samples from Colombia(Fig. 1.9).

PCR with G35p/m primer pair resulted in amplification of the expected fragment length of about 1,200 bp only from 9 carrots out of 15 symptomatic samples tested with this system that showed no polymorphisms in RFLP analyses with *Tru*I and *Alu*I restriction enzymes (Table 1.4).

Figure 1.9. Polyacrylamide gel 5% showing the *Tru*I RFLP patterns of phytoplasma putative aa kinase gene and ribosomal recycling factor amplified with BB88F1/R1 primer pair from carrot samples. Sample abbreviations: 2006/1-2006/15, carrot samples; see Fig. 2 for abbreviations of reference strains; MBSC1 and MBSC2, maize bushy stunt from Colombia; phiX174, marker ϕX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72. pBR322, marker pBR322 *Hae*III digested; fragment sizes in base pairs from top to bottom: 587; 540; 502; 458; 434; 267; 234; 213; 192; 184; 124; 123; 104; 89; 80; 64; 57 and 51.

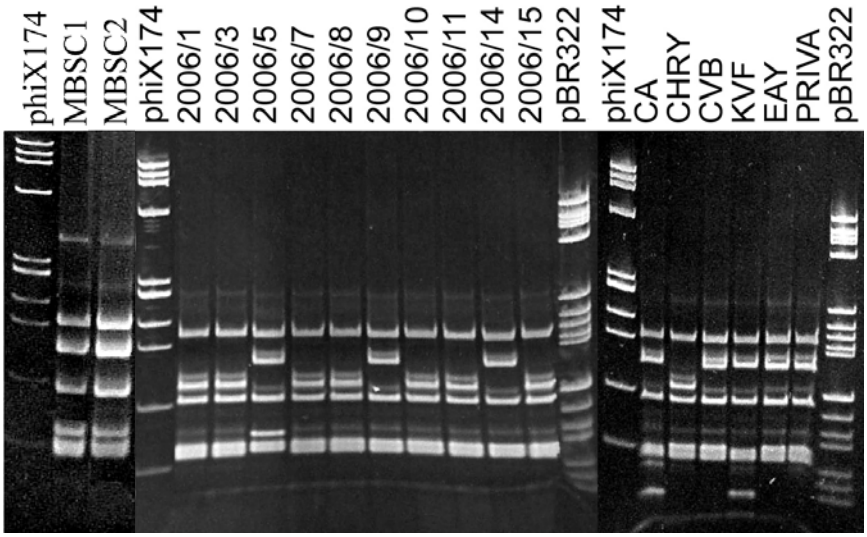


Table 1.4. PCR-RFLP characterization based on different genes of phytoplasmas from carrot samples.

Gene/ Pseudo gene	16S ribosomal in R16(I)F1/R1 plus spacer region (P1/P7)					Ribosomal protein *l22* and *s3*			*tuf*	*aak+rrf**		DNA helicase
Primers	P1/P7				R16(I)F1/R1	rpF1C/rp(I)R1A			TufAYf/r	BB88F1/R1		G35p/m
Strain**	*Hha*I	*Tru*I	*Tsp509*I	*Taq*I	*Hha*I, *Tsp509*I	*Alu*I	*Tsp*509I, *Tru*I	*Dde*I	*Tru*I, *Tsp509*I	*Tsp*509I	*Tru*I	*Tru*I, *Alu*I
2006/1	A	A	A	A	A	A	A	A	A	A	A	A
2006/2	A	A	A	A	A	A	A	A	A	A	A	A
2006/3	A	A	A	A	A	A	A	A	A	A	A	-
2006/4	A	A	A	A	A	A	A	A	A	A	A	-
2006/5	B	A	C	B	B	B	C	A	B	B	C	-
2006/6	B	A	C	B	B	B	C	A	B	B	C	-
2006/7	A	A	A	A	A	A	A	A	A	A	A	A
2006/8	A	A	A	A	A	A	A	A	A	A	A	A
2006/9	B	A	B	A	B	B	B	A	B	B	B	A
2006/10	A	A	A	A	A	A	A	A	A	A	A	A
2006/11	A	A	A	A	A	A	A	A	A	A	A	A
2006/12	A	A	A	A	A	A	A	A	A	A	A	A
2006/13	A	A	A	A	A	A	A	A	A	A	A	A
2006/14	B	A	B	A	B	B	B	A	B	B	B	-
2006/15	A	A	A	A	A	A	A	A	A	A	A	-

*, aaK, aminoacid kinase; rrf, ribosomal recycling factor;

**, in bold strains sequenced

A, B, C, distinct RFLP profiles;

Amplification, sequencing and alignment of Amp and Imp genes. The amplification of Amp genes from carrot strains 2006/5 and maize bushy stunt strain MBSC1 resulted in expected length DNA that were aligned and produced two 3.7 bp sequences:

Carrot 2006/5 amp 3.7 kb sequence

TTTTGAGCtcgCGGCCGCATGAAACAAAAAACAATTATCCCTTTACATGACAATGTTGTTTTAAAATTGAAAATGGAAGAAACTAAAACAGCTAGCGGAATT
ATTTTGGCTTTATCAGAAAAAGAAAAATCCTCTGTTGGGGTTGTTGTTGGAGTAGGTTCCAAAGTTGAAGGTTTAAAAAAAGATGATGAAGTAGTTTATAA
AAGCTATTCTGGTAGCAAAGTTACTTTAGGAGAAAAAGATTATTTAATTGTTGCTGTAAAAGATATTTTGGCACAAATTAAATAAGATAATATAGATAAGAT
AAATAATTATAAACTAAGTATTAATAAATAAAAAGACAATATTTTAAACTTTTTTAAAATTATAAATCATAAAATTATAAATATTAATTAAATATCAATAAATC
AATAAATAAAGATATAATAATCAATAATCAAAATATTTAAGGAGTAATAAAATGAGTAAAAAAATACTTTATGGCAAAGAAGCAAGAAAAGCTTTATTACA
AGGAGTAGATGAAATTGCAAATACTGTTAAAGTGACTTTAGGACCTAAAGGACGTAATGTTATTTTAGAAAAAGCCTATGATTCACCTGCTATTGTAAATG
ATGGTGTTTCTATTGCTAAAGAAATTGAATTAAAAAATCCTTATCAAAATATGGGAGCAAAGTTAGTATATGAAGTAGCTTCCAAAACTAACGATAAAGCA
GGAGATGGAACAACTACRGCAACTGTTTTGGCACAAAGTATGATTCATCGTGGGTTTGATGCAATTGATGCAGGAGCTAATCCTGTTTTAGTAAAAGAAGG
AATTGAGTTAGCAGCATTAACAGTTGCCAAAAAACTTTTAGTTAAATCTAAAAAAGTAGACGCCCAAGAAGATATTCAAAATGTGGCTGCTGTTTCATCAG
GTAGTCAAGAAATTGGTAAAATCATTGCCCAAGCGATGCAAAAAGTAGGAAAAGATGGAGTTATTAATGTTGATGAATCCAAAGGTTTTGAAACAGAATT
AGAAGTTGTTGAAGGATTGCAGTACGATAAAGGATATGCTTCTCCTTATTTTGTCTCTGATAGAGAAAGTATGACAGTACAGTTAGAAAATGCGTTAGTTTT
AGTAACTGATCATAAAATTAGTACTGTGCAAGAAATTGTACCTATTTTGGAAGAAGTAGTAAAAGCATCTAGACCTTTATTAATTGTAGCTGAAGCTGTGGA
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CTAATTATAAAGAATTTAGCGAAAAAAGATGGTTTC

MBSC1 amp 3.7 kb sequence

ggcCGCATGAAACAAAAAACAATTATCCCTTTACATGACAATGTTGTTTTAAAATTGAAAATGGAAGAAACTAAAACAGCTAGCGGAATTATTTTGGCTTTAT
CAGAAAAAGAAAAATCCTCTGTTGGGGTTGTTGTTGGAGTAGGTTCCAAAGTTGAAGGTTTAAAAAAAGATGATGAAGTAGTTTATAAAAGCTATTCTGGT
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GAAGTTTTAGGGGTTTTGGTAGCTAATAAATTAAGAGGAACTTTTAATGTAGTTGTAACTAATGCTCCTGGTTTTGGTGATAATCAAAAAGAAATGTTACAA
GATATTGCAGTACTTACAAAAGCTAATTTTGTTTCTAAAGAACTTAATATGAAATTAGCAGATTTAAAAATGGATGATTTAGGAAATATCAATAAAGCTATT
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GTAACAAAAGATTCTGTAAAAGTAGCGGTTTCTGATAATAAAGTAACAGTTACACCTGCAGATGGTGATGCTGGAAAAGCTTTATCAGGTTCAAAAGTATT
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AAATATTTATCAATTTATTTAACATCATAAAAAATTATTAAATTATTTAAATATAAACAATAAAAATTATAGTTATTATTATTAATTATCTTAATAAAAAATATT
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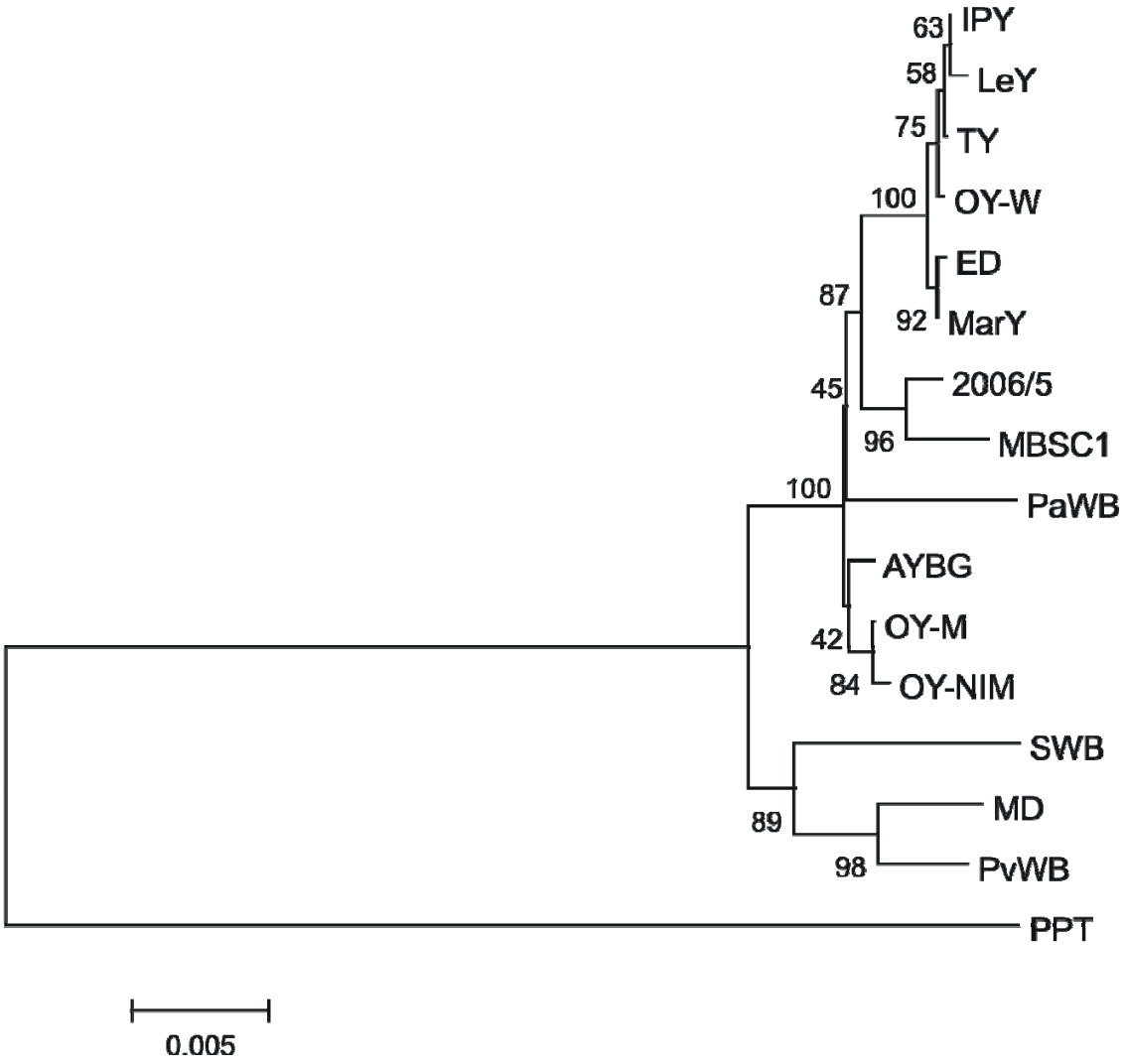
TATATTTTAGGTATGCCTCTTGCTTTGCATGAAA

The phylogenetic tree constructed using 3.7 kb sequences allowed to confirm the very close relationship between the carrot strain and the MBSC1 samples that resulted very closely associated also for the Amp gene.

Fig. 1.10. Phylogenetic tree constructed using neighbor joining algorithm of amplified ES-1/Nad-2 sequences from carrot sample 2006/5 from Serbia, one MBSC1 from Colombia and 14 reference phytoplasma sequences. Phytoplasma sequences are described in Table 1.2.



The amplification of Imp genes from carrot strains 2006/5 and maize bushy stunt strain MBSC1 resulted in expected length DNA fragments that were aligned and produced two 5.0 bp sequences:

Carrot 2006/5 Imp 5 kb sequence

TCTTTTTCAAACCCTCAATATCACTCCCCAAAACCTTGCCCTTTACAAACAAGCTCTTACCCACAGCTCTTATTCTAACGAACAAAACCCACCCCAAGAAGACA
ACGAAAGACTTGAATTTTTAGGAGATGCCATCGTAGGCTTACTTATGGCTGACTATCTTTATTCCCAATCCAAAGAAGATGAAGGCATTATGACTAAAAAAA
GAGCTCAAGCGGTGTGTGAAAGGTCACTTACCATCTACGCTCAAAACATTGAATTGCAAAATTATCTTCTTTTAGGCAAAGGCGAAAAAAACAAAGATTTT
AATGCTAAAAGTATTATGGCAGATACTTTTGAAGCCTTATTTGGTGCTATTTATCTTGATTTAGGATATTTGACAGCCAAAAAAGTTTTTCATAATATTGTCTT
ACCTCACTTAGCCAAAACTATCTATATTATTGATTTTAAAACTCAATTACAAGAAATAGTTCAGTCAGAAAAAAAAACTATCCAATATAAAATAGTTCAAGAG
CAAGGACCTGCTCATTCTAAAAATTTTGTAGCAGAAGTTTATTTAGAAAAAAATCTTTTAGGAACAGGTGAAGGCAGCACTAAAAAAGCCGCTGAGCAAAA
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CAAAAACTTTACGAAGATGGTTTTTTACAAGTAGAAAAAATGTTAATTCAAGAATACCAAAAATTAGGATTAACCTACCCTGAACTAACTATTTTGCTATTCT
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CAAAGGTTTTTTTTGACCTTTCCCAAGAAACTAAAAACCATAAAATTATTGAAGTTTTTTCACTACACCCCACTTTTGCCAAGCTAGAAAAAATCTATCAAGA
CCAAATTCGCGAATCCAAACGCCAAAACCAACTTACCTTAATCCAACAAACTATTGAATATTTAGAACACAACAAAGGACAAACTCTTACATCTAACGAATT
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CATCAACACTTTGCTTGGCAAAAAGAAATTTTCKACAGTTATNCCCGATGAAAAAGCAGACAAAGCCTTAGCAAAACTTTTTAAAAAAATAAAATAACACTC
AGTTTGGCAAACAAAAAGTTAAGCTTACTCTTTTTATGTGTTATAATTACTTATGTAATTATTTTTTTAGTATTTCAATTATTTTATTTATGAAAAATAACTAGT
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GGGTTCAGAGAAGGATTTATAAGTGATTGGATGGGGCTTTGTAGGAGTTATTTTTTTACCAATAACAGTTAAAATAGGGATTTGGGTTTCATATCTTTTTTG
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TCGGCTAAAGAATCAATTTGAATGCCATAGCTTTTTTCTGTATGACTGCGATTTTTTTTGGTTCGGCTTACCACACCATCAAACATATCACAAACGC

MBSC1 imp 5 kb sequence

GGTAATAAATATTTTAATAAGATTTTAAGCAACATAAATTGGATAAAATTTAGTGCTAATAATAAAATAATTGTTMTTGTAGTTTTGGGTTTNGGCTTGGGG
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GTCTGATGGCTTCTAAAAAAGGCGATGATTCAATGTCTCCTACGGTTCCTCCAATTTCTACAATAACAACATCTGATTTGTGAAAAAGAGCAGCATCAATTA
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CTCCTACTTCTATTTGAACTATTTTACCAAAATTTTTGGTTTGTAAGATACTATATTCGCGAGTGTTTGTTTTAAAAACATCAAAACTTTCAAAGGCAATGGGA
TTTACTGTATGTAATTTTCCTTTGATTTTGCAAGGAAGCATTTGAAAAAAACCTTCGTCTACAAAAAGATAACGGTGATAATCATGGGGTTCTAAGCGGAAA
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AAGAACTAAATTTTTGTTTTTCAAATAAGTTTAAATCAATTTGGTGTTGTTCGATGATTTTCAAAGCGTATTGGCGTGACCAAGGAGAATAAAAATACAAAT
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TGAGTCTTGACAAATAATTTTTCCTTCTAAATTAACTATTTTCATATTGTAAATCGCTCACTTTGTAATTGCCATTGGTAATAAATATTTTAATAAGATTTTAAG
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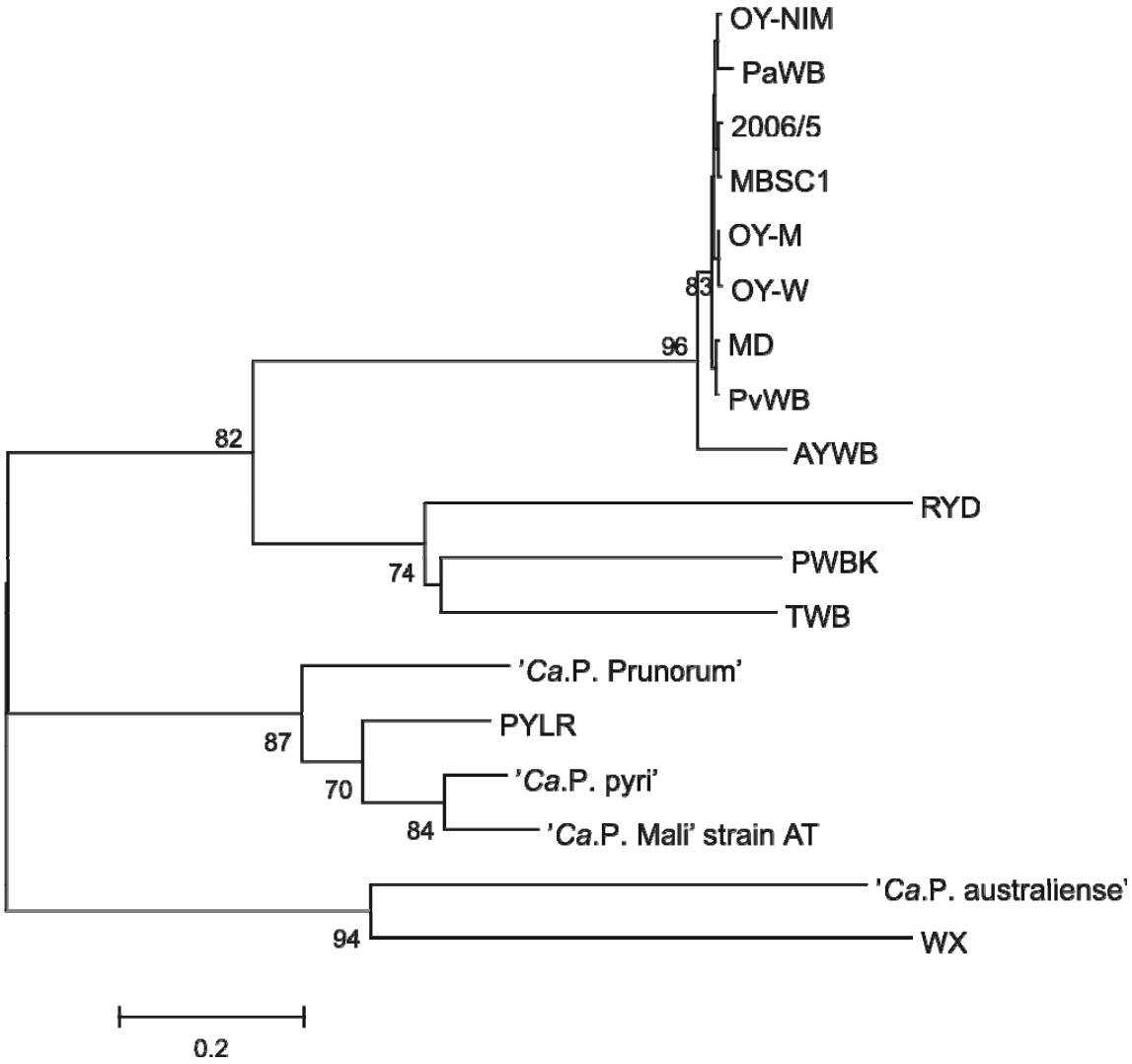
CTGCGATTTTTTTTGGTTCGGCTTACCACACCATCAAACATATCACAAAMAMC

The phylogenetic tree constructed using imp gene sequences allowed to confirm the very close relationship between the carrot strain 2006/5 and the MBSC1 sample that resulted very closely associated also for the Imp gene.

Fig. 1.11. Phylogenetic tree constructed using neighbor joining algorithm of imp gene sequences from carrot sample 2006/5 from Serbia, MBSC1 from Colombia and 14 reference phytoplasma sequences. Phytoplasma sequences are described in Table 1.2.



1.4. Discussion

RFLP analyses of 16S rDNA and of 16S rDNA plus spacer regions confirmed phytoplasma presence all symptomatic carrot sample tested. Phytoplasmas were identified as belonging to ribosomal subgroups 16SrI-A and 16SrI-B. They were detected in the same carrot field but never in mixed infection. Collective RFLP profiles on putative aminoacid kinase plus ribosome recycling factor, *tuf* and *rpS3* genes distinguished phytoplasmas belonging to the two 16SrI subgroups confirming results on 16S ribosomal gene.

Lack of amplification of putative DNA helicase gene of some carrot is in agreement with published data indicating that G35p/m primers failed to amplify 16SrI-A phytoplasmas as well as a number of 16SrI-B strains (Botti and Bertaccini, 2003). Since primers were developed from 16SrXII-A phytoplasmas (Davis *et al*., 1992), variability of this region could be a reason for inconsistent amplification of aster yellows-related phytoplasma strains.

The obtained results confirmed that the symptoms observed in carrot plants are associated with the presence of aster yellows phytoplasmas ('*Ca*. P. asteris'), in particular, with strains belonging to 16SrI-A and 16SrI-B subgroups. Phytoplasmas belonging to the same 16SrI subgroups had also been identified together with beet leafhopper-transmitted virescence agent (BLTVA) and *Spiroplasma citri* (SC) in North American carrot fields, where plants with symptoms similar to those observed in Serbia were described (Lee *et al*., 2006a). This study presents the first molecular identification of phytoplasmas in carrot in Serbia obtained with different molecular markers in order to characterize phytoplasma population infecting carrots in the field.

The results obtained in this work indicated the presence of three different phytoplasmas in symptomatic carrot: 16SrI-A, 16SrI-B and 16SrXII-A, confirming the previous phytoplasma finding in the same area (Duduk *et al*., 2007; 2009a), and also showing for the first time a stolbur infection in carrot.

The diverse phytoplasmas detected can not be differentiated by specific symptomatology as often reported for carrot, other vegetables, and woody hosts such as grapevine (Bertaccini *et al*., 1995, 1999; Lee *et al*., 2006b). Orenstein *et al*. (1999) and Weintraub and Orenstein (2004) monitored in Israel commercial and experimental carrot fields with the plants showing symptoms of phytoplasma disease and detected phytoplasmas belonging to 16SrI, 16SrIII and 16SrV ribosomal groups confirming that different phytoplasmas can infect carrot plants.

Based on the RFLP analyses on 16S ribosomal RNA gene, spacer region, and beginning of 23S ribosomal gene (P1/P7 amplicons), two carrot samples (2006/5 and 2006/6) were identified as members of group 16SrI, but collective RFLP profiles differed from those of 16SrI reference strains. The DNA fragments obtained with *Taq*I restriction enzyme were

approximately 0.8, 0.5, 0.4, 0.35, 0.15 kb in size, which is in total larger than P1/P7 expected PCR products (approximately 1.7 kb). That leads to a hypothesis that those profiles represent two differentiable ribosomal operons, and the hypothesis is supported by the DNA sequence chromatogram indicating the presence of several probable point mutations. The possibility that two phytoplasma strains are present in a sample as a mixed infection is unlikely, since the other analyzed genes clearly indicated the presence of a single sequence with unique RFLP pattern. Comparison of 16SrDNA of this sample with MBSC1 sample, that was the closely related strain for the majority of tested genes, has shown that the operon 2006/5 rrnB is the one which is closely related to MBSC1, while rrnA operon is different. This is in agreement with results of RFLP analyses because both 2006/5 rrnB and MBSC1, with all employed restriction enzymes, have shown restriction profiles referable to 16SrI-B subgroup, while 2006/5 rrnA operon, with several restriction enzymes, has shown different restriction profiles.

The presence of 16S rRNA interoperon sequence heterogeneity is not uncommon, and has been reported in 16SrI and several other ribosomal groups (Schneider and Seemüller, 1994; Liefting *et al*., 1996; Lee *et al*., 1998a; Jomantiene *et al*., 2002b; Jung *et al*., 2003c; Davis *et al*., 2003a). Although the difference in homology between two operons is relatively small (0.2-0.7%), when differences occur in restriction sites, misidentification or erroneous assignment of the same phytoplasma to two different 16S rRNA subgroups is possible because classification is mainly based on the RFLP analyses of 16SrDNA (Davis *et al*., 2003a; IRPCM, 2004). In those cases, cloning of PCR products and analyses of both rRNA operons separately was helping in understanding the problem (Davis *et al*., 2003a). However the use of other genes present as single copy in the phytoplasma genome can be more helpful in discriminating when different phytoplasma populations are present in mixed infection from when it is the case of interoperon sequence heterogeneity.

After cloning the strain 2006/5 two different sequences were obtained, correlating with two different *Taq*I restriction enzyme RFLP profiles. Comparison of sequence homology among the sequences clustered in 16SrI-B clade (Fig. 1.5) showed that 2006/5 cloned sequences, even if they have only 6 substitutions and 1 deletion in 16SrDNA (1,540 bp), are phylogenetically closer to some other 16SrI-B sequences than to each other. The phylogenetic tree is showing that while 2006/5rrnB is clustering in the 16SrI-B clade, most close to MBSC1 strain, 2006/5rrnA is clustering out of the 16SrI-B clade, what is in agreement with RFLP analyses that differentiated 2006/5rrnA from other described members of the 16SrI-B ribosomal subgroup.

Difference in homology of 0.004 reported here for the two operons of strain 2006/5 is inside the range (0.2-0.7%) already reported for difference between two operons of one phytoplasma, and also inside the range of homology found here for ribosomal subgroup 16SrI-B (0-0.009). In this case different operons of same phytoplasma can be affiliated to

different 16SrI subgroups according to RFLP analyses and this fact can be supported also by phylogenetic analyses. This situation lead to conclusion that 16S rDNA is a gene that is not reliable to use for subgroup differentiation for 16SrI, and in general for phytoplasma subgrouping; for that purpose other genes, more variable and present in only one copy in the phytoplasma genome, as those employed in the present research, can be used.

The presence of a close relationship between the carrot strain 2006/5 and the MBSC1 phytoplasma also for the Amp gene, suggests that there is the possibility to produce specific antisera for fast and mass detection of these pathogens in both crops (Arashida *et al*., 2008a).

Based on the RFLP analyses with *Tsp509*I restriction enzyme on ribosomal protein genes *l22* and *s3* [rpF1C/rp(I)R1A amplicons], all phytoplasmas within the subgroup 16SrI-A were classified as rp subgroup rpI-A, all phytoplasmas within the subgroup 16SrI-B were classified as rp subgroup rpI-B, while the two phytoplasmas showing interoperon heterogeneity were not classified at rpI subgroup level (Lee *et al*., 2003; Martini *et al*., 2007). The RFLP analyses with *Tru*I restriction enzyme on the putative aa kinase gene and ribosomal recycling factor (BB88F1/R1 amplicon) confirmed that these two phytoplasmas, in these genes, are also different from those already described as members of aster yellows group. These results confirm the importance of multiple gene analyses for differentiation among field-collected phytoplasma strains and are in agreement with results reported for 16SrI group phytoplasmas in periwinkle samples artificially infected (Botti and Bertaccini, 2003).

The RFLP analyses of *tuf* gene (TufAYf/r amplicon) is in agreement with the results obtained with 16Sr DNA, ribosomal protein genes and BB88F1/R1 amplicons showing no difference among the strains from carrot identified as belonging to 16SrI-A ribosomal subgroup and CHRY reference phytoplasma strain representing 16SrI-A ribosomal subgroup. On the contrary, the RFLP analyses on *tuf* gene showed no difference among the strains classified as 16SrI-B, the two strains not classified at the subgroup level and EAY reference phytoplasma strain representing 16SrI-B ribosomal subgroup.

Sequence homology, phylogenetic and virtual restriction analyses of ribosomal protein genes confirmed that 16SrI-A rpI-A and 16SrI-B rpI-B phytoplasmas from carrot samples cluster together with the strains described in the same ribosomal and ribosomal protein subgroup respectively, while the previously unreported strain shows major homology to maize bushy stunt phytoplasma and MBSC1 strain, from which it can however be distinguished.

These results are consistent with RFLP and virtual RFLP analyses, confirming that this strain is genetically close to 16SrI-B and rpI-L subgroups, but distinct from both. The fact that 16SrDNArrnB and ribosomal protein genes both shows major homology to maize bushy stunt phytoplasma, however this disease was never reported in corn in Serbia neither in Europe (Duduk and Bertaccini, 2006). Similar conclusions could be drawn from the very

strong phylogenetic relationships between sample MBSC1 and carrot 2006/5 the show how these strains are more closely related than all other tested belonging to the same 16SrI-B ribosomal subgroup.

Aster yellows phytoplasmas have been described to infect a wide variety of host plants worldwide and studies of 16Sr DNA alone in some cases of interoperon heterogeneity are not sufficient or suitable to resolve molecular differences among strains. In our case data obtained with RFLP and sequence analyses of less-conserved genes substantiated those obtained through RFLP and sequence analyses of rDNA (16S rRNA gene and 16S/23S rDNA spacer region).

However since interoperon sequence heterogeneity is present, the clear molecular differentiation and definition of these strains was achieved by studying other less conserved genes such as ribosomal protein, amp, imp or putative aa kinase gene and ribosomal recycling factor. These differences can be relevant for epidemiological studies that are the basis for controlling the spreading of phytoplasma diseases in the fields.

The identification of a new phytoplasma strain in carrot fields in which infection was high, but not at epidemic level, indicates that a certain amount of genetic mutation can occur under such conditions, leading to appearance of new strains with possibly different epidemic capacities and very closely related to a phytoplasma strain infecting corn in Colombia.

The use of more genes, in addition to 16Sr DNA, for phytoplasma classification was shown to be relevant for studying the epidemiology of other phytoplasma diseases such as "flavescence dorée" in which the presence of phytoplasma strains, differentiated on the basis of several genetic markers, was proved to be useful for determining their epidemic ability (Martini *et al*., 2002; Botti and Bertaccini, 2007, Arnaud *et al*., 2007). It is clear from the results presented here that more genes beside 16Sr DNA should be applied for phytoplasma identification, as it has already been suggested by other authors (Liefting *et al*., 1996; Lee *et al*., 2006b). For aster yellows phytoplasmas at least the rp and aa kinase genes plus ribosomal recycling factor can be used for this purpose but short conserved regions of amp and imp genes could be further explored for similar purposes.

Chapter 2

Phytoplasmas associated with corn reddening in Serbia

2.1. Introduction

Reddening of corn (*Zea mays* L.) was observed for the first time in 1957 in south and middle Banat region of Serbia in Europe (Marić and Savić, 1965). After the first sporadic appearance in 1957, the disease entered into an epidemic phase during the late 1950s and early 1960s. Another epidemic phase in the same region was reported forty years later, during the late 1990s and early 2000s. Between these epidemic phases the disease was always present sporadically in the region (Šutić *et al.*, 2003), although it appeared to be more widespread in dry years, and it was reported as the most important disease of corn in the region due to its impact on yield (Blaženčić, 1982; Šutić *et al.*, 2003).

During the epidemic phase, disease symptoms can be present in up to 90% of the plants, and yield losses can be over 50% (Starović *et al*., 2004). Symptoms of reddening begin in the second half of July on the main leaf midrib, then they spread to the stalk, and eventually affect the whole plant (Fig. 2.1), the most obvious symptoms are present in August and September (Šutić, 1983). Symptomatic plants have the same size and shape as asymptomatic ones, but disease reduces grain filling and corn cob weight. Symptomatic plants contain poor shriveled grains that ripen and dry earlier than asymptomatic ones.

The reddening symptoms observed on corn, the presence of recurrent epidemic outbreaks, and the characteristics of disease development, in particularly the fact that symptoms always appear first around the border of fields, suggesting infections from outside of the field through vectors, indicate a possible association of the syndrome with phytoplasmas, even though phytoplasma presence in corn has generally been associated with stunting symptoms.

Throughout the world, two diseases associated with *Mollicutes* have been reported in corn: maize bushy stunt (MBS) and corn stunt (CS), both present in North, Central, and South America (Harrison *et al*., 1996; Ebbert *et al*., 2001; Lee *et al.*, 2004). MBS is associated with

a phytoplasma that is a member of the aster yellows 16SrI-B ribosomal subgroup (Lee *et al*., 2004). CS is a disease caused by *Spiroplasma kunkelii* (Chen and Liao, 1975; Williamson and Whitcomb, 1975). These pathogens are both transmitted by the cicadellid leafhopper *Dalbulus maidis* and by other *Dalbulus* spp. (Legrand and Power, 1994; Moya-Raygoza and Nault, 1998; Ebbert *et al*., 2001).

Figure 2.1. Symptomatic corn plants (up and down left), shriveled corn knob on the left next to the healthy one on the right (down right).

Preliminary experiments indicating the possible infective nature of reddening of corn (Starović *et al*., 2004), and suggesting association of this syndrome with phytoplasmas by

other authors (Blaženčić, 1982; Šutić, 1974) stimulated research to verify the possibility of association of this disease with a phytoplasma.

Experiments performed during 2005 confirmed relation between stolbur phytoplasma and corn reddening syndrome (Duduk and Bertaccini, 2006). A role of *Reptalus panzeri* in epidemiology was also reported recently (Jović *et al*., 2007); moreover *Pentastiridius beierii* and *Macrosteles quadripunctulatus* were also reported as stolbur phytoplasma vectors in France and in Spain respectively (Gatineau *et al*., 2001; Battle *et al*., 2008).

2.2. Material and Methods

Sample collection and nucleic acid extraction

Sixteen samples of symptomatic, and 4 of asymptomatic corn plants were collected during the second half of August 2005 in the south Banat region of Serbia (Table 2.1). Total nucleic acids were extracted from 1 g of main leaf midribs and phloem stalk tissues following the protocol described by Prince *et al*. (1993), dissolved in TE buffer, and maintained at 4°C. Before performing polymerase chain reaction (PCR) assays, nucleic acids were quantified and diluted in sterile distilled water to a final concentration of 20 ng/µl.

Reference phytoplasma strains

The eighteen phytoplasmas used as reference strains were primula phyllody (PRIVA, ribosomal subgroup 16SrI-B), clover phyllody (KVG, ribosomal subgroup 16SrI-C), aster yellows from apricot (AY-A, ribosomal subgroup 16SrI-F), faba bean phyllody (FBPSA, ribosomal subgroup 16SrII-C), Green Valley X disease (GVX, ribosomal subgroup 16SrIII-A), elm yellows (EY-C, ribosomal subgroup 16SrV-A), *Catharanthus* phyllody from Sudan (CPS, ribosomal subgroup 16SrVI-C), ash yellows (ASHY1, ribosomal subgroup 16SrVII-A, '*Candidatus* Phytoplasma fraxini'), *Pichris echioides* yellows (PEY, ribosomal group 16SrIX), flower stunting (BVK, ribosomal subgroup 16SrXI-C), apple proliferation (AP-15, ribosomal subgroup 16rX-A, '*Ca*. P. mali'), German European stone fruit yellows (GSFY/1, ribosomal subgroup 16SrX-B, '*Ca.* P. prunorum'), pear decline (PD, ribosomal subgroup 16SrX-C, '*Ca*. P. pyri'), and stolbur from pepper from Serbia (STOL, ribosomal subgroup 16SrXII-A).

These strains represent the majority of ribosomal phytoplasma groups described in the literature (Lee *et al*., 1998a; b) and have been employed for phytoplasma identification in other studies. They were maintained in periwinkle [*Catharanthus roseus* (G.) Don.] (Bertaccini, 2003). Further phytoplasma positive controls were strain VE16, from grapevine cv. Chardonnay collected in Venice province, Italy; strains MO-2 and MO-47 from grapevine cv. Lambrusco collected in Modena province, Italy; and strain P1 from grapevine cv. Chardonnay collected in Serbia (Duduk *et al*., 2004). These samples represent the nucleic acids extracted from grapevine infected by 'Bois noir' disease phytoplasmas (ribosomal subgroup 16SrXII-A), and were only employed for restriction fragment length polymorphism (RFLP) characterization of the *tuf* gene, which is described below.

16S Ribosomal DNA

Detection of phytoplasmas was done using PCR assays with phytoplasma universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) in direct PCR reaction, followed by nested PCR with F1/B6 primers (Davis and Lee 1993; Padovan *et al*., 1995). Further nested PCR reactions on P1/P7 amplicons were performed with R16(I)F1/R1 primer pair, specific for phytoplasmas belonging to aster yellows (16SrI) (Lee *et al*., 1994), faba bean phyllody (16SrII) (Tolu *et al*., 2006) and stolbur (16SrXII) groups.

Each 25 µl PCR reaction mix contained 20 ng template DNA, 2.5 µl 10X PCR buffer, 0.8 U *Taq* polymerase (Polymed, Italy, EU), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.4 mM each primer. Samples lacking DNA were run as negative controls in each PCR reaction. One µl of amplicon from direct PCR, diluted 1: 30 in sterile distilled water, was used as template in nested PCR reactions. Thirty-five PCR cycles were performed under the following conditions: 1 min (2 min for the first cycle) for denaturation at 94°C, 2 min for annealing at 50°C, and 3 min (10 min for the last cycle) for primer extension at 72°C. Six µl of PCR products were separated in 1% agarose gel, stained with ethidium bromide and photographed under UV at 312 nm using a transilluminator.

Identification of detected phytoplasmas was done using RFLP analyses with *Tru*I and *Taq*I restriction enzymes on amplified ribosomal DNA sequences. Visualization of RFLP products was performed in a 5% polyacrylamide gel, stained with ethidium bromide and visualized with UV transilluminator.

P1/P7 amplified product (about 1,800 bp) of sample 2005/2 was purified using Qiagen PCR Purification Kit (Qiagen GmbH, Hilden, Germany, EU). The PCR product was sequenced in both directions using the BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). Sequence of the purified sample was obtained from PCR products

generated by amplification with primers P1, R16F2, and P7 in the Centro Ricerche Interdipartimentale Biotecnologie Innovative (C.R.I.B.I. Padua, Italy, EU). The sequences were then aligned using BLAST engine for local alignment (version Blast N 2.2.12). The gene sequence (1,648 bp) obtained was deposited in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) NCBI under accession No. DQ222972.

The public available alignments of '*Candidatus* Phytoplasma' species 16S rDNA sequences were downloaded from TreeBase (http://www.treebase.org/treebase/console.html, matrix accession No. M1788) and 16S rDNA sequences of additional phytoplasmas were aligned using BioEdit (Hall, 1999), which was also used to calculate sequence identity values for the spacer region.

Table 2.1. Phytoplasma 16S rDNA sequences retrieved from GenBank including DQ222972 - Corn sample 2005/2 from Serbia – and employed for phylogenetic analysis.

Accession number	Phytoplasma strain	Origin	Plant host	16S rRNA group[b]
AY265208	Maize bushy stunt (MBS)	Ohio (USA)	Corn	16SrI-B
AF487779	Maize bushy stunt (MBS)	Mexico	Corn	16SrI-B
M30790	'*Ca*. P. asteris'	Michigan (USA)	*Oenothera* spp.	16SrI-B
U15442	'*Ca*. P. aurantifolia'	Oman	Lime	16SrII-B
L04682	'*Ca*. P. pruni'[a]	California (USA)	Peach	16SrIII-A
U18747	'*Ca*. P. palmae'[a]	Florida (USA)	*Veitchia merrillii*	16SrIV
X80117	'*Ca*. P. cocostanzianae'[a]	Tanzania	*Cocos nucifera*	16SrIV
Y14175	'*Ca*. P. cocosnigerianae'[a]	Nigeria	*Cocos nucifera*	16SrIV
AF515637	'*Ca*. P. phoenicium'	Iran	Almond	16SrIX
AF122910	'*Ca*. P. ulmi'	New York (USA)	Elm	16SrV-A
AF305240	'*Ca*. P. ziziphi'	South Korea	Jujube	16SrV-B
X76560	'*Ca*. P. vitis'[a]	France (EU)	Grapevine	16SrV-C
AY390261	'*Ca*. P. trifolii'	Canada	Clover	16SrVI-A
AF092209	'*Ca*. P. fraxini'	New York (USA)	Ash	16SrVII-A
AF086621	'*Ca*. P. luffae'[a]	Taiwan	Luffa	16SrVIII-A
AJ542541	'*Ca*. P. mali'	Italy (EU)	Apple	16SrX-A
AJ542544	'*Ca*. P. prunorum'	Germany (EU)	Peach	16SrX-B
AJ542543	'*Ca*. P. pyri'	Germany (EU)	Pear	16SrX-C
X92869	'*Ca*. P. spartii'	Italy (EU)	*Spartium junceum*	16SrX-D
D12581	'*Ca*. P. oryzae'	Japan	Rice	16SrXI-A
AF248959	'*Ca*. P. solani'[a]	Serbia	Periwinkle from pepper	16SrXII-A
L76865	'*Ca*. P. australiense'	Australia	Grapevine	16SrXII-B
AF147708	'*Ca*. P. brasiliense'	Brazil	Hibiscus	16SrXV

DQ222972	Corn 2005/2	Serbia	Corn	16SrXII-A
AJ310849	'*Ca*. P. pini'	Germany (EU)	*Pinus sylvestris*	n.d.
AB010425	'*Ca*. P. japonicum'	Japan	Hydrangea	n.d.
X76431	'*Ca*. P. rhamni'	Germany (EU)	*Rhamnus frangula*	n.d.
AY135523	'*Ca*. P. allocasuarinae'	Australia	*Allocasuarina muelleriana*	n.d.
AJ550984	'*Ca*. P. cynodontis'	Italy (EU)	*Cynodon dactylon*	n.d.
AB054986	'*Ca*. P. castaneae'	South Korea	Chestnut	n.d.

[a] '*Candidatus*' names proposed at the X International Congress of the International Organization of Mycoplasmology, 1994, held in Bordeaux, France, but not yet formally described, reported here as incidental citations which do not constitute prior citations, according to rule 28b of the bacteriological code (Lapage *et al*., 1992).
[b] n.d.= ribosomal subgroup not determined.

A phylogenetic tree was then constructed using CLUSTALX program (Thompson *et al*., 1997) for aligning corn sample 2005/2 16S sequence with 16S ribosomal gene sequences from 29 representative strains of the genus '*Candidatus* Phytoplasma' (IRPCM, 2004); bootstrap analysis was also performed and replicated 100 times (Table 2.1). *Acholeplasma palmae*, a cultivable Mollicute that is phylogenetically related to phytoplasmas, was designated as the out-group to root the tree.

Corn 2005/2 phytoplasma spacer region sequence was compared to the spacer region sequences of *A. palmae* and of 12 other phytoplasma strains retrieved from GenBank and phylogenetically related to 16SrXII group phytoplasmas or to corn diseases associated phytoplasmas: Australian grapevine yellows (AGY, ribosomal subgroup 16SrXII-B); papaya dieback disease (PDD); Mexican periwinkle virescence (MPV, ribosomal group 16SrXIII); strawberry lethal yellows from Australia (StrawLY, ribosomal subgroup 16SrXII-B); strawberry green petals from Australia (StrawGP, ribosomal subgroup 16SrXII-B); maize bushy stunt (MBS, ribosomal subgroup 16SrI-B); barley deformation phytoplasma (BaD, ribosomal subgroup 16SrI-B); chinaberry yellows from Bolivia (CbY1); stolbur phytoplasma from pepper from Serbia (STOL, ribosomal subgroup 16SrXII-A); grapevine yellows phytoplasma from Chile clone b (GYb, ribosomal group 16SrXII); grapevine yellows phytoplasma from Spain (GY-S; ribosomal subgroup 16SrXII-A); grapevine yellows phytoplasma from Chile clone a (GYa; ribosomal subgroup 16SrXII-A) (Table 2.2).

Tuf gene

Further molecular characterization of detected phytoplasmas was carried out on the *tuf* gene. Direct PCR reaction with the Tuf1f/Tuf1r primer pair was followed by nested PCR with

TufAyf/TufAyr primer pair (Schneider *et al*., 1997). Direct PCR was performed for 35 cycles under the following conditions: 30 sec denaturation at 95°C, 30 sec annealing at 45°C and 1 min primer extension at 72°C. The conditions for nested PCR were the same except that the annealing temperature was 55°C (Schneider *et al*., 1997); each 25 µl PCR of master mix was as described above. Six µl of PCR products were separated in 1% agarose gel and PCR products were subjected to RFLP analyses with *Tru*I and *Hpa*II restriction enzymes (Fermentas, Vilnius, Lithuania, EU); fragments were separated in 5% polyacrylamide gel and photographed as described above.

Other chromosomal DNA

G35p/m primer pair, obtained by random cloning of DNA from phytoplasmas belonging to 16SrXII-A ribosomal subgroup (Davis *et al*., 1992), was used in direct PCR under the following conditions: 1 min (2 min for the first cycle) of denaturation at 94°C, 2 min of annealing at 50°C, and 3 min (10 min for the last cycle) of primer extension at 72°C; each 25 µl PCR of master mix was as described above. PCR amplification was followed by RFLP analyses with *Tru*I, *Tsp509*I, and *Alu*I restriction enzymes (Fermentas, Vilnius, Lithuania, EU). G35p/m amplified product (about 1200 bp) of sample 2005/2 was purified, sequenced using primer G35p and G35m, and aligned as described above. The gene sequence (751 bp) obtained (C.R.I.B.I. Padua, Italy, EU), was deposited in the NCBI under accession No. DQ239437, and aligned with those of phytoplasmas and other prokaryotes available in GenBank using the World Wide Web service BLAST (http://www.ncbi.nlm.nih.gov) for Nucleotide-nucleotide BLAST (blastn), and translated query vs. protein database (blastx).

Virtual RFLP analyses on 16S ribosomal gene

Virtual RFLP analyses on R16F2/R2 amplicon of Corn sample 2005/2 was performed, using pDRAW32 program (AcaClone Software). The sequenced strain was also compared with selected sequences of 16SrXII phytoplasmas available in GenBank (Table 3.2).

2.3. Results

16S ribosomal DNA

After direct amplification with P1/P7 primer pair, fragments of 1,880 bp were obtained from 12 of 16 samples collected from symptomatic corn plants, while none of the four samples collected from asymptomatic plants gave amplification (Table 2.2). Nested PCR reactions with F1/B6 and R16(I)F1/R1 primer pairs both resulted in expected length fragment amplification for phytoplasmas (respectively about 1,700 and 1,100 bp) from all the symptomatic samples tested; no amplification was obtained from asymptomatic samples (Table 2.2). All the 12 samples positive in direct PCR with P1/P7 primer pair showed restriction profiles when subjected to RFLP analyses with *Tru*I and *Taq*I restriction enzymes that were identical (Fig. 2.2a) and referable to the profile of stolbur phytoplasma, strain STOL, belonging to 16SrXII-A ribosomal group (Fig. 2.2b).

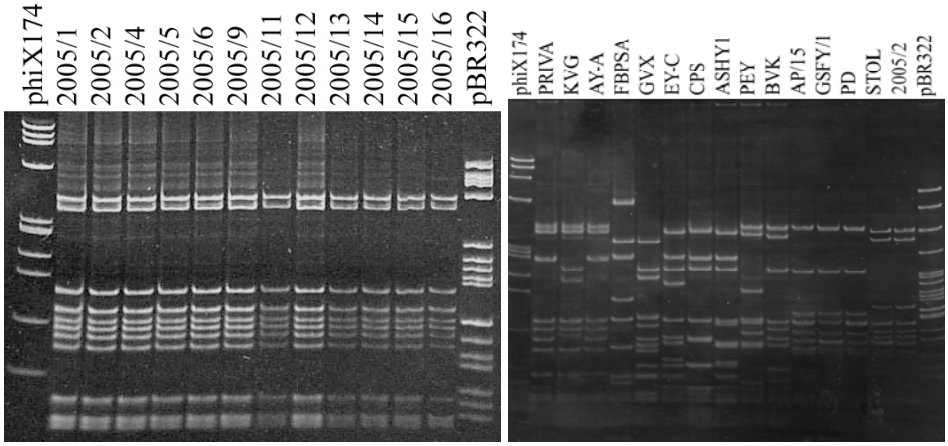


Fig. 2.2a. Polyacrylamide gel 5% showing the *Tru*I RFLP patterns of phytoplasma 16S rDNA and spacer region obtained with P1/P7 primer pair in direct PCR from corn samples (2005/1 to 2005/16); corn sample acronyms are as reported in Table 2.2; phiX174, marker ΦX174 *Hae*III digested, fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72; pBR322, marker pBR322 *Bsu*RI digest, fragment sizes in base pairs from top to bottom: 587, 540, 502, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, and 51.

Fig. 2.2b. Polyacrylamide gel 5% showing the *Tru*I RFLP patterns of phytoplasma16S rDNA and spacer region obtained with P1/P7 primer pair in direct PCR from a corn sample and from phytoplasma reference strains in periwinkle. Sample acronyms: PRIVA, primula phyllody; KVG, clover phyllody; AY-A, aster yellows from apricot; FBPSA, faba bean phyllody; GVX, Green Valley X disease; EY-C, elm yellows; CPS, *Catharanthus* phyllody; ASHY1, ash yellows; PEY, *Pichris echioides* yellows; BVK, flower stunting; AP/15, apple proliferation; GSFY/1, German European stone fruit yellows; PD, pear decline; and STOL, stolbur; 2005/2, corn sample 2005/2 (see Table 2.2); phiX174, marker ΦX174 *Hae*III digested; fragment sizes as above; pBR322, marker pBR322 *Msp*I digest, fragment sizes in base pairs from top to bottom: 622, 527, 404, 307, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, and 67.

Table 2.2. Phytoplasmas detected in corn with reddening and the different phytoplasma DNA fragments amplified using PCR.

Gene/	16Sr DNA		Tuf gene		Chromosomal DNA
Primer pair	P1/P7	F1/B6[n]; R16(I)F1/R1[n]	Tuf1f/r	TufAYf/r[n]	G35p/m
2005/1 [S]	+	+	+	+	+
2005/2 [S]	**+**	**+**	**+**	**+**	**+**
2005/3 [S]	-	+	-	+	-
2005/4 [S]	+	+	+	+	+
2005/5 [S]	+	+	+	+	+
2005/6 [S]	+	+	+	+	+
2005/7 [S]	-	+	-	+	-
2005/8 [S]	-	+	-	+	-
2005/9 [S]	+	+	+	+	+
2005/10 [S]	-	+	-	+	-
2005/11 [S]	+	+	+	+	+
2005/12 [S]	+	+	+	+	+
2005/13 [S]	+	+	+	+	+
2005/14 [S]	+	+	+	+	+
2005/15 [S]	+	+	+	+	+
2005/16 [S]	+	+	+	+	+
2005/17 [A]	-	-	-	-	-
2005/18 [A]	-	-	-	-	-
2005/19 [A]	-	-	-	-	-
2005/20 [A]	-	-	-	-	-
Total symptomatic[b]	12/16	16/16	12/16	16/16	12/16
Total asymptomatic[b]	0/4	0/4	0/4	0/4	0/4

[n] nested PCR; [b] number of positive/number tested; [S] symptomatic; [A] asymptomatic.

The strain subjected to sequencing is in bold.

The four samples providing positive results in nested PCR assays with F1/B6 and R16(I)F1/R1 primer pairs digested with *Tru*I restriction enzyme showed restriction profiles identical to each other, and referable to those of stolbur phytoplasma, strain STOL, belonging to 16SrXII-A ribosomal group.Phylogenetic comparison of the 16S rRNA gene of corn sample 2005/2 with 29 representative strains of the genus '*Candidatus* Phytoplasma' revealed that the phytoplasmas detected in corn are very closely related to the stolbur phytoplasmas (Fig. 2.3) and to other members of the 16SrXII group as defined previously (Lee *et al*., 1998a; b). The web search with blast-n on 16S sequence (1,370 bp) of sample 2005/2 showed identity values of 99% with stolbur strains from Spanish, Chilean, and German grapevine yellows respectively (AJ964960, AY739653, and X76428), with strain STOL from Serbian pepper (AF248959), and with stolbur strain from weed from Germany (X76427); 98% similarity was

observed with a clone of the stolbur strain from Chilean grapevine yellows (AY739654); and 97% similarity with '*Ca.* P. australiense'.

Calculation of percent similarity values of spacer region sequences (168 bp) showed 100% identity between the phytoplasma detected in corn sample 2005/2 and the STOL strain (AF248959), isolated from pepper in Serbia, while different degrees of similarities, always above 90%, were present with other phytoplasmas in 16SrXII group. Similarity lower than 90% was observed with the phytoplasma strain from maize bushy stunt (AF487779, 70%) and from barley deformation (AY734453, 71%) only (Table 2.3).

Table 2.3. Analysis of sequence similarities among 14 spacer region sequences from phytoplasma detected in Corn sample 2005/2 and 12 phytoplasmas, most of which belonged to 16SrXII group.

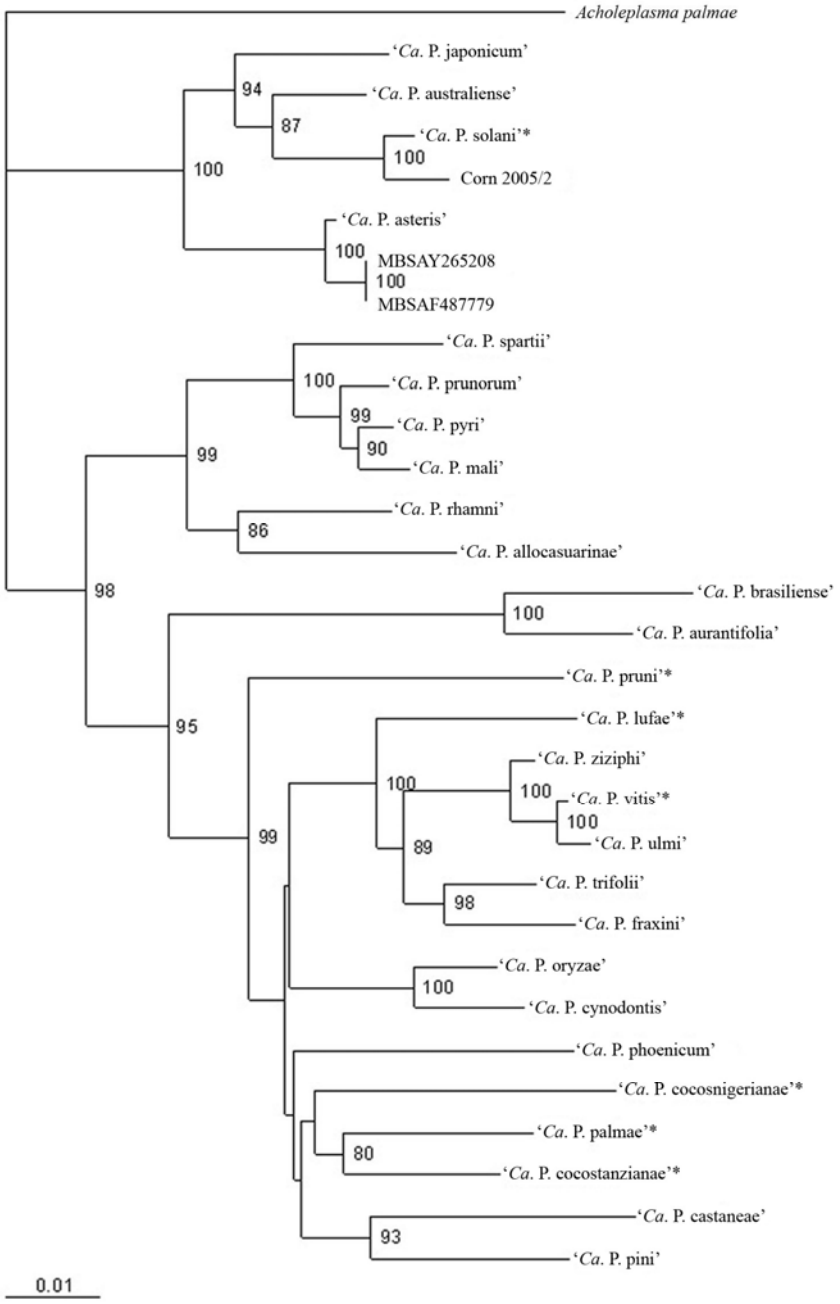
GeneBank strains[a]	16Sr grouping[b,c]	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 X95706-AGY	16SrXII-B	ID	0,994	0,923	0,994	0,994	0,72	0,725	0,896	0,905	0,894	0,905	0,911	0,911	0,586
2 Y10095-PDD	n.d.	0,994	ID	0,929	1	1	0,72	0,725	0,896	0,905	0,894	0,905	0,911	0,911	0,591
3 AF025428-MPV	16SrXIII	0,923	0,929	ID	0,929	0,929	0,701	0,706	0,894	0,921	0,909	0,921	0,927	0,927	0,574
4 AJ243045 StrawLY	16SrXII-B[c]	0,994	1	0,929	ID	1	0,72	0,725	0,896	0,905	0,894	0,905	0,911	0,911	0,591
5 AJ2430444 StrawGP	16SrXII-B[c]	0,994	1	0,929	1	ID	0,72	0,725	0,896	0,905	0,894	0,905	0,911	0,911	0,591
6 AF487779 MBS	16SrI-B	0,72	0,72	0,701	0,72	0,72	ID	0,99	0,741	0,703	0,698	0,703	0,708	0,708	0,545
7 AY734453 BaD	16SrI-B	0,725	0,725	0,706	0,725	0,725	0,99	ID	0,746	0,708	0,703	0,708	0,712	0,712	0,549
8 AF495882 CbY1	n.d.	0,896	0,896	0,894	0,896	0,896	0,741	0,746	ID	0,904	0,903	0,904	0,91	0,91	0,572
9 AY739654 GYa	16SrXII-A	0,905	0,905	0,921	0,905	0,905	0,703	0,708	0,904	ID	0,987	0,987	0,993	0,993	0,558
10 AY739653 GYb	16SrXII	0,894	0,894	0,909	0,894	0,894	0,698	0,703	0,903	0,987	ID	0,975	0,981	0,981	0,558
11 AJ964960 GY-S	16SrXII-A	0,905	0,905	0,921	0,905	0,905	0,703	0,708	0,904	0,987	0,975	ID	0,993	0,993	0,558
12 AF248959 STOL	16SrXII-A	0,911	0,911	0,927	0,911	0,911	0,708	0,712	0,91	0,993	0,981	0,993	ID	1	0,563
13 DQ222972 Corn 2005/2	16SrXII-A	0,911	0,911	0,927	0,911	0,911	0,708	0,712	0,91	0,993	0,981	0,993	1	ID	0,563
14 *A. palmae*		0,586	0,591	0,574	0,591	0,591	0,545	0,549	0,572	0,558	0,558	0,558	0,563	0,563	ID

[a] AGY, Australian grapevine yellows; PDD, Papaya dieback disease; MPV, Mexican periwinkle virescence; StrawLY, Strawberry lethal yellows; StrawGP, Strawberry green petals; MBS, Maize bushy stunt; BaD, Barley deformation phytoplasma; CbY1, Chinaberry yellows; GYa, Grapevine yellows phytoplasma clone a; GYb, Grapevine yellows phytoplasma clone b; GY-S, Grapevine yellows from Spain; STOL, stolbur phytoplasma from pepper from Serbia.

[b] n.d. ribosomal subgroup not determined.

[c] ribosomal subgroup proposed here.

Fig. 2.3. Phylogenetic tree reporting the placement of the phytoplasma associated with corn sample 2005/2 within the genus '*Candidatus* Phytoplasma'. The asterisk (*) marks '*Candidatus*' names proposed at the X International Congress of the International Organization of Mycoplasmology, 1994, held in Bordeaux, France, but not yet formally described, and are reported here as incidental citations which do not constitute prior citations, according to rule 28b of the bacteriological code (Lapage *et al.*, 1992). *Acholeplasma palmae* was employed as the out-group taxon. Abbreviations are given in Table 2.1. The scale bar represents a phylogenetic distance of 1%.



Tuf gene

In direct PCR assays with Tuf1f/Tuf1r primer pair, expected length amplicons (1,080 bp) were obtained from 12 of 16 symptomatic samples, while no amplification was observed in the four asymptomatic samples. Through nested PCR with TufAyf/TufAyr primer pair, expected length fragments (940 bp) were obtained from all 16 symptomatic samples and no asymptomatic ones (Table 2.2). Positive samples in nested PCR were subjected to RFLP analyses with *Tru*I, and *Hpa*II restriction enzymes. All restriction profiles of corn samples obtained with *Hpa*II were identical to each other and to control strains STOL, MO-2 and P1, and showed different profile from those of control strains VE-16 and MO-47 (Fig. 2.4). All restriction profiles of corn samples and of all control strains after RFLP analyses with *Tru*I restriction enzyme were identical to each other.

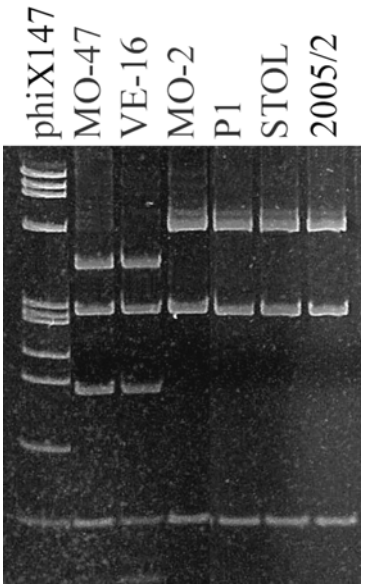


Fig. 2.4. Polyacrylamide gel showing RFLP results with *Hpa*II restriction of the corn samples with reddening from Serbia infected with 16SrXII-A phytoplasmas and amplified in nested PCR with TufAyf/r primers. Sample acronyms are: 2005/2, corn sample 2005/2 (see Table 2.2); STOL, stolbur; P1, grapevine yellows from Serbia; VE-16, MO-47 and MO-2 grapevine yellows from Venice and Modena provinces in Italy; phiX174, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Other chromosomal DNA

In direct PCR assays performed using G35p/m primer pair, expected length amplicons (1,200 bp) were obtained from 12 of 16 symptomatic samples, while four symptomatic and all four asymptomatic samples gave negative results (Table 2.2). RFLP profiles obtained with *Tru*I, *Tsp509*I, and *Alu*I restriction enzymes were identical to each other and to phytoplasma strain STOL used as control. The 751 bp sequence of corn sample 2005/2 obtained from G35p/m amplicon showed 93% in nucleotide sequence similarity, and about 80% in amino acid sequence similarity with parts of genome of '*Ca*. P. asteris' (replicative DNA helicase of onion yellows phytoplasma, AP006628.1) (Oshima *et al*., 2004).

Virtual RFLP analyses on 16S ribosomal gene

Virtual restriction analyses showed that *Hpy188*I restriction enzyme differentiate Serbian Corn 2005/2 from all other stolbur strains tested (Fig 3.5).

2.4. Discussion

Presence of stolbur phytoplasmas in corn samples with reddening symptoms is a new finding not only for Serbia, this is in fact the first report of stolbur phytoplasma in this species, worldwide. Corn was reported to be infected with maize bushy stunt phytoplasma in the Americas where leaf reddening is a primary symptom associated with MBS disease (Nault, 1980). It is not uncommon, however, for different pathogens to be associated with often indistinguishable symptoms in corn (Bradfute *et al*., 1981; Harrison *et al.*, 1996; Nault, 1980).

Presence of phytoplasmas was demonstrated by the positive results of direct PCR on different phytoplasma genomic sequences, for example 16S rDNA and *tuf*, which are phylogenetically conserved genes, and G35p/m sequence, which is specific for phytoplasmas belonging to ribosomal groups 16SrI and 16SrXII (Davis *et al*., 1992; Vibio *et al*., 1996). All direct PCR assays (with P1/P7, Tuf1f/r and G35p/m primer pairs) yielded consistent positive results, while nested PCR reactions (with primers F1/B6, 16S(I)F1/R1 and TufAyf/r) increased the sensitivity of the systems employed, allowing detection of stolbur phytoplasmas in all examined symptomatic corn samples. Negative results obtained by direct PCRs with template DNAs from symptomatic plants can be result of low titer or/and uneven phytoplasma distribution.

The phytoplasmas detected in corn with reddening symptoms were identified as belonging to stolbur, 16SrXII-A ribosomal subgroup, on the basis of RFLP analyses of 16S rDNA. Phylogenetic similarities of both 16S rDNA and spacer region sequences showed that corn 2005/2 strain has very close molecular relationship with 16SrXII-A phytoplasmas (98% to 99% identity in 16S and 98.7% to 100% identity in spacer region). The highest similarity was found between the phytoplasmas detected in corn and STOL phytoplasma reference strain from Serbian pepper, for which similarity was 99% in 16S rDNA and 100% in spacer region sequences.

Tuf gene RFLP comparison was employed since it was shown to have more high genetic variability in phytoplasma molecular characterization than other genes (Schneider *et al*.,

1997). From RFLP analyses with *Hpa*II, phytoplasmas detected in corn have restriction profile identical to reference strain STOL, strain P1 from grapevine with yellows from Serbia, and strain MO-2 from Italy. The reference strains VE-16 and MO-47 from grapevine with yellows from Italy had a different RFLP profile corresponding to the one described in stolbur phytoplasmas from grapevine and *Urtica dioica* in Germany and France (Langer and Maixner, 2004).

The amplification of G35p/m sequence further confirmed data obtained with other genes in direct PCR, and therefore phytoplasma presence in symptomatic samples. The high homology of this sequence only with '*Ca.* P. asteris', belonging to ribosomal subgroup 16SrI-B retrieved by BLAST searches, reflects the fact that there are no additional sequences available in GenBank of this part of the genome belonging to any other phytoplasmas. '*Ca.* P. asteris' is, at present time, the only full phytoplasma sequence available in GenBank (Oshima *et al*., 2004).

Since stolbur phytoplasmas were detected only in samples from symptomatic corn plants, it can be suggested that these phytoplasmas likely are involved in the reddening syndrome, however, more biological data must be obtained to confirm etiology of this corn reddening by fulfilling Koch's postulates.

Stolbur disease in Serbia was first noticed in 1949 on pepper (Martinović and Bjegović, 1950). Since then outbreaks of stolbur periodically have reduced productivity of *Solanaceae* plants, especially in dry years. Natural reservoir of stolbur disease in Serbia is the perennial, herbaceous weed *Convolvulus arvensis* (bindweed) (Aleksić *et al*., 1969). The most significant vector of stolbur was determined to be the Cixiid *Hyalesthes obsoletus* (Aleksić *et al*., 1967; 1969; Šutić, 1983), which overwinters on roots of *C. arvensis*. Activity of *H. obsoletus*, in Serbia, starts during the end of June or beginning of July with maximum flight in the middle of July, and is followed by stolbur symptoms appearing on pepper at the end of July and becoming severe by the second half of August (Aleksić *et al*., 1969).

Recurrent epidemic outbreaks of corn reddening in Banat region, especially in dry years as occurs for stolbur disease of *Solanaceae* plants, development of reddening on corn approximately at the same time as stolbur on pepper (Aleksić *et al*., 1969; Šutić, 1983), and the highest similarity (99% in 16S and 100% in spacer regions) being between stolbur phytoplasma detected in corn in 2005 and stolbur phytoplasma from pepper from Serbia must be taken into consideration in further epidemiological studies. More research is needed to verify possibility of the unique restriction profile of corn 2005/2 strain to be used as a tool for differentiation of stolbur strain from corn from stolbur phytoplasmas detected in other plant species or insect vectors.

The results of these findings could contribute to the start of corn reddening control. Knowledge about genetic relationship of this stolbur strain of phytoplasma with other stolbur

strains described previously only on different plant species can help in further epidemiological work to identify insect vectors and possible weed or woody plants as reservoir hosts of this prokaryote in the region where corn is grown. Knowing that corn is another host plant can also help with control measures to reduce stolbur phytoplasma spreading in other crops in Serbia.

Chapter 3

‘Flavescence dorée’ and Bois noir phytoplasmas associated with grapevine yellows in Serbia

3.1. Introduction

Grapevine Yellows (GY) are widespread diseases of grapevine associated with molecularly distinguishable phytoplasmas in the most important grape growing areas world wide (Caudwell, 1957; 1961; 1983; Boudon-Padieu, 2003; Bertaccini *et al*., 2004). During the 1990s strong symptoms of GY were reported in the Župa Aleksandrovac region of Serbia (Ivanović and Ivanović, 2000), and subsequently the presence of flavescence dorée (FD) phytoplasmas and of their vector *Scaphoideus titanus* Ball., have been reported (Duduk *et al*., 2003a; b; 2004; Magud and Toševski, 2003). Phytoplasmas were also detected in diseased grapevine samples from the same area by the electron microscopy (Kuzmanović *et al*., 2003).

Flavescence dorée is a quarantine infectious disease of grapevine associated with yellows symptoms still dangerous in spite all the quarantine measures that over the last 20 years allow to reduce its impact in affected European viticultural areas. Symptoms of disease are similar to those associated with other phytoplasma diseases in grapevine, and mainly involve plant decline, leaf rolling, shriveled grapes, unripened shoots and reddening or yellowing of leaves on red or white cultivars respectively (Fig. 3.1).

The severity and increasing presence of this disease in Europe has prompted extensive efforts for its detection and differentiation from the phytoplasmas associated to bois noir (BN) disease and for the fine identification and characterization of strains of both phytoplasmas. The major problem viticulturists are facing is the great ability of phytoplasmas, associated with this disease, to differentiate new strains in short periods of time. The molecular differentiation of FD strains present in diverse grape growing areas where the disease is present, is therefore of major relevance towards a correct disease management.

During the last ten years, the use of molecular methods has enabled the detection and characterization of phytoplasmas associated with these and other grapevine yellows. In

particular, PCR/RFLP and DNA sequencing of conserved phytoplasma genetic regions such as 16SrDNA and ribosomal protein genes have lead to identification and classification schemes for several plant phytoplasmas (Lee *et al*., 1998a, Seemüller *et al*., 1998a, Martini *et al*., 1999).

Phytoplasma strains FD associated belong to ribosomal subgroups 16SrV-C and 16SrV-D and they are further differentiated using polymorphisms detected in *rpS3*, *SecY* genes as well as other genes (Bertaccini *et al*., 1997; Angelini *et al*., 2001; Martini *et al*., 2002; Lee *et al*., 2004b; Botti and Bertaccini, 2007; Arnauld *et al*., 2007). Strains of FD 16SrV-D have been detected in Northern Italy (Martini *et al*., 1999; Bertaccini *et al*., 2001, Bianco *et al*., 2003), France, and Spain (Angelini *et al*., 2003; Torres *et al*., 2003; 2005) where the disease showed the highest epidemic outbreaks. In other grape producing areas such as North-central Italy and Serbia the flavescence dorée strains associated with disease outbreaks belong to ribosomal subgroup 16SrV-C (Marzachì *et al*., 2001; Duduk *et al*., 2004; Botti and Bertaccini, 2006b). Both FD type resulted to be experimentally transmissible by the same vector *S. titanus* (Mori *et al*., 2002).

In the same areas as well as in almost all other grapevine growing regions worldwide the presence of bois noir phytoplasmas, belonging to ribosomal subgroup 16SrXII-A and associated with symptoms undistinguishable from those caused by flavescence dorée, was also reported. BN phytoplasmas were transmitted to grapevine by *Hyalesthes obsoletus* Signoret (Homoptera, Cixiidae) using as source of inoculum *Convolvulus arvensis* L. (Maixner, 1994; Maixner *et al*., 1995; Sforza *et al*., 1998) and *Urtica dioica* L. (Alma *et al*., 2002; Bressan *et al*., 2006). However, these phytoplasmas were also detected in other plants and auchenorrhyncha species that are supposed to be involved in BN epidemiology (Palermo *et al*., 2004; Maixner *et al*., 2006).

Over the last decade severe spreading of bois noir (BN) disease was described in several European grapevine-growing areas and the usefulness of *tuf* gene for epidemiological studies was shown (Langer and Maixner, 2004; Mori *et al*., 2008). Three strains specific for different plant species were differentiated: type I was detected only in *U. dioica*, type II was found in *C. arvensis* and in *Calystegia sepium* L. and type III was recorded only in *C. sepium*.

Recent findings indicate however that there is a molecular variability also inside the 16S gene of BN and other stolbur-related phytoplasmas (Quaglino *et al*., 2008; Duduk *et al*., 2009b) that could be indicative for the presence of diverse strains, possibly relevant to study BN epidemic outbreaks.

Figure 3.1. Symptoms of flavescence dorée and bois noir in vineyards and in grapevine plants in Serbia. In the infected plants the production is dramatically reduced, and the frequent lack of lignification also reduces the possibility to harvest the canes for producing cuttings.

3.2. Flavescence dorée

3.2.1 Material and Methods

During 2006-2008 samples were collected in three areas of Serbia (11 samples in Irig, Niš, and Aleksandrovac), and in two areas of Italy (7 samples in Veneto and Tuscany) (Table 3.1). As reference strains TV-54, Liguria 3, FD-70, and FD88 (Martini *et al*., 2002) obtained from infected grapevines in Italy and in France respectively were employed.

Total nucleic acid extraction was carried out from 1 g of fresh phloem and leaf midrib tissues following either a chloroform/phenol procedure (Prince *et al*., 1993) or a CTAB based procedure (Angelini *et al*., 2001).

PCR amplification and sequence analyses

16S ribosomal DNA. A direct PCR amplification with P1/P7 universal phytoplasma primer pair (Deng and Hiruki, 1991; Schneider *et al*., 1995), amplifying 16S rDNA, the spacer region between 16S and 23S rDNA and the 5' portion of 23S rDNA, was performed for the phytoplasma detection. Nested PCR amplification was also carried out with P1A/P7A (Lee *et al*., 2004b; Martini, 2004) primers internal to the above described on P1/P7 amplicons diluted 1:30 in sterile distilled water and with F1/B6 (Davis and Lee, 1993; Padovan *et al*., 1995) or M1/B6 (Martini *et al*., 1999).

Each 25 µl PCR reaction mix contained 2.5 µl 10X PCR buffer, 0.8 U of *Taq* polymerase (Polymed, Florence, Italy), 0.2 mM dNTPs, 1.5 mM MgCl2 and 0.4 µM each primer. PCR conditions were: 35 cycles (Biometra, Uno Thermoblock, Gottingen, Germany as thermal cycler), 1 min (2 min for the first cycle) denaturation step at 94 ^{o}C, 2 min for annealing at 50°C and 3 min (10 min for the last cycle) for primer extension at 72 ^{o}C. A total of 6 µl of PCR products were analysed in 1% agarose gel, stained with ethidium bromide, and visualized under UV with a transilluminator. RFLP analyses of P1/P7 amplified product with *Taq*I (Fermentas, Vilnius, Lithuania) were performed for identification of the detected phytoplasmas; DNA fragments were separated in 5% polyacrylamide gels, stained with ethidium bromide and visualized with UV transilluminator.

The P1/P7 amplified products of grapevine samples Niš (RS) 155/08 and Niš (RS) 158/08 were, after cleaning with Qiagen PCR Purification Kit, according to manufacturers instruction, sequenced in both directions using primers P1, P7 and M1 or P1A, B6 and M1 (Deng and Hiruki, 1991; Schneider *et al*., 1995; Padovan *et al*., 1995; Lee *et al*., 2004b) with

the BIG DYE sequencing terminator kit. The sequences were assembled using DNA STAR software, and compared with selected sequences of phytoplasmas in GenBank database using BLAST (version Blast N 2.2.18) at the National Center for Biotechnology Information.

Traslocase (SecY) gene. A further molecular characterization was performed by RFLP analyses on *SecY* (traslocase) gene using primers FD9f2/FD9r followed by FD9f3/FD9r2 in nested PCR, amplifying the entire *SecY* gene and a portion of *rpl15* gene (Angelini *et al*., 2001). The final amplicons obtained are of 1,150 bp following sequences of primers:

FD9f2 5'-GCTAAAGGTGATTTAAC-3' (Angelini *et al.,* 2001)
FD9f3 5'-GGTAGTTTTATATGACAAG-3' (Angelini *et al.,* 2001)

FD9r 5'-TTTGCTTTCATATCTTGTATCG-3' (Daire *et al.,* 1997)
FD9r2 5'-GACTAGTCCCGCCAAAAG-3' (Angelini *et al.,* 2001)

Amplification conditions are as follows:

- 1 µl template DNA 20 ng/µl;
- 17,09 µl deionized, distilled, 0.2 µm filtered water;
- 2.5 µl 10X PCR buffer (Subtherm Fisher, Hampton, NH, USA);
- 1 µl $MgCl_2$ (50mM);
- 2 µl d-NTPs 2.5 mM (Fermentas, MBI, Vilnus, Lithuania);
- 0.625 µl each primer 20 µM;
- 0.16 µl Taq Polymerase (5 U/µl) (Subtherm, Fisher, Hampton, NH, USA).

For direct PCR 35 cycles with FD9f2/r primers are carried out while 40 cycles are used for primers FD9f3/r2 in nested-PCR. Each cycle is as follows: denaturation of 30 sec at 92°C (1 min 30 sec for first cycle), annealing for 30 sec at 47°C when FD9f2/r primers are employed and at 48°C when FD9f3/r2 primers are used, finally elongation at 72°C for 1 min and 15 sec. Aliquots of 6 µl of PCR amplicons are loaded in 1% agarose gel, stained with ethidium bromide and visualized under UV in transilluminator.

RFLP analyses of amplified product with with *Tru*I, *Taq*I and *Tsp509*I restriction enzymes (Fermentas, Vilnius, Lithuania) were performed; DNA fragments were separated in 5% polyacrylamide gels, stained with ethidium bromide and visualized with UV transilluminator.

3.2.2 Results and discussion

16S ribosomal DNA. The molecular characterization on 16S ribosomal DNA by RFLP analyses with *Taq*I restriction enzyme allow to confirm that all samples collected in the three areas of Serbia and in the two areas of Italy were infected by 16SrV-C phytoplasmas (Fig. 3.2). Sequencing of phytoplasma strains 155/08 and 158/08 from Serbia allow to obtain 1,747 bp and 1,653 bp sequences respectively both showing the highest homology of 99% with strain FD-C from France (AF176319) (Davis and Dally, 2001), but also showed high homology with several phytoplasma strains belonging to diverse subgroups of 16SrV group. In particular strain 155/08 showed 99% homology with FD-D strain FD1487 (AJ548787) from Spain (Torres *et al*., 2005), but also with alder yellows from Italy (Y16387) (Seemüller *et al*., 1998) and 98% homology with FD strain from France (X76560) (Seemüller *et al*., 1998) and other FD strains retrievable in GenBank. Serbian strain 158/8 showed 99% homology with all the above listed strains and with all the FD strains in Genbank. The two strains also had 99% homology between them represented by three gaps (see below).

Niš (RS) 155/08

ACCTTTCAAAGGTCTTAGTGGCGAACGGGTGAGTAACACGTAAGTAACCTACCTTTAAGACGAGGATAACAATCGGAAACGGTTGCTAAGACTGGATAGG
AAACAGAAAGGCATCTTTTTGTTTTTAAAAGACCTTCTTCGGAGGGTATGCTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTGAGGTAAAGGCTTAC
CAAGATTATGATGTGTAGCTGGACTGAGAGGTTGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCG
GCAATGGAGGAAACTCTGACCGAGCGACGCCGCGTGAACGATGAAGTATTTCGGTATGTAAAGTTCTTTTATTGAAGAAGAAAAAATAGTGGAAAAACTA
TCTTGACGTTATTCAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAAGACATAGGGGGCGAGCGTTATCCGGAATTATTGGGCGTAAAGG
GTGCGTAGGCGGTTAGATAAGTCTATAATTTAATTTCAGTGCTTAACGCTGTCTTGTTATAGAAACTGTCTTGACTAGAGTGAGATAGAGGCAAGCGGAAT
TCCATGTGTAGCGGTAAAATGTGTAAATATATGGAGGAACACCAGAAGCGTAGGCGGCTTGCTGGGTCTTTACTGACGCTGAGGCACGAAAGCGTGGGG
AGCAAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTACTAAGTGTCGGGGTAACTCGGTACTGAAGTTAACACATTAAGTACTCCGCC
TGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTAC
CAGGTCTTGACATACTCTGCAAAGCTATAGAAATATAGTGGAGGTTATCAGGGATACAGGTGGTGCATGGTTGTCGTCAGTTCGTGTCGTGAGATGTTAG
GTTAAGTCCTAAAACGAACGCAACCCCTGTCGCTAGTTGCCAGCACGTAATGGTGGGGACTTTAGCGAGACTGCCAATTAAACATTGGAGGAAGGTGGGG
ATAACGTCAAATCATCATGCCCCTTATGATCTGGGCTACAAACGTGATACAATGGCTATTACAAAGAGTAGCTGAAACGCGAGTTTTTAGCCAATCTCAAAA
AGGTAGTCTCAGTACGGATTGAAGTCTGCAACTCGACTTCATGAAGCTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGT
TTGTACACACCGCCCGTCAAACCACGAAAGTTAGCAATACCCGAAAGCAGTGGCTTAACTTCGCAAGAAGAGGGAGCTGTCTAAGGTAGGGTTGATGATT
GGGGTTAAGTCGTAACAAGGTATCCTTACCGGAAGGTGAGGATGGATCACCTCCTTTCTAAGGACATACATATAAAAATCATCATCTTCAGTTTTGAAAGA
CTTAGGTTAAAATATAAGTTTTTCTTTTTATAAAAAAAAAGTGTTTCTCTTATATAAAAGACCAAAGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGATA
AGCGTGAGGACGGTGGTTCAAGTCCACTTAGGCCCACCAATTTTATATCAGAAAAATATTTACTTCGAAGAAAGTTCTTTGAAAAGTAGATAAACATGATT
GCTTAATCAATTAATTAAGAAGAAGGGGATTAAAAAA

Niš (RS) 158/08

ACCTTCAAAGGTCTTAGTGGCGAACGGGTGAGTAACACGTAAGTAACCTACCTTTAAGACGAGGATAACAATCGGAAACGGTTGCTAAGACTGGATAGGA
AACAGAAAGGCATCTTTTTGTTTTTAAAAGACCTTCTTCGGAGGGTATGCTTAAAGAGGGGCTTGCGCCACATTAGTTAGTTGGTGAGGTAAAGGCTTACC
AAGATTATGATGTGTAGCTGGACTGAGAGGTTGAACAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGG
CAATGGAGGAAACTCTGACCGAGCGACGCCGCGTGAACGATGAAGTATTTCGGTATGTAAAGTTCTTTTATTGAAGAAGAAAAAATAGTGGAAAAACTAT
CTTGACGTTATTCAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAAGACATAGGGGGCGAGCGTTATCCGGAATTATTGGGCGTAAAGGG
TGCGTAGGCGGTTAGATAAGTCTATAATTTAATTTCAGTGCTTAACGCTGTCTTGTTATAGAAACTGTCTTGACTAGAGTGAGATAGAGGCAAGCGGAATT
CCATGTGTAGCGGTAAAATGTGTAAATATATGGAGGAACACCAGAAGCGTAGGCGGCTTGCTGGGTCTTTACTGACGCTGAGGCACGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTACTAAGTGTCGGGGTAACTCGGTACTGAAGTTAACACATTAAGTACTCCGCCTG
AGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTTACCA
GGTCTTGACATACTCTGCAAAGCTATAGAAATATAGTGGAGGTTATCAGGGATACAGGTGGTGCATGGTTGTCGTCAGTTCGTGTCGTGAGATGTTAGGTT
AAGTCCTAAAACGAACGCAACCCCTGTCGCTAGTTGCCAGCACGTAATGGTGGGGACTTTAGCGAGACTGCCAATTAAACATTGGAGGAAGGTGGGGATA
ACGTCAAATCATCATGCCCCTTATGATCTGGGCTACAAACGTGATACAATGGCTATTACAAAGAGTAGCTGAAACGCGAGTTTTTAGCCAATCTCAAAAAG
GTAGTCTCAGTACGGATTGAAGTCTGCAACTCGACTTCATGAAGCTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTT
GTACACACCGCCCGTCAAACCACGAAAGTTAGCAATACCCGAAAGCAGTGGCTTAACTTCGCAAGAAGAGGGAGCTGTCTAAGGTAGGGTTGATGATTGG
GGTTAAGTCGTAACAAGGTATCCTTACCGGAAGGTGAGGATGGATCACCTCCTTTCTAAGGACATACATATAAAAATCATCATCTTCAGTTTTGAAAGACTT
AGGTTAAAATATAAGTTTTTCTTTTTATAAAAAAAAGTGTTTCTCTTATATAAAAGACCAAAGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGATAAG
CGTGAGGTCGGTGGTTCAAGTCCACTTAGGCCCACCAATTTTAT

```
Identities = 1652/1655 (99%), Gaps = 2/1655 (0%)

Niš (RS) 158/08  1     ACC-TTCAAAGGTCTTAGTGGCGAACGGGTGAGTAACACGTAAGTAACCTACCTTTAAGA  59
                       ||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1     ACCTTTCAAAGGTCTTAGTGGCGAACGGGTGAGTAACACGTAAGTAACCTACCTTTAAGA  60

Niš (RS) 158/08  60    CGAGGATAACAATCGGAAACGGTTGCTAAGACTGGATAGGAAACAGAAAGGCATCTTTTT 119
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  61    CGAGGATAACAATCGGAAACGGTTGCTAAGACTGGATAGGAAACAGAAAGGCATCTTTTT 120

Niš (RS) 158/08  120   GTTTTTAAAAGACCTTCTTCGGAGGGTATGCTTAAAGAGGGGCTTGCGCCACATTAGTTA 179
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  121   GTTTTTAAAAGACCTTCTTCGGAGGGTATGCTTAAAGAGGGGCTTGCGCCACATTAGTTA 180

Niš (RS) 158/08  180   GTTGGTGAGGTAAAGGCTTACCAAGATTATGATGTGTAGCTGGACTGAGAGGTTGAACAG 239
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  181   GTTGGTGAGGTAAAGGCTTACCAAGATTATGATGTGTAGCTGGACTGAGAGGTTGAACAG 240

Niš (RS) 158/08  240   CCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCG 299
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  241   CCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATTTTCG 300

Niš (RS) 158/08  300   GCAATGGAGGAAACTCTGACCGAGCGACGCCGCGTGAACGATGAAGTATTTCGGTATGTA 359
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  301   GCAATGGAGGAAACTCTGACCGAGCGACGCCGCGTGAACGATGAAGTATTTCGGTATGTA 360

Niš (RS) 158/08  360   AAGTTCTTTTATTGAAGAAGAAAAAATAGTGGAAAAACTATCTTGACGTTATTCAATGAA 419
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  361   AAGTTCTTTTATTGAAGAAGAAAAAATAGTGGAAAAACTATCTTGACGTTATTCAATGAA 420

Niš (RS) 158/08  420   TAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAAGACATAGGGGGCGAGCGTTATCC 479
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  421   TAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAAGACATAGGGGGCGAGCGTTATCC  480

Niš (RS) 158/08  480   GGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTAGATAAGTCTATAATTTAATTTCAGT  539
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  481   GGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTAGATAAGTCTATAATTTAATTTCAGT  540

Niš (RS) 158/08  540   GCTTAACGCTGTCTTGTTATAGAAACTGTCTTGACTAGAGTGAGATAGAGGCAAGCGGAA  599
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  541   GCTTAACGCTGTCTTGTTATAGAAACTGTCTTGACTAGAGTGAGATAGAGGCAAGCGGAA  600

Niš (RS) 158/08  600   TTCCATGTGTAGCGGTAAAATGTGTAAATATATGGAGGAACACCAGAAGCGTAGGCGGCT  659
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  601   TTCCATGTGTAGCGGTAAAATGTGTAAATATATGGAGGAACACCAGAAGCGTAGGCGGCT  660

Niš (RS) 158/08  660   TGCTGGGTCTTTACTGACGCTGAGGCACGAAAGCGTGGGGAGC-AAACAGGATTAGATAC  718
                       |||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||
Niš (RS) 155/08  661   TGCTGGGTCTTTACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAAACAGGATTAGATAC  720

Niš (RS) 158/08  719   CCTGGTAGTCCACGCTGTAAACGATGAGTACTAAGTGTCGGGGTAACTCGGTACTGAAGT  778
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  721   CCTGGTAGTCCACGCTGTAAACGATGAGTACTAAGTGTCGGGGTAACTCGGTACTGAAGT  780

Niš (RS) 158/08  779   TAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTG  838
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  781   TAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTG  840

Niš (RS) 158/08  839   ACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTT  898
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  841   ACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACACGAAAAACCTT  900

Niš (RS) 158/08  899   ACCAGGTCTTGACATACTCTGCAAAGCTATAGAAATATAGTGGAGGTTATCAGGGATACA  958
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  901   ACCAGGTCTTGACATACTCTGCAAAGCTATAGAAATATAGTGGAGGTTATCAGGGATACA  960

Niš (RS) 158/08  959   GGTGGTGCATGGTTGTCGTCAGTTCGTGTCGTGAGATGTTAGGTTAAGTCCTAAAACGAA 1018
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  961   GGTGGTGCATGGTTGTCGTCAGTTCGTGTCGTGAGATGTTAGGTTAAGTCCTAAAACGAA 1020

Niš (RS) 158/08  1019  CGCAACCCCTGTCGCTAGTTGCCAGCACGTAATGGTGGGGACTTTAGCGAGACTGCCAAT 1078
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1021  CGCAACCCCTGTCGCTAGTTGCCAGCACGTAATGGTGGGGACTTTAGCGAGACTGCCAAT 1080

Niš (RS) 158/08  1079  TAAACATTGGAGGAAGGTGGGGATAACGTCAAATCATCATGCCCCTTATGATCTGGGCTA 1138
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1081  TAAACATTGGAGGAAGGTGGGGATAACGTCAAATCATCATGCCCCTTATGATCTGGGCTA 1140
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Niš (RS) 158/08  1139  CAAACGTGATACAATGGCTATTACAAAGAGTAGCTGAAACGCGAGTTTTTAGCCAATCTC  1198
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1141  CAAACGTGATACAATGGCTATTACAAAGAGTAGCTGAAACGCGAGTTTTTAGCCAATCTC  1200

Niš (RS) 158/08  1199  AAAAAGGTAGTCTCAGTACGGATTGAAGTCTGCAACTCGACTTCATGAAGCTGGAATCGC  1258
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1201  AAAAAGGTAGTCTCAGTACGGATTGAAGTCTGCAACTCGACTTCATGAAGCTGGAATCGC  1260

Niš (RS) 158/08  1259  TAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCC  1318
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1261  TAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCC  1320

Niš (RS) 158/08  1319  GTCAAACCACGAAAGTTAGCAATACCCGAAAGCAGTGGCTTAACTTCGCAAGAAGAGGGA  1378
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1321  GTCAAACCACGAAAGTTAGCAATACCCGAAAGCAGTGGCTTAACTTCGCAAGAAGAGGGA  1380

Niš (RS) 158/08  1379  GCTGTCTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCTTACCGGAAG  1438
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1381  GCTGTCTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCTTACCGGAAG  1440

Niš (RS) 158/08  1439  GTGAGGATGGATCACCTCCTTTCTAAGGACATACATATAAAAATCATCATCTTCAGTTTT  1498
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1441  GTGAGGATGGATCACCTCCTTTCTAAGGACATACATATAAAAATCATCATCTTCAGTTTT  1500

Niš (RS) 158/08  1499  GAAAGACTTAGGTTAAAATATAAGTTTTTCTTTTTATaaaaaaaaGTGTTTCTCTTATAT  1558
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1501  GAAAGACTTAGGTTAAAATATAAGTTTTTCTTTTTATAAAAAAAAAGTGTTTCTCTTATAT  1560

Niš (RS) 158/08  1559  AAAAGACCAAAGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGATAAGCGTGAGGTC  1618
                       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| | |
Niš (RS) 155/08  1561  AAAAGACCAAAGGGCCTATAGCTCAGTTGGTTAGAGCACACGCCTGATAAGCGTGAGGAC  1620

Niš (RS) 158/08  1619  GGTGGTTCAAGTCCACTTAGGCCCACCAATTTTAT  1653
                       ||||||||||||||||||||||||||||||||||
Niš (RS) 155/08  1621  GGTGGTTCAAGTCCACTTAGGCCCACCAATTTTAT  1655
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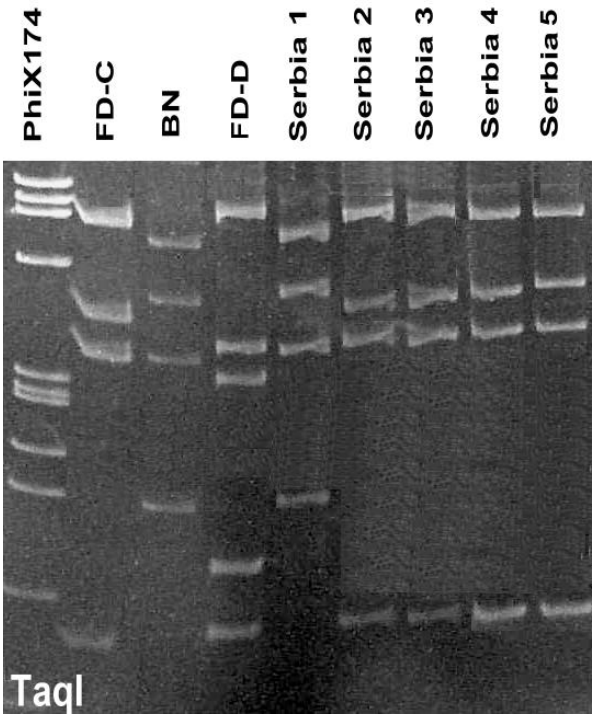

Fig. 3.2. Polyacrylamide gel showing the RFLP profiles of F1/B6 products obtained in nested PCR on P1/P7 amplicons from phytoplasma detected in grapevine from Serbia (Serbia 1 to 5) and in reference strain FD-C, BN and FD-D from Italy after digestion with *Taq*I restriction enzyme. PhiX174, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Molecular characterization of FD-C strains. The examined strains showed RFLP polymorphisms with *Tru*I and *Taq*I restriction enzymes on *SecY* gene that resulted partially related to their geographic origin (Table 3.1, Fig. 3.3a and 3.3.b). In particular strain differentiation was achieved from samples from Serbia and Italy and their tentative grouping showed identity between strains from Aleksandrovac (RS) and Tuscany (I) (profile IV in Table 3.1).

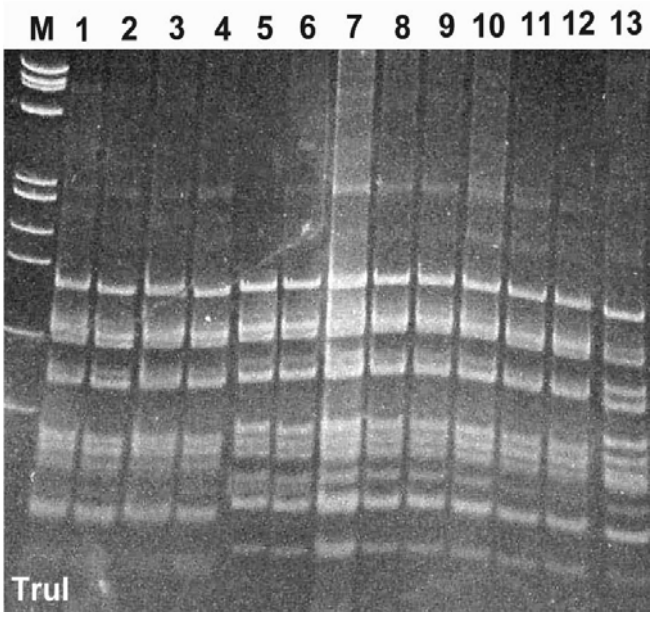

Fig. 3.3a. Polyacrylamide gel showing the RFLP profiles of FD9f3/r2 products obtained in nested PCR on FD9f2/r amplicons from phytoplasma detected in grapevine from Serbia and Italy after digestion with *Tru*I restriction enzyme. Samples: 1, Niš (RS) 154/08; 2, Niš (RS) 155/08; 3, Niš (RS) 157/08; 4, Niš (RS) 158/08; 5, Aleksandrovac (RS) AP1/06; 6, Tuscany (I) 66/08; 7, Irig (RS) 64/08; 8, Irig (RS) 66/08; 9, Irig (RS) 68/08; 10, Veneto (I) 38/08; 11, Veneto (I) 53/08; 12, Veneto (I) 87/08; 13, reference strain FD70. M, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

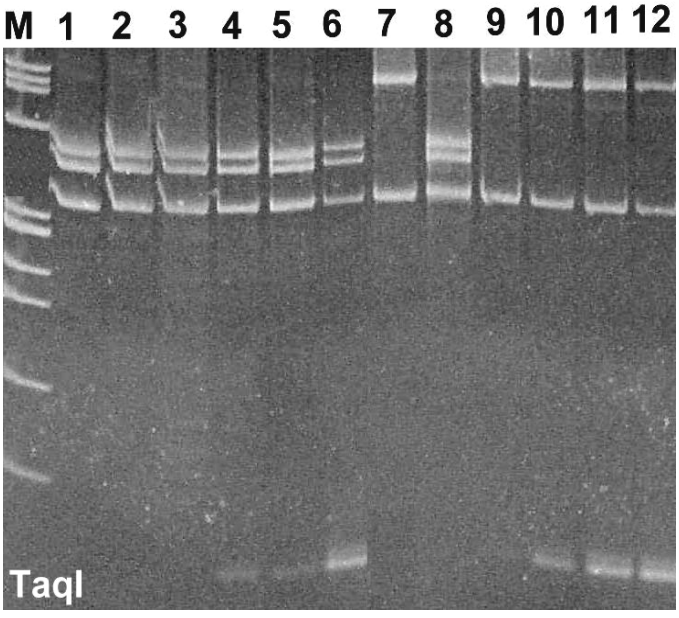

Fig. 3.3b. Polyacrylamide gel showing the RFLP profiles of FD9f3/r2 products obtained in nested PCR on FD9f2/r amplicons from phytoplasma detected in grapevine from Serbia and Italy after digestion with *Taq*I restriction enzyme. Samples: 1, Niš (RS) 154/08; 2, Niš (RS) 157/08; 3, Veneto (I) 38/08; 4, Aleksandrovac (RS) AS2/06; 5, Tuscany (I) 66/08; 6, Aleksandrovac (RS) AP1/06; 7, reference strain FD-70; 8, Irig (RS) 67/08; 9, reference strain FD-D from Veneto; 10-12, reference strains FD-D from Emilia. M, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Table 3.1. RFLP results on FD-C amplicons obtained with primers FD9f3/r2 in nested PCR on FD9f2/r products. Identical letter = identical profile.

Location (Country) sample/year	Restriction enzyme		Profiles
	*Tru*I	*Taq*I	
Niš (RS) 154/08	A	A	I
Niš (RS) 155/08	A	A	I
Niš (RS) 156/08	B	B	II
Niš (RS) 157/08	C	A	III
Niš (RS) 158/08	C	A	III
Aleksandrovac (RS) ARR5/06	E	C	IV
Aleksandrovac (RS) AS2/06	E	C	IV
Aleksandrovac (RS) AP1/06	E	C	IV
Irig (RS) 64/08	C	A	III
Irig (RS) 66/08	C	A	III
Irig (RS) 67/08	A	B	VI
Veneto (I) 38/08	C	A	III
Veneto (I) 53/08	C	A	III
Veneto (I) 87/08	C	A	III
Veneto (I) 107/06	C	A	III
Veneto (I) 108/06	C	A	III
Tuscany (I) 1/08	E	C	IV
Tuscany (I) 66/08	E	C	IV
TV-54 (I)	C	A	III
FD-70 (F)	D	A	V
Liguria 3 (I)	C	A	III

RFLP profiles identity was present also among samples from Niš, Irig, and Veneto (mainly Treviso province) (profile III in Table 3.1). Further RFLP profiles differentiable from each other and from all the previous were also identified in samples from Niš and Irig (profiles I, II and VI in Table 3.1). These results confirmed that *SecY* gene is reliable marker to identify FD-C strains as it was previously reported in Northern Italy and in Tuscany as well (Martini *et al.*, 2002; Botti and Bertaccini, 2006b).

The presence of differentiable strains in just one Serbian locality (Niš) could indicate the presence of long-term infection transmitted by the insect vector, while strains showing *SecY* similarities detected in geographically far areas could be associated with transmission by propagating materials.

3.3.Bois noir

3.3.1 Material and Methods

During summer 2008, routine surveys carried out to verify identity of phytoplasmas associated with yellows symptoms in some grapevine growing areas in Hungary, Serbia, and Italy allow to identify BN phytoplasmas by RFLP analyses with *Tru*I restriction enzyme on R16F2/R2 amplicons (Bertaccini *et al*., 1995). In Serbia eight plants were employed for BN characterization from Bela Crkva, Radmilovac, and Čoka (Table 3.2), representing some of the major viticultural areas of the Country. In Hungary six plants from two vineyards of variety Zweigelt both located in Sopron area (near to the Austrian border) were selected for further molecular characterization (Table 3.2). In Italy four samples collected in Veneto and Tuscany regions were studied (Table 3.2). Reference strains employed maintained in periwinkle were STOL (from Serbia), STOLC and STOL-PO (from France).

PCR amplification and sequence analyses

16S ribosomal DNA/spacer region amplification and sequencing. Total nucleic acids were extracted from midribs and petioles as described above and direct PCR amplification with P1/P7 universal phytoplasma primer pair (Deng and Hiruki, 1991; Schneider *et al*., 1995), amplifying 16S rDNA, the spacer region between 16S and 23S rDNA and the 5' portion of 23S rDNA, was performed for the phytoplasma detection. Nested PCR amplification was also carried out with P1A/P7A (Lee *et al*., 2004b; Martini, 2004) primers internal to the above described on P1/P7 amplicons diluted 1:30 in sterile distilled water, with F1/B6 (Davis and Lee, 1993; Padovan *et al*., 1995), M1/B6 (Martini *et al*., 1999) and R16F2/R2 (Lee *et al*., 1995) (Table 3.2). RFLP analyses with *Hpy8*I, *Mbo*I, *Mbo*II, *Tru*I, *Rsa*I, *Alu*I, and *Tsp509*I restriction enzymes were carried out on the majority of amplicons produced (Table 3.2).

Reference strains STOL, STOLC, and STOL-PO plus seven samples (Langeveildeground (H) L2; Brandmajor (H) B4; Bela Crkva (RS) BC2; Radmilovac (RS) R1; Čoka (RS) C1; Veneto (I) 39, and Tuscany (I) 3) showing polymorphisms in the 16S plus spacer region amplicons were sequenced in both directions using primers P1, P7 and M1 or P1A, B6 and M1 (Deng and Hiruki, 1991; Schneider *et al*., 1995; Padovan *et al*., 1995; Lee *et al*., 2004) after purification using Qiagen PCR Purification Kit with the BIG DYE sequencing terminator kit. The sequences were assembled using DNA STAR software, and compared

with selected sequences of phytoplasmas in GenBank database using BLAST (version Blast N 2.2.18) at the National Center for Biotechnology Information.

Virtual RFLP analyses on R16F2/R2 amplicons were carried out, using pDRAW32 program (AcaClone Software), to compare results obtained in real RFLP analyses for sequenced strains adding *Hpy188*I to the enzymes used in regular RFLP analyses. The sequenced strains were also compared with selected sequences of 16SrXII phytoplasmas available in GenBank and showing 99% homology with them (Table 3.2).

Table 3.2. 16SrXII phytoplasma 16S rDNA sequences retrieved from GenBank and employed for virtual RFLP analyses.

Genbank accession Number	**Species infected**	**Isolate/clone/ strain**	**Country**	**Literature/submission**
AF248959	Periwinkle from grapevine	STOL#11	France	Davis and Dally, 2001
AJ964960	Grapevine	2642BN	Spain	Torres *et al*., 2005
DQ160244	Rhododendron	RHOD-CZ	Czech Republic	Mertelik *et al*., 2004
X76428	*Vitis vinifera* L.	VK	Germany	Seemüller *et al*., 1994
EU836649	*Vitis vinifera* L. cv. Chardonnay	BN-Fc13	Italy	Quaglino *et al*., 2009
EU661607	Potato	Iranian potato purple top phytoplasma	Iran	Hosseini *et al*., 2008
EU344889	Potato	Rus-PPT111	Russia	Girsova *et al*., 2007
EU086529	Grapevine Grenache clone 70 on rootstock 3309 imported into Canada from Europe in 2006	06PS085	Canada	Rott *et al*., 2007
DQ222972	Corn	Corn-reddening phytoplasma 2005/2	Serbia	Duduk and Bertaccini, 2006
Iran	Grapevine	Strains C, D	Iran	Karimi *et al*., 2009

Other genes. Further nested-PCR/RFLP characterization was carried out on *tuf* gene (Langer and Maixner, 2004; Andersen *et al*., 2004; Bertaccini *et al*., 2006) and on *rpS3* gene (Martini *et al*., 2007).

3.3.2 Results and discussion

Tuf and *rpS3* genes characterization. The amplification of *tuf* gene was achieved for all samples using nested-PCR procedures, and restriction analyses with *Hpa*II enzyme showed

that all the samples belong to tuf type II, enclosing the reference strains STOL, STOLC and STOL-PO (Langer and Maixner, 2004).

RFLP analyses with *Ssp*I, *Hpy8*I and *Tai*I on samples positive for *rpS3* gene showed clear polymorphism in sample BC1 from Serbia (the only one amplified among Serbian samples), and possible polymorphisms in some of the strains from Hungary and Italy with *Ssp*I restriction enzyme.

16S ribosomal gene/spacer region amplification and sequencing. RFLP analyses showed different profiles in several of the examined samples, according with amplicon employed; overall restriction profiles indicate the possible presence of interoperon heterogeneity and/or of mixed phytoplasma population in some of the samples (Fig. 3.4, Table 3.2).

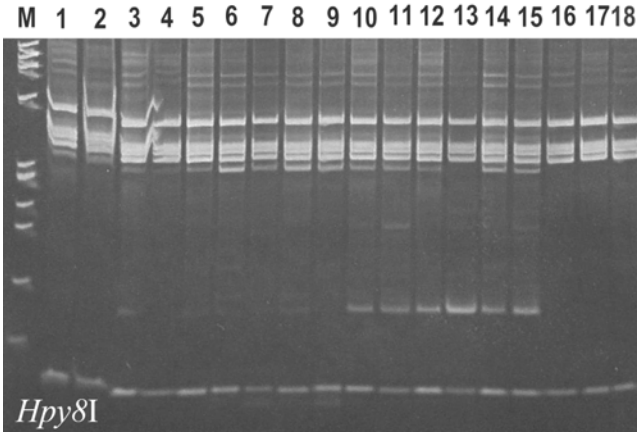

Figure 3.4. Polyacrylamide gel showing some of the RFLP profiles of P1A/P7A products obtained in nested PCR on P1/P7 amplicons from BN phytoplasmas. 1-9, grapevine samples from Serbia; 10-15, grapevine samples from Hungary; 16, STOL; 17, STOLC; 18, STOL-PO. M, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Sequencing of phytoplasma from reference strains and from grapevine from Serbia, Hungary and Italy allow obtaining sequences ranging from 1,405 to 1,737 bp, all showing 99% homology with several of the 16SrXII-related strains deposited in Genbank. The aligned sequences obtained are listed below.

STOL (1,564 bp)

TAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATTTTTAAAAGACC
TAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTACCAAGACGATGATGTGTAGCCGGGCTGAGAGGTCG
AACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAATGCCGCG
TGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACCATTATGACGGTACCTAATGAATAAGCCCCGGCTAAC
TATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGT
GCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAATTCCGTGTGTAGTGGTAAAATGCGTAAATATACGGA
GGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAAACTGACGCTGAGGCACGAAAGCGTGGGGGAGCAAAMMAGGATTAGATACCCTGGTAGTCC
ACGCCCTAAACGATGAGTACTAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAA
GGAATTGACGGGACTCCGCACAAGCGGTGGATCWWGTTGTTTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAG
AAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
TGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTG

GGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTGAAGTCTGCAA
CTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAACCACGAAAGT
TGGCAATACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTAC
CGGAAGGTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTCATTTTTTAAGATTCGGGCCTATA
GCTCAGCTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGC

STOLC (1,563 bp)

TAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTCTTATTTTTAAAAGACC
TAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTACCAAGACGATGATGTGTAGCCGGGCTGAGAGGTCG
AACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAATGCCGCG
TGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACCATTATGACGGTACCTAATGAATAAGCCCCGGCTAAC
TATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGT
GCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAATTCCGTGTGTAGTGGTAAAATGCGTAAATATACGGA
GGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
CTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAA
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CAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTA
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CAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGAC
TTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAACCACGAAAGTTGGCAA
TACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAG
GTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAGC
TGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCC

STOL-PO (1,641 bp)

AGTAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTCTTATTTTTAAAAG
ACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTACCAAGACGATGATGTGTAGCCGGGCTGAGAGGT
CGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAATGCCG
CGTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACCATTATGACGGTACCTAATGAATAAGCCCCGGCTA
ACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAA
GTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAATTCCGTGTGTAGTGGTAAAATGCGTAAATATACG
GAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGG
AATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAA
TACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTT
AATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCT
ACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGA
CTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAACCACGAAAGTTGGCA
ATACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAA
GGTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAG
CTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAATAGGTCACATCTTAAAAAAGCTCTTtGAAAAGTAGA
TAAACAAASGTTAGAAAATTAAAGGAACTAAG

Radmilovac (RS) R1 (1,665 bp)

AGTTTAAGCATTAAACTTTAGTGGCGAACGGGTGAGTAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAGACTGGAT
AGGAGATAAGAAGGCATCTTTTTATTTTTAAAAGACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTA
CCAAGACGATGATGTGTAGCCGGGCTGAGAGGTCGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTC
GGCAATGGAGGAAACTCTGACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACC
ATTATGACGGTACCTAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
GGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAA
TTCCGTGTGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGAAGAAGTTAACACATTAAGTACTCCGCC
TGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAAACCTCA
CCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTGGGGA
CGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAATCTCAAAA
AAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGT
TTGTACACACCGCCCGTCAAACCACGAAAGTTGGCAATACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGATGA
TTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAAGA
AAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAA
ATAGGTCACATCTTAAAAAAGCTCTTTGAAAAGTAGATAAACAAAGGTAGAAAATAAAG

Bela Crkva (RS) BC2 (1,663 bp)

GCAGTGAGCATTAAACTTTAGTGGCGAACGGGTGAGTAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAGACTGGAT
AGGAGATAAGAAGGCATCTTTTTATTTTTAAAAGACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTA
CCAAGACGATGATGTGTAGCCGGGCTGAGAGGTCGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTC
AGCGATGGAGGAAACTCTGACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACC
ATTATGACGGTACCTAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
GGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAA

TTCCGTGTGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCGCC
TGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAAACCTCA
CCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTGGGGA
CGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAATCTCAAAA
AAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGT
TTGTACACACCGCCCGTCAAACCACGAAAGTTGGCAATACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGATGA
TTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAAGA
AAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAA
ATAGGTCACATCTTAAAAAAGCTCTTTGAAAAGTAGATAAACAAAGGTAGAAAATAA

Čoka (RS) C1 (1,674 bp)

GAGTTTAGCATTAAACTTTAGTGGCGAACGGGTGAGTAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAGACTGGAT
AGGAGATAAGAAGGCATCTTTTTATTTTTAAAAGACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTA
CCAAGACGATGATGTGTAGCCGGGCTGAGAGGTCGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTC
GGCAATGGAGGAAACTCTGACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACC
ATTATGACGGTACCTAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
GGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAA
TTCCGTGTGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGG
GAGCAAACAGGATTAGATAcCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCGCC
TGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAAACCTCA
CCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGgTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTGGGGA
CGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAATCTCAAAA
AAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGT
TTGTACACACCGCCCGTCAAACCACGAAAGTTGGCAATACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGATGA
TTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAAGA
AAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAA
ATAGGTCWCATCTTAAAAAAGCTCtTTGAAAAGTAGATAAACAAAGGTAGAAAATTAAAGGAACTAAA

Langeveildeground (H) L2 (1,737 bp)

AGGAGCCTCGCACGGGGAGTTAAGCAATTAAACTTTAGTGGCGAACGGGTGAGTAACGCGTAAGCGAATCTGCCCCTAAGACGAGGATAACAGTTGGAA
ACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATTTTTAAAAGACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAG
TTGGTGGGGTAATGGCCTACCAAGACGATGATGTGTAGCCGGGCTGAGAGGTCGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAG
GCAGCAGTAAGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAG
AAAAGATGGTGGAAAAACCATTATGACGGTACCTAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCC
GGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTT
GGATAGAGGCAAGTGGAATTCCGTGTGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCT
GAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTT
AACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGA
AGGTACCCGAAAAACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATA
AATTGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAA
GCTTCTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGC
GGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAACCACGAAAGTTGGCAATACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCC
GTCTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTT
CAGTTTTGAGAGACTTAAGAAAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCA
AGTCCATTTAGGCCCACCAAAATAGGTCACATCTTAAAAAAGCTCTTTGAAAAGTAGATAAACAAAGGTTAGAAAATTAAAGGAACTAAGGGCGCACAGT
GGATGCCTTGGCACTAGAGAGCCCGATGAAAGGA

Brandmajor (H) B4 (1,666 bp)

GTAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAGACTGGATAGGAGATAAGAAGGCATCTTTTTATTTTTAAAAGA
CCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCCTACCAAGACGATGATGTGTAGCCGGGCTGAGAGGTC
GAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAAGGAATTTTCGGCAATGGAGGAAACTCTGACCGAGCAATGCCGC
GTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGAAAAACCATTATGACGGTACCTAATGAATAAGCCCCGGCTA
ACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAA
GTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTGGAATTCCGTGTGTAGTGGTAAAATGCGTAAATATACG
GAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGG
AATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAA
TACAGTGGAGGTTATCAAAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTT
AATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCT
ACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGA
CTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAACCACGAAAGTTGGCA
ATACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAA
GGTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAG
CTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAATAGGTCACATCTTAAAAAAGCTCTTTGAAAAGTAGA
TAAACAAAGGTTAAAAAATTAAAGGAACTAAGGGCGCACAGTGGATGCCTTGGCACTA

Veneto (I) 39 (1,669 bp)

GGCAGTTTAGCATTAAACTTTAGTGGCGAACGGGTGAGTAACGCGTAAGCAATCTGCCCCTAAGACGAGGATAACAGTTGGAAACGACTGCTAAGACTGG
ATAGGAGATAGGAAGGCATCTTTTTATTTTTAAAAGACCTAGCAATAGGTATGCTTAGGGAAGAGCTTGCGTCACATTAGTTAGTTGGTGGGGTAATGGCC
TACCAAGACGATGATGTGTAGCCGGGCTGAGAGGTCGAACGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCACTAAGAGCCTA
TGAAGGATGGAGGAAACTCTGACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAAGATGGTGGAAAA
ACCATTATGACGGTACCTAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAGCGTTATCCGGAATTATTGGGCGTA
AAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAAAAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTG
GAATTCCGTGTGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCGAAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCC
GCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAAACC
TCACCAGGTCTTGACATGCTTTTGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTGGG
GACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTTCTGGCGAATCTCAA
AAAAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCTCGGG
GTTTGTACACACCGCCCGTCAAACCACGAAAGTTGGCAATACCCAAAGCCGGTGGCCTAACTTGAGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGAT
GATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGATCACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAA
GAAAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTGGTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCA
AAATAGGTCACACTATTAAAAAAGCTCTTGAAAAGTAGATAAACAAAGGTAGAAAATAAGAAT

Tuscany (I) 3 (1,405 bp)

TTTGAGGGATGGAGGAAACTCTGACCGAGCAATGCCGCGTGAACGATGAAGTATTTCGGTACGTAAAGTTCTTTTATTAGGGAAGAAAA
GATGGTGGAAAAACCATTATGACGGTACCTAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAATACATAGGGGGCAAG
CGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTAGGCGGTTAAATAAGTTTATGGTCTAAGTGCAACGCTCAACGTTGTGATGCTATAA
AAACTGTTTAGCTAGAGTTGGATAGAGGCAAGTAGAATTCCGTGTGTAGTGGTAAAATGCGTAAATATACGGAGGAACACCAGAAGCG
AAGGCGGCTTGCTGGGTCTTAACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAA
ACGATGAGTACTAAACGTTGGATAAAACCAGTGTTGAAGTTAACACATTAAGTACTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTT
AAAGGAATTGACGGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGGTACCCGAAAAACCTCACCAGGTCTTGACATGCTTT
TGCAAAGCTGTAGAAATACAGTGGAGGTTATCAGAAGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTGTTGTTAATTGCCATCATTAAGTTGGGGACTTTAGCAAGACTGCCAATGATAAATTGGAGGAAGGTG
GGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAAACGTGATACAATGGCTGTTACAAAGGGTAGCTAAAGCGTAAGCTT
CTGGCGAATCTCAAAAAAGCAGTCTCAGTTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAATCAGC
ATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCGTCAAACCACGAAAGTTGGCAATACCCAAAGCCGGTGGCCTAACTTG
AGCAATCAAGAAGGAGCCGTCTAAGGTAGGGTTGATGATTGGGGTTAAGTCGTAACAAGGTATCCCTACCGGAAGGTGGGGATGGATC
ACCTCCTTTCTAAGGATAAAGTTATCATCTTCAGTTTTGAGAGACTTAAGAAAGTTTTTCATTTTTTAAGATTCGGGCCTATAGCTCAGCTG
GTTAGAGCACACGCCTGATAAGCGTGAGGTCGGTGGTTCAAGTCCATTTAGGCCCACCAAAATAGGTCACATCTTAAAAAAGCTCTTTTG
AAAAGTAGATAAACAAAGGTTAGAAAATTAAAGGAACTAAGGGCGCACAGTGGATGCCTTGGCACT

All sequences showed 99% homology among themselves, however it was possible to distinguish BN strains after virtual RFLP analyses on R16F2/R2 amplicons. The comparison between real and virtual RFLP analyses showed in some cases differences between real and virtual RFLP profiles (Table 3.2) confirming the possible presence of interoperon heterogeneity and/or of mixed phytoplasma population in some of the samples.

After Genbank search, virtual RFLP analyses on R16F2/R2 amplicons of BN-infected and the other 16SrXII group phytoplasma strains listed in the Table 3.2, showed identical *Hpy188*I profiles among sample B4 from Hungary and BN-infected samples from Canada and Spain (EU086529 and AJ964960) (Fig. 3.5). Corn sample from Serbia (DQ222972) also showed a different profile as described in chapter 2 as well as strain from grapevine from Iran (Karimi *et al.*, 2009).

Table 3.2. RFLP results on BN infected samples from Hungary, Serbia and Italy and from reference strains. Identical letter = identical profile; *, almost identical profiles; bold, phytoplasma strains sequenced; small characters, results of virtual RFLP.

Samples (Country) acronym	Primers P1A/P7A° (27-1,755/1,847 bp)			Primers F1/B6 (130-1,669/1,814 bp)		Primers M1/B6 (758-1,669/1,814 bp)			Primers R16F2/R2 (149-1,397 bp)				
	*Hpy8*I	*Mbo*I	*Mbo*II	*Tru*I	*Mbo*II	*Rsa*I	*Mbo*II	*Alu*I	*Tru*I	*Mbo*II	*Tsp509*I	*Hpy8*I	*Hpy188*I
Langeveildeground (H) L1	C	A	B	A	B	A	A	A	A	A	A	A	
Langeveildeground (H) L2	C	A	B	A	A	A	A	A	Aa	Aa	Aa	Aa	a
Brandmajor (H) B1	C	B	C	A*	C	A	A	A	A	C	A	A	
Brandmajor (H) B2	B	B	C	A	C	-	-	-	A	C	A	A	
Brandmajor (H) B3	C	B	C	A	C	-	-	-	A	C	A	A	
Brandmajor (H) B4	C	-	C	A*	C	-	-	-	Aa	Ca	Aa	Aa	b
Bela Crkva (RS) BC1	D	A	A	A	A	B	A	A	A	C	A	A	
Bela Crkva (RS) BC2	D	B	B	-	-	-	-	-	Aa	A*a	Aa	Aa	a
Bela Crkva (RS) BC3	E	B	A	-	-	-	-	-	A	A	A	A	
Bela Crkva (RS) BC4	D	B	C	-	-	-	-	-	A	A	A	A	
Radmilovac (RS) R1	D	B	B	-	-	-	-	-	Aa	Ac	Aa	Aa	a
Radmilovac (RS) R2	D	B	B	-	-	-	-	-	A	A	A	A	
Radmilovac (RS) R3	D	A	A	A	A	A	A	A	A	C	A	A	
Čoka (RS) C1	D	B	B	A	A	A	A	A	Aa	Aa	Aa	Aa	a
Veneto (I) 39	D	B	B	A	C	A*	B	B	Aa	Ca	Bb	Aa	a
Veneto (I) 41	D	A	B	A	C	A*	B	B	A	C	A	A	
Tuscany (I) 2	D	A	B	-	C	A*	A	A	A	C	A	A	
Tuscany (I) 3	D	A	A	-	C	A*	A	A	Aa	Ba	Aa	A	a
STOL (RS)	A	A	A	A*	B	B	A	A	Ab	Aa	Aa	Ab	a
STOLC (F)	A	A	B	-	B	A	B	B	Aa	Ba	Aa	Aa	a
STOL-PO (F)	A	A	A	-	-	A	A	A	Aa	Ba	Aa	Aa	a

°, Martini, 2004

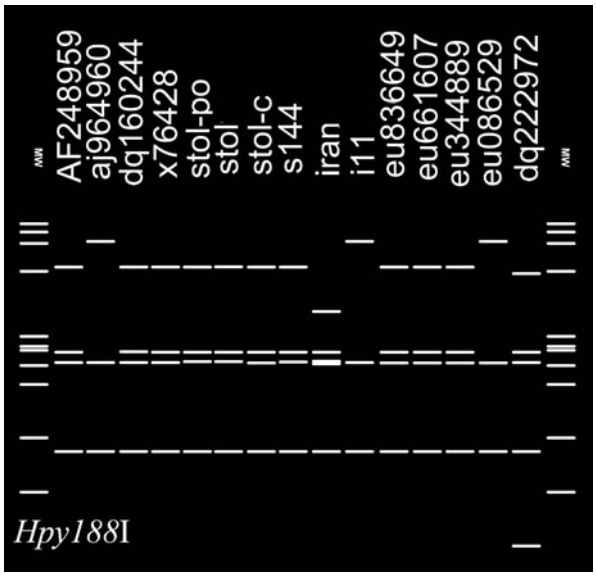


Figure 3.5. Virtual polyacrylamide 5% gel showing some of the RFLP profiles obtained with *Hpy*188I of R16/F2/2 products obtained from some sequenced BN strains from Serbia and Hungary and from reference strains STOL, STOLC and STOL-PO (Table 3.2) and from Genbank sequences (list in materials and methods) MW, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

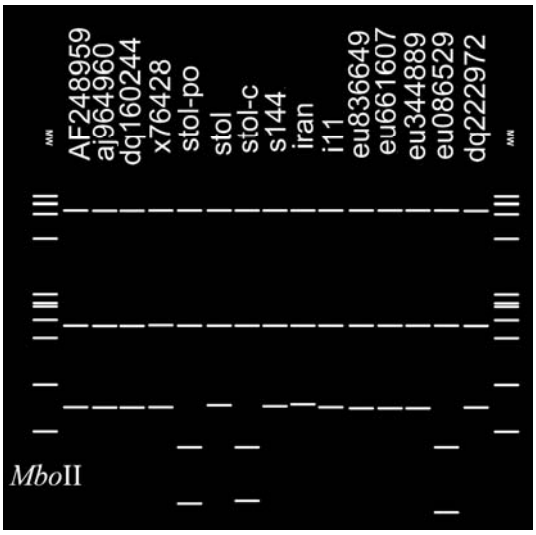
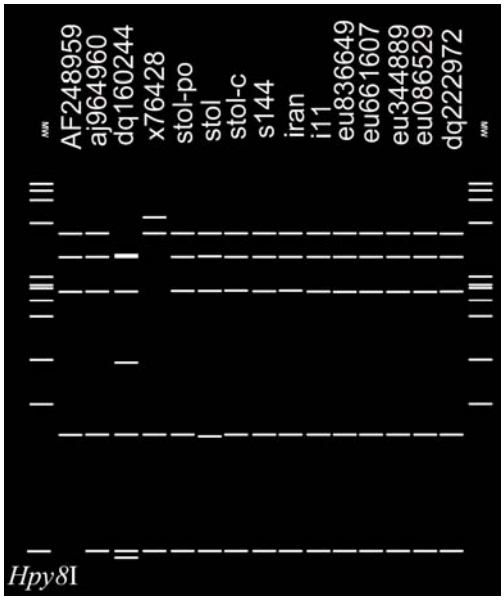


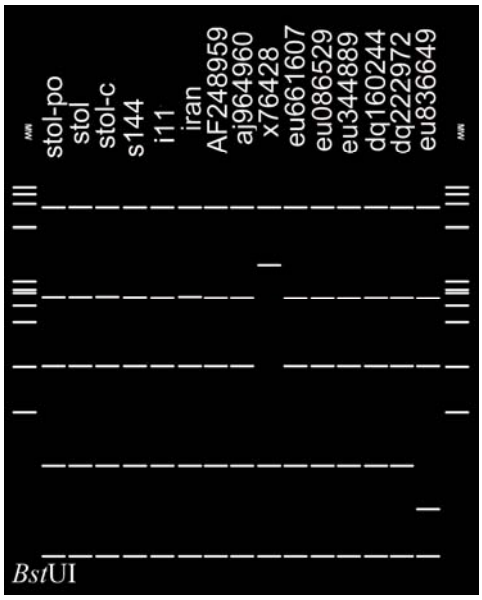
Figure 3.6. Virtual polyacrylamide 5% gel showing some of the RFLP profiles obtained with *Mbo*II of R16/F2/2 products obtained from some sequenced BN strains from Serbia and Hungary and from reference strains STOL, STOLC and STOL-PO (Table 3.2) and from Genbank sequences (list in materials and methods) MW, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Canadian isolated strain (EU086529) showed a unique profile with *Mbo*II restriction enzyme slightly different from those of the reference strains STOL-PO and STOLC: These latter however showed a profile with indistinguishable form the majority of strains employed and different from the other reference strain STOL (Fig. 3.6). A BN strain from Germany (VK) (X764281) showed unique *Bstu*I and *Hpy8*I profiles, and the BN strain EU836649 from Lombardy showed an unique *Bstu*I profile (Fig. 3.7a and b).

Figure 3.7. Virtual polyacrylamide 5% gels showing some of the RFLP profiles of R16/F2/2 products obtained in a) with *Hpy8*I and ion b) with *BstU*I from some sequenced BN strains from Serbia and Hungary and from reference strains STOL, STOLC and STOL-PO (Table 3.2) and from Genbank sequences (list in materials and methods) MW, marker ΦX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.



a



b

These results show that among phytoplasmas associated with grapevine Bois noir disease it is possible to distinguish strains based on 16S ribosomal gene inside phytoplasmas all classified as belonging to tuf type II and previously classified as subgroup 16SrXII-A. Further research is necessary, on higher number of strains from grapevine, on other host plants and possibly on other specific genes (Davis *et al*., 1993; Cimerman *et al*., 2006), to further verify the presence, the epidemiological relevance and the geographical distribution of differentiated strains.

Chapter 4

Apple proliferation phytoplasmas in Serbia

4.1 Introduction

Phytoplasma diseases of fruit trees in Europe include three economically important disorders: Apple Proliferation (AP), Pear Decline (PD) and European Stone Fruit Yellows (ESFY). AP is only known in Europe and it was for the first time described in Trentino in the fifties (Rui, 1950), and its major impact on the agriculture is that the infected plants continue to vegetate producing small and unmarketable fruits. Affected apple cultivars are almost all those present in the main apple growing areas of Europe (Refatti and Ciferri, 1954; Bovey, 1961; Brcak *et al*., 1972; Minoiu and Craciun, 1983; Bliefernicht and Krczal, 1995, Marcone *et al*., 1996a; Seemüller *et al*., 1998), and are Golden Delicious, and Renetta of Canada grafted on different rootstocks. Recently the disease was also reported in Serbia (Duduk *et al.,* 2008c). Psyllids play a crucial role in the transmission of fruit trees phytoplasmas: *Cacopsylla pyricola* Forster in England and *Cacopsylla pyri* (L) in France and Italy have been reported as vectors of PD (Tedeschi *et al*., 2002), whereas *C. pruni* (Scopoli) is able to transmit ESFY (Carraro *et al.*, 2001). Two species, *C. melanoneura* and *C. picta,* have also been reported as vectors for transmission of AP (Frisinghelli *et al*., 2000; Tedeschi *et al.*, 2002).

Phylogenetic analyses revealed that the 16S rDNA sequences of strains of each of these pathogens are identical or nearly identical. Seemüller and Schneider (2004) showed that the differences between the three phytoplasmas range from 1.0 to 1.5% of nucleotide positions and are thus below the recommended threshold of 2.5% for assigning species rank to phytoplasmas under the provisional status '*Candidatus*'. However, supporting data for distinguishing the AP, PD and ESFY agents at the '*Candidatus'* species level were obtained by examining other molecular markers, including the 16S-23S rDNA spacer region, protein-encoding genes and randomly cloned DNA fragments. Moreover the three phytoplasmas showed clear differences in vector transmission and host-range specificity. From these results, it can be concluded that the AP, PD and ESFY phytoplasmas are coherent but discrete taxa

that can be distinguished at the putative species level, for which the names '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', respectively, were proposed by Seemuller and Schneider (2004).

Apple proliferation is one of the most important phytoplasma diseases of apple, affecting almost all cultivars, reducing size (by about 50%), weight (by 63-74%) and quality of fruit, as well as reducing tree vigor and increasing susceptibility to powdery mildew. The disease was also detected in genotypes resistant to apple scab (Loi *et al*., 1995). Apple proliferation has only been reported from the EPPO region.

Fig. 4.1. Apple proliferation symptoms in an old tree (top left) and in productive trees showing witches' broom and reddening of the leaves (top right). Low row from left to right: stipules enlargement in infected plants, reddening and reduction of size in infected apple shoot, flowering of infected plants in September.

Apple is the main host of '*Ca.* P. mali'. Cultivars vary in reaction but most, including seedlings, appear to be susceptible. The disease can be observed either on cultivars or on rootstocks, as well as on wild and ornamental *Malus*. Also, '*Ca.* P. mali' was found in hazelnut (*Corylus* spp.) (Marcone *et al*., 1996b), cherry (*Prunus avium*), apricot (*P. armeniaca*) and plum (*P. domestica*) (Mehle *et al*., 2007).

The most typical symptom caused by '*Ca.* P. mali' is witches' broom at the end of shoots. On diseased trees, leaves are generally smaller and more dented, with unusually enlarged

stipules. Fruits are smaller and flattened, and peduncles longer. Early leaf reddening is a good indication of the presence of the disease (Fig. 4.1).

Frisinghelli *et al.* (2000) and Jarausch *et al.* (2003) reported that the psyllid *Cacopsylla picta* (Forster) (synonym: *C. costalis)* is a vector of apple proliferation in north-eastern Italy and in Germany, respectively. Field surveys carried out in apple orchards located in north-western and north-eastern Italy revealed that *C. melanoneura* is the most abundant psyllid (Alma *et al*., 2000; Tedeschi *et al.*, 2002). Besides, Tedeschi and Alma (2004) found that the overwintering adults of *C. melanoneura* are the responsible of the diffusion of AP in apple orchards. Another leafhopper, *Fieberiella florii* Stal (Homoptera: Cicadellidae), has been implicated as a vector of AP in Germany (Krczal *et al.*, 1989). Recently, Tedeschi and Alma (2006) confirmed the ability of this leafhopper to carry and transmit the phytoplasma in Italy. Despite the high infection rate of specimens, the risk of apple tree infection by *F. florii* in nature is probably low because of the very low insect density (polyphagy) recorded on apple. In spite of the low number of specimens collected, the presence of the leafhopper in apple orchards in summer, when the main vector, the psyllid *C. melanoneura*, feeds on alternative hosts, is meaningful (Tedeshi and Alma, 2006).

Distribution of phytoplasmas in the tree is not constant over the year. In winter the content of phytoplasmas declines in the tree due to sieve tube degeneration. They also concentrate more in the roots but, during April to May, reinvade the stem from the roots and reach a peak in late summer or early autumn (Seemüller *et al.*, 1984a and b). The distribution pattern of the phytoplasmas in the tree is also dependent on temperature. In France, phytoplasmas could be found throughout the trees at temperatures of 21-25°C, causing symptoms; at 29-32°C symptoms were inhibited and phytoplasmas were found only in the roots, but reinvaded the stems when plantlets were stored at lower temperature.

When a tree is inoculated with an infected bud, the first symptoms appear the following year, mostly on the inoculated branches. When carried in the rootstock, the causal agent produces symptoms on the first growth of the scion. It appears to be localized mainly in suckers and terminal shoots, where it has been observed in the phloem of leaf petioles, midribs and stipules.

Although in Europe AP disease affects most or all varieties of apple trees, it is caused by a relatively homogeneous pathogen that is closely related to the phytoplasmas including PD and ESFY. Jarausch *et al.* (2000) by RFLP analysis of a 1.5 kb chromosomal DNA fragment amplified by PCR (PCR/RFLP) from various isolates of '*Ca.* P. mali' distinguished three different subtypes named AP, AT-1 and AT-2. Sequence analysis of an 846 bp fragment of each subtype showed that the sequences differed only in the restriction sites responsible for the observed polymorphism. Thus, the '*Ca.* P. mali' subtypes are very closely related. The observed point mutations were responsible for specific amino acid changes in the putative

protein PR3. Physical map of AT strain chromosome was constructed by Lauer and Seemüller (2000). In this work, they showed genome size and *Bss*HII restriction profiles of strain AT is different from strain AP15, pear decline and European stone fruit yellows phytoplasmas. Kison *et al.* (1997) showed different profiles of *Hin*dIII-digested DNA of strains AT and AP15 by southern blot following hybridization with probe IH184 and IH196.

4.2. Material and Methods

During 2005-2008 apple plants showing proliferation symptoms were observed in diverse cvs in different areas of north eastern Italy, Hungary and Serbia. Samples employed are listed in Table 4.1 and are as follows: six samples from Hungary collected in small field destroyed from AP, near to the Austrian border (samples H-1 trough H-6). Two samples from Serbia (RS-135 and RS-151) from Bela Crkva where the disease was only observed in scattered plants. Five samples from Italy respectively three from different areas of Trentino region (I-TN1 to I-TN3) where the disease is epidemically present since more than 15 years and two samples from Veneto region (I-VE11 and I-VE16) from areas where the disease is starting to appear in these years.

As reference strains the three AP strains reported in literature as AP-15, AT-1 and AT-2 were employed. The last one was in apple Golden Delicious kindly provided by S. Grando (E. Mach Foundation, S. Michele all'Adige, TN; Italy) while the other two are from the collection at DiSTA (Bertaccini, 2003).

To evaluate the possible presence of molecularly differentiable strains PCR/RFLP analyses were carried out on three diverse regions of the AP genome such as 16S rDNA plus spacer region and beginning of 23S (Khan *et al*., 2002b; Casati *et al*., 2007); ribosomal protein (rp) gene sequences *rpl22* and *rps3,* and nitroreductase genes (Martini *et al*., 2008; Bertaccini *et al*., 2008; Jarausch *et al*., 2000; 2004).

Leaves and young shoots were collected from June to October in orchards with epidemic presence of apple proliferation, and in others where the symptomatic plants were present in a scattered way. DNA was extracted from fresh leaf midribs and phloem by a chloroform/phenol procedure (Prince *et al.*, 1993) or CTAB (Angelini *et al*., 2001).

PCR experiments were carried out on the nucleic acid samples resuspended in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] and diluted in distilled sterile water to obtain a final concentration of 20 ng/µl. In total 25 µl reaction mixtures contained 1.0 µl of nucleic acid, 200 µM of each dNTP, 1.25 U Taq polymerase, and 0.4 µM of primers.

PCR amplification and RFLP analyses

16S ribosomal DNA plus spacer region. A direct PCR amplification with P1/P7 universal phytoplasma primer pair (Deng and Hiruki, 1991; Schneider *et al*., 1995), amplifying 16S rDNA, the spacer region between 16S and 23S rDNA and the 5' portion of 23S rDNA, was performed for the phytoplasma detection. Nested PCR amplification was also carried out on P1/P7 amplicons diluted 1:30 in sterile distilled water with R16F2/R2 (Lee *et al*., 1995) and F1/B6 (Davis and Lee, 1993; Padovan *et al*., 1995).

Each 25 µl PCR reaction mix contained 2.5 µl 10X PCR buffer, 0.8 U of *Taq* polymerase, 0.2 mM dNTPs, 1.5 mM MgCl2 and 0.4 µM each primer. PCR conditions were: 35 cycles (Biometra, Uno Thermoblock, Gottingen, Germany as thermal cycler), 1 min (2 min for the first cycle) denaturation step at 94 oC, 2 min for annealing at 50°C and 3 min (10 min for the last cycle) for primer extension at 72°C.

DNAs extracted from periwinkle plants infected by phytoplasma strains from the micropropagated collection of DiSTA (University of Bologna) (Bertaccini, 2003) were used as positive control samples. Samples with the reaction mixture devoid of DNA templates were included in each experiment as negative controls. PCR products were subjected to electrophoresis in a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination.

Three µl of PCR products were digested using enzymes *Ssp*I and *Rsa*I for R16F2/R16R2 amplicons following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). Further strain characterization was carried out using RFLP analyses with *Hpa*II and *Fau*I on F1/B6 amplicons. The restriction patterns were then compared with those of control strains after electrophoresis through a 5% polyacrylamide gel in 1X TBE buffer followed by staining with ethidium bromide and visualization under an UV transilluminator.

Ribosomal protein (rp) gene sequences *rpl22* and *rps3*. *RpS3* gene was amplified in direct PCR reactions using primers rpAP15f/rpAP15r. This detection method is specific for '*Ca*. P. mali' and can distinguish 4 different RFLP-subtypes (rpX-A, B, C and D) on the basis of a sequence-12 nucleotide long (AAGAAATTAAAG) that can be present in variable copy number (ranging from 2, in the case of rpX-B, to 6, in the case of rpX-C) (Martini *et al*., 2008).

rpAP15f 5'-AGTGCTGAAGCTAATTTGG-3'
rpAP15r 5'-TGCTTTTTATAGCAAAAGGTT-3'

PCR conditions were as follows: initial denaturation for 2 min at 94°C; 40 cycles (1 min at 94°C, 45 s at 55°C and 90 s at 72°C); final extension for 8 min at 72°C.

Three µl of PCR products were digested using enzyme *Alu*I following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction patterns were then observed after electrophoresis through a 5% polyacrylamide gel in 1X TBE buffer followed by staining with ethidium bromide and visualization under an UV transilluminator. Samples with the reaction mixture devoid of DNA templates were included in each experiment as negative controls. PCR products were subjected to electrophoresis in a 1% agarose gel and visualised by staining with ethidium bromide and UV illumination.

Non ribosomal DNA fragment. For the amplification of the non ribosomal DNA fragment (nitroreductase gene, Jarausch *et al.*, 2000) a semi-nested PCR was employed using 1:30 diluted products of the direct amplification as templates with primers AP8/AP10 followed by primers AP13/AP10. PCR cycle was as follows: 95°C for 1 min, followed by 45 cycles with 94°C for 1 min, 53°C for 45 sec, 72°C for 1.30 min, the extension of last cycle was at 72°C for 8 min.

Three µl of PCR products were digested using enzymes *Rca*I and *Hinc*I following the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction patterns were then compared with those of control strains after electrophoresis through a 5% polyacrylamide gel in 1X TBE buffer followed by staining and visualization as described above.

4.3 Results and discussion

Nested PCR amplification with R16(X)F1/R1 primers followed by RFLP analyses with *Rsa*I and *Ssp*I allow to confirm that all sample analyzed were infected with '*Ca*. P. mali' (Fig. 4.2).

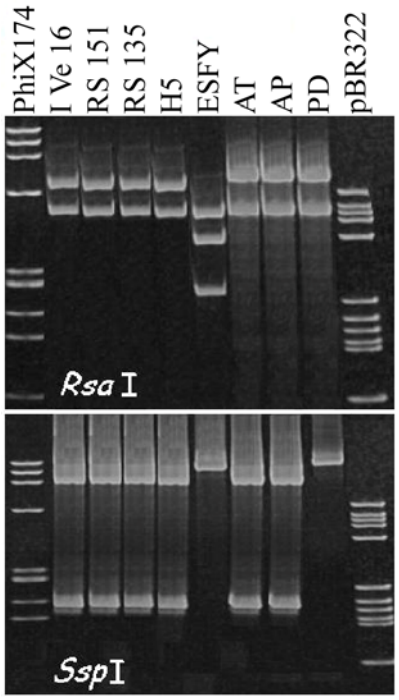


Fig. 4.2. Polyacrylamide gels showing RFLP profiles with *Rsa*I and *Ssp*I of nested-PCR prducts amplified from selected samples from Serbia, Hungary and Italy (27, 13, 21 and 23) with primers R16F2/R2. Control samples ESFY, European stone fruit yellows (16SrX-B); AT and AP, apple proliferation from Germany and from Italy respectively (16SrX-A); PD, pear decline (16SrX-C). Markers: phiX174 *Hae*III digested, fragment sizes in base pairs (top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, and 194; pBR322: *Bsu*RI digest, fragment sizes in base pairs from top to bottom: 587, 540, 502, 458, 434, 267, 234, 213, 192, 184, and 124.

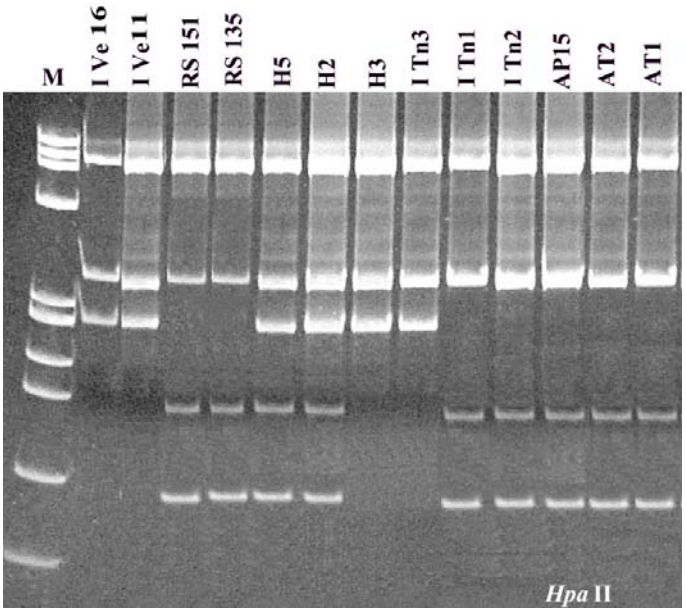


Fig. 4.3. Polyacrylamide gel showing RFLP profiles with *Hpa*II of nested-PCR amplicons amplified from selected samples from Serbia, Hungary and Italy (see list in Table 4.1) with primers F1/B6. Control samples AP15, AT1 and AT2, apple proliferation strains from Germany and from Italy (16SrX-A). M, marker phiX174 *Hae*III digested, fragment sizes in base pairs (top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, and 194.

Also primers F1/B6 amplified all tested samples and RFLP analyses on these amplicons distinguished two phytoplasma profiles (P-I and P-II) (Fig. 4.3). P-I was detected in reference strains AP, AT1, AT2, in samples from Serbia, and in two out of the three samples from Trentino. The P-II profile was detected in samples from Hungary, where the majority of samples show both profiles together (Table 4.1).

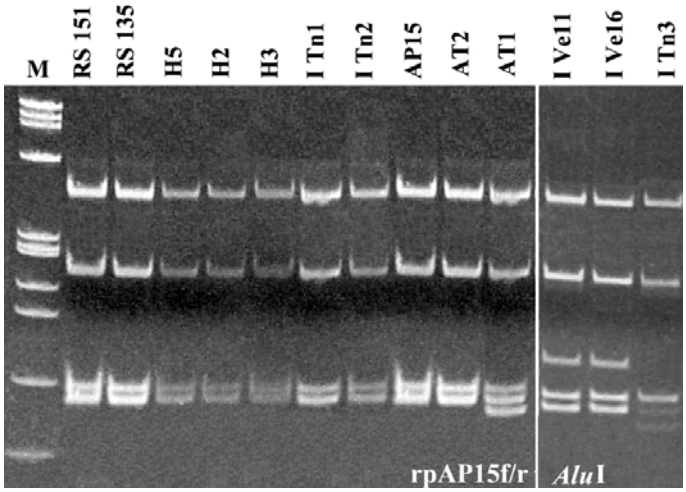


Figure 4.4. Polyacrylamide gel electrophoresis of RFLP results from selected positive samples (see Table 4.1) obtained with *Alu*I restriction enzyme on amplicons from rp primers. Control samples AP15, AT1 and AT2, apple proliferation strains from Germany and from Italy (16SrX-A). M, marker phiX174 *Hae*III digested, fragment sizes in base pairs (top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, and 194.

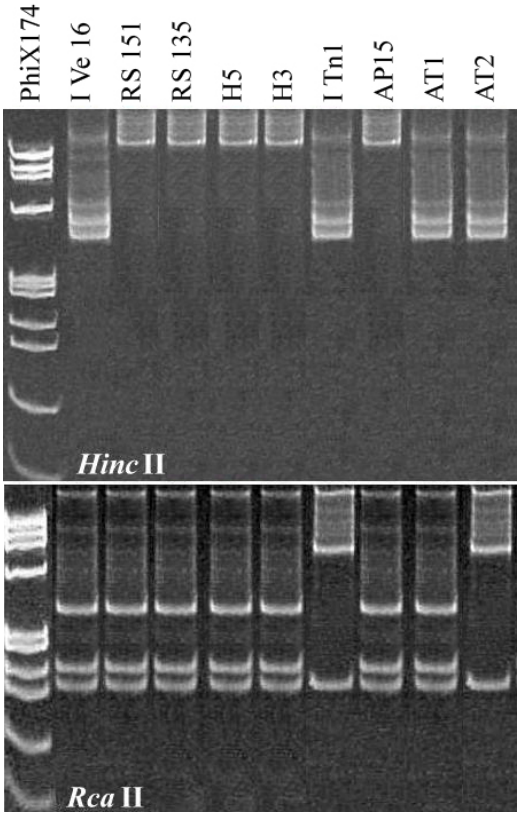


Figure 4.5. Polyacrylamide gels electrophoresis of RFLP results from selected positive samples obtained with *Rca*I and *Hinc*I restriction enzymes on AP13/AP10 amplicons. Reference strains AT and AP, apple proliferation from Germany and from Italy respectively (16SrX-A). phiX174, marker phiX174 *Hae*III digested, fragment sizes in base pairs (top to bottom): 1353, 1078, 872, 603, 310, 281, 271, 234, and 194.

The RFLP analyses of *rpl22-s3* genes allow to identify in all the samples showing P-I profile the presence of phytoplasmas belonging to rpX-A subgroup, while in samples showing P-II profile it was possible to distinguish other described rp subgroups. The rpX-B and X-C subgroups were identified in one of the samples from Trentino and in the two samples from Veneto respectively (Fig. 4.4, and Table 4.1).

Further RFLP characterization on AP13/AP10 amplicons differentiates among strains belonging to rpX-A subgroup: the two Serbian samples show AP profiles, while those from Trentino show AT-2 profiles (Fig. 4.5). In the samples from Hungary the presence of AT1, AT2, and AP profiles was identified apparently with lack of correlation with rp strain identification but probably due to the fact that mixed phytoplasma strain infection was quite common in these samples (Table 4.1).

Table 4.1. Results of molecular analyses to characterize AP strains from the different geographic origin.

Strain acronyms	Primers F1/B6		Group	Primers AP13/AP10		Group	Primers rpAP15f/rpAP15r	Group
	*Hpa*II	*Fau*I		*Rca*I	*Hinc*II		*Alu*I	
H-1	-	-	nd	A	A	AT2	A	rpX-A
H-2	A+B	A	PI+PII	-	-	nd	A	rpX-A
H-3	B	A	PII	B	B	AP	A	rpX-A
H-4	A+B	A	PI+PII	B	A	AT1	A	rpX-A
H-5	B	A	PI+PII	B	B	AP	A	rpX-A
H-6	A+B	A	PI+PII	B	B	AP	A	rpX-A
RS-135	A	A	PI	B	B	AP	A	rpX-A
RS-151	A	A	PI	B	B	AP	A	rpX-A
I-VE11	B	A	PII	-	-	nd	C	rpX-C
I-VE16	B	A	PII	B	B	AT1	C	rpX-C
I-TN1	A	A	PI	A	A	AT2	A	rpX-A
I-TN2	A	A	PI	-	-	nd	A	rpX-A
I-TN3	B	A	PII	?	B	nd	B	rpX-B
AP-15	A	A	PI	B	B	AP	A	rpX-A
AT-1	A	A	PI	B	A	AT1	B	rpX-B
AT-2	A	A	PI	A	A	AT2	A	rpX-A

Nd, group not determined

The combined use of these molecular markers allows differentiating '*Ca*. P. mali' strains according with geographical and epidemic distribution. It was shown that in the epidemic areas of Trentino, a homogeneity of these three molecular markers was present (Bertaccini *et al*., 2008) so the epidemic could be linked with just one phytoplasma population. However, some strain differentiation was detected in both Italian areas studied indicating the population variability was present. Considering that rp-C and rp-D strains detected in scattered plants in other areas of Italy were negative in ELISA (Martini *et al*., 2008). It is evident that AP phytoplasmas are differentiating new strains that could not be easily detected and requires further study also at the epidemiological level to verify identity of insect vector as well as the possible role of infected nursery material in the long distance spreading of the diverse strains.

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