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A MOLECULAR INVESTIGATION TO IDENTIFY PHYTOPLASMAS ASSOCIATED WITH FRUIT TREES

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Phytoplasmas are cell wall-less and phloem-restricted plant pathogenic bacteria. They are known to be associated with diseases in several hundred plant species and have been shown to be transmitted in propagative manner by sap-sucking insect vectors. Because of the inability to culture them *in vitro*, detection and identification of phytoplasmas have been largely based on their molecular characteristics. Prior to the development of molecular probes, 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. Phytoplasma strains were differentiated and identified by their biological properties, such as the similarity and difference in symptoms they induced in infected plants and their insect vectors. Determination of these biological properties was laborious and time-consuming, and often the results were inconsistent. In many cases, identities of insect vectors also remained unknown, further complicating identification based on biological criteria. The development of serological and nucleic acid-based molecular probes and polymerase chain reactions (PCR) technology in 1980s has advanced the art of phytoplasmal diagnostics. Phylogenetic analysis of 16S rDNA gene and ribosomal protein sequences showed that phytoplasmas belong to a monophyletic clade within the class Mollicutes. The causal agents of Apple Proliferation (AP), Pear Decline (PD) and European Stone Fruit Yellows (ESFY) are closely related to each other and clustered in a very homogenous phylogenetic subclade. Although these phytoplasmas are classified as quarantine organisms in Europe, a rapid, specific and sensitive diagnostic method for large-scale analyses is still lacking. The design of primers based on various conserved sequences such as 16S rRNA gene, ribosomal protein gene operon, *tuf* gene and other chromosomal DNA fragments was the major breakthrough in detection, identification, and classification of phytoplasmas. PCR-based assays provide so far the most sensitive method for phytoplasma detection. The majority of PCR protocols employ primers that amplify a fragment of the 16S ribosomal RNA gene. This study describes a suitable technique for detection and identification of phytoplasmas associated with fruit trees in Europe and an analysis of chromosomal DNA polymorphism to compare these phytoplasmas.

The present study include some samples of apple, pear and stone fruit trees from France (Dr. B. Pradier, Station de Quarantaine des Ligneux, Lempdes, France) showing symptoms potentially caused by phytoplasmas (yellowing, depression, decline, leaf reddening). Total nucleic acid was extracted from fresh leaf midribs and phloem tissues by modifications of Zhang *et al.* (1998) method. Total DNA preparations were quantified by absorbance at 260 nm and were adjusted to a concentration of 100 ng/μl for all experiments. DNA

amplifications with direct and nested PCR were conducted using primer pair fU5/rU3 and R16F1/R16mR1, derived from phytoplasma rDNA sequences (Lorenz K.H. *et al.* 1995 and Gundersen D.E. *et al.* 1996). Specific primers AP5/AP4 were used for detection of Apple Proliferation (AP) in apple samples (Jarausch W. *et al.* 1995). The detection of European Stone Fruit Yellows (ESFY) in stone fruit samples was also carried out with specific primers ECA1/ECA2 (Jarausch W. *et al.* 1998). DNA samples of AP, PD and ESFY served as controls in all experiments. For more precise identification of these phytoplasmas, restriction fragment length polymorphism (RFLP) analysis was also carried out with three restriction enzymes (*SspI*, *RsaI* and *TaqI*) on the PCR fragments amplified in nested PCR (Lee I.M. *et al.* 1998).

The specificity of the primer pairs AP5/AP4 and ECA1/ECA2 was tested with DNA extracts obtained from plants infected with closely related phytoplasmas. Primer pair AP5/AP4 gave a PCR product of expected size (483bp) only with DNA from infected *Malus* and primer pair ECA1/ECA2 gave a PCR product of expected size (237 pb) only with infected *Prunus*. Direct PCR amplification with universal primer pairs, fU5/rU3 and R16F1/R16mR1 resulted in products with the expected size of 0.9 kb and 1.4 kb, respectively, but only in case of some symptomatic samples. Nested PCR with universal primer pair R16F2n/R2 was then applied on dilution (1/25) of each of the latter products. An expected PCR product of 1.2kb was observed for more samples in comparison with the result of direct PCR. No amplification was observed for total nucleic acid extracts from healthy plants. RFLP analyses of the amplified nested-PCR products using *SspI*, *RsaI* and *TaqI*, resulted in three different 16S rDNA RFLP patterns depending on the host plant group. The isolates of AP and PD were differentiated from each other with *SspI* restriction endonuclease. Furthermore, the isolates of ESFY were distinct from AP and PD with the use of the other enzymes. These analyses also revealed identical patterns of infected apple, pear and stone fruit samples with reference strains of APP, PDP and ESFYP.

Totally, phytoplasmas could be detected by PCR assays, the most sensitive detection method for phytoplasmas at present. Specific and sensitive detection of phytoplasmas affecting apple and stone fruit trees could be achieved using primers derived from a chromosomal DNA fragment, confirming already obtained results. No cross reaction was obtained with DNA from plant diseased with other phytoplasmas, e.g. the closely related phytoplasmas. Nested PCR assay using universal primer pairs increased sensitivity in phytoplasmal detection. The result of RFLP analyses showed that the phytoplasmas associated with symptomatic apple, stone fruit and pear trees belong respectively to subgroups 16SrX-A, 16SrX-B and 16SrX-C. Thus, the approach using RFLP analyses of amplified 16S rDNA sequences provided a simple and quick alternative to differentiate such very closely related organisms. All strains of AP and PD showed the same restriction profiles with most endonucleases, confirming that the phytoplasmas infecting apple and pear are closely related.

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