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Interception of '*Candidatus* Phytoplasma solani' in Brazil in apple tree propagative material imported from France

*Interceptação de '***Candidatus** *Phytoplasma solani' no Brasil em material propagativo de macieira importado da França*

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ABSTRACT: Apple plants from France introduced in Brazil for research purposes were subjected to a phytosanitary analysis at the Plant Quarantine Laboratory of Embrapa Genetic Resources and Biotechnology (Cenargen). After grafting onto healthy apple rootstock, some plants showed phytoplasma-infection symptoms. Polymerase Chain Reaction (PCR) tests yielded DNA fragments of the expected size for phytoplasmas. DNA sequencing revealed an identity of the 16S rDNA nucleotide sequence of 98-99% with '*Candidatus* Phytoplasma solani'. This phytoplasma species is responsible for losses in European apple orchards and has not been reported in Brazil. According to the Federal Legislation on Plant Protection, the plants were incinerated to avoid the introduction of this exotic pest in Brazil.

KEYWORDS: *Malus* spp.; '*Candidatus* Phytoplasma solani'; 16SrXII phytoplasma; quarantine; molecular diagnosis.

RESUMO: Plantas de macieira originárias da França introduzidas no Brasil para fins de pesquisa foram submetidas à análise no Laboratório de Quarentena Vegetal da Embrapa Recursos Genéticos e Biotecnologia (Cenargen). Após realizar a enxertia em porta-enxertos sadios, algumas plantas apresentaram sintomas típicos de infecção por fitoplasmas. Os testes por meio da técnica de reação em cadeia da polimerase (PCR) obtiveram fragmentos de DNA do tamanho esperado para fitoplasmas. O sequenciamento de DNA revelou uma identidade de 98-99% da região 16 rDNA dos fitoplasmas encontrados com o fitoplasma '*Candidatus* Phytoplasma solani'. Essa espécie de fitoplasma é responsável por perdas em pomares de maçã europeus e não foi relatada no Brasil. De acordo com a Legislação Federal de Proteção de Plantas, as plantas de macieira foram incineradas para evitar a entrada dessa praga exótica no Brasil.

PALAVRAS-CHAVE: *Malus* spp.; '*Candidatus* Phytoplasma solani'; fitoplasma 16SrXII; quarentena; diagnóstico molecular.

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The safe exchange of plant germplasm has been an indispensable tool for the improvement of genetic resources, and hence, the generation of new varieties that contribute to agriculture. This is essential for agriculture in Brazil, where the majority of cultivated crops belongs to species from other regions of the world (MARINHO et al., 2003). In Brazil, from the point of view of economic development, apple crops have a great capacity to generate income and employment and, as a consequence, rural development. Fresh fruits and juice have also been designated for exportation (BRASIL, 2013).

Several apple breeding programs based on imported germplasm have been developed in Brazil. The movement of germplasm inevitably involves the risk of accidentally introducing pests along the host plant material, and quarantines are the first line of defense against the movement of economically significant plant pests between, and within, countries (YADAV; TIAGY, 2006).

Phytoplasmas are plant-pathogenic bacteria associated with diseases in several hundreds of plant species, many of which are economically important crops (BERTACCINI et al., 2014). These bacteria can easily be unknowingly disseminated over long distances in the vegetative parts of plants. This mainly happens, because in several cases, the diseased plant is symptomless and the difficulty involved in cultivating these bacteria *in vitro* hampers their detection. They are detected by the analysis of their 16S rRNA region, which is also used to classify them into groups based on the Restriction Fragment Length Polymorfism (RFLP) analysis (LEE et al., 1998) and into putative species through the sequence similarity of this gene (IRPCM, 2004).

In 2012, the Plant Quarantine Laboratory of Embrapa Genetic Resources and Biotechnology (Cenargen) received 11 apple tree samples (*Malus* spp.) from France. On average, each sample consisted of five stems. After the initial inspection, the stems were treated with insecticides and fungicides and grafted onto healthy apple rootstocks. The plants were kept in a quarantine greenhouse. After two months, typical symptoms of phytoplasma infection were observed in six samples, such as plant stunting, small leaves, and severe foliar distortion (Fig. 1). The symptomatic leaves were subjected to DNA extraction with a DNeasy® Plant Mini Kit (Qiagen, Netherlands) according to the manufacturer's instructions. Nested PCR reactions were performed using universal primer pairs P1/P7, followed by R16F2n/R16R2 for the 16S rRNA region of phytoplasmas (LEE et al., 1998). We obtained fragments of the expected size (approximately 1,200 bp - data not shown) from two of the six suspect samples, which were sent for sequencing at Macrogen (South Korea). The obtained sequences were compared with the Genbank sequences by employing the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/Blast), and phylogenetic analyses were performed using MEGA 6 (TAMURA et al., 2013).

Phytoplasmas were amplified from two of the six suspect apple samples, named "Dalinette" and "Galiwa", through nested PCR (data not shown). The negative results obtained in other symptomatic accessions may be related to the well-known and uneven distribution of phytoplasmas in woody hosts, and to low phytoplasma concentration, as previously reported (DUDUK et al., 2010).

The nucleotide comparisons between the phytoplasmas detected in apple samples showed an 97.7% identity and therefore may be member of the same putative '*Candidatus* Phytoplasma' species, according to the IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group standards. The partial 16S rDNA sequence analyses using BLAST showed that both phytoplasmas detected in apple samples shared more than 98% homology with the sequence of the putative '*Candidatus* Phytoplasma' solani members (accession number KP864674), and of the 16SrXII group, subgroup A, according to the RFLP classification scheme.

The phylogenetic analyses confirmed the results of the sequence comparisons; therefore, two phytoplasmas detected in imported apple samples clustered in the same phylogenetic branch that encompasses 16SrXII group phytoplasmas and are more closely related to the member of the putative '*Candidatus* Phytoplasma solani' species (Fig. 2).

Phytoplasmas classified in 16S rDNA RFLP group 16SrXII infect a wide range of wild and cultivated plants in different regions of the world (QUAGLINO et al., 2013). Four '*Candidatus* Phytoplasma' species have been formally described within group

Figure 1. Apple plant with foliar reduction and stunting surrounded by healthy apple plants.

16SrXII, including '*Ca*. Phytoplasma solani' (QUAGLINO et al., 2013), '*Ca.* Phytoplasma australiense' (DAVIS et al., 1997), '*Ca*. Phytoplasma japonicum' (SAWAYANAGI et al., 1999), and '*Ca*. Phytoplasma fragarie' (VALIUNAS; STANIULIS; DAVIS, 2006). Strains of '*Candidatus* Phytoplasma solani' are relatively widespread around the world, and new reports continue to emerge (QUAGLINO et al., 2014). They are associated with both *bois noir* disease of grapevines and with *stolbur* disease in peppers, tomatoes, potatoes, and peas, as well as with other herbaceous and woody plant diseases (QUAGLINO et al., 2013). In South America, however, there are still few reports of this Phytoplasma putative species, including data of grapevines in Chile (GAJARDO et al., 2009) and potatoes in Colombia (MEJIA et al., 2011). There was no report of '*Candidatus* Phytoplasma solani' affecting apple trees or other plant species in Brazil. However, its presence in the continent, as well as in many other parts of the world, in several distinct hosts, shows that strains of this putative phytoplasma species may develop well in different conditions. It is possible that once introduced in Brazil, they can spread, considering Brazil has hundreds of distinct genera insects belonging to the Cixiidae and Cicadellidae families, which encompass the known vectors for this putative phytoplasma species.

To date, in Brazil, only phytoplasmas belonging to the group 16SrIII were associated with apple trees, causing apple rubbery wood disease (RIBEIRO et al., 2007). However, no great losses have been registered. Owing to the risk of this phytoplasma introduction in Brazil, considering its potential to affect agriculture that presents large areas cultivated with hosts for group 16SrXII phytoplasmas, associated with the inefficiency of antibiotic treatment or other control measures to recover the diseased plants, all accessions were incinerated according to the Federal Legislation on Plant Protection (Decree 24.114 of April 12, 1934). This communication reports the first interception of this phytoplasma species by the Plant Quarantine Laboratory of Cenargen.

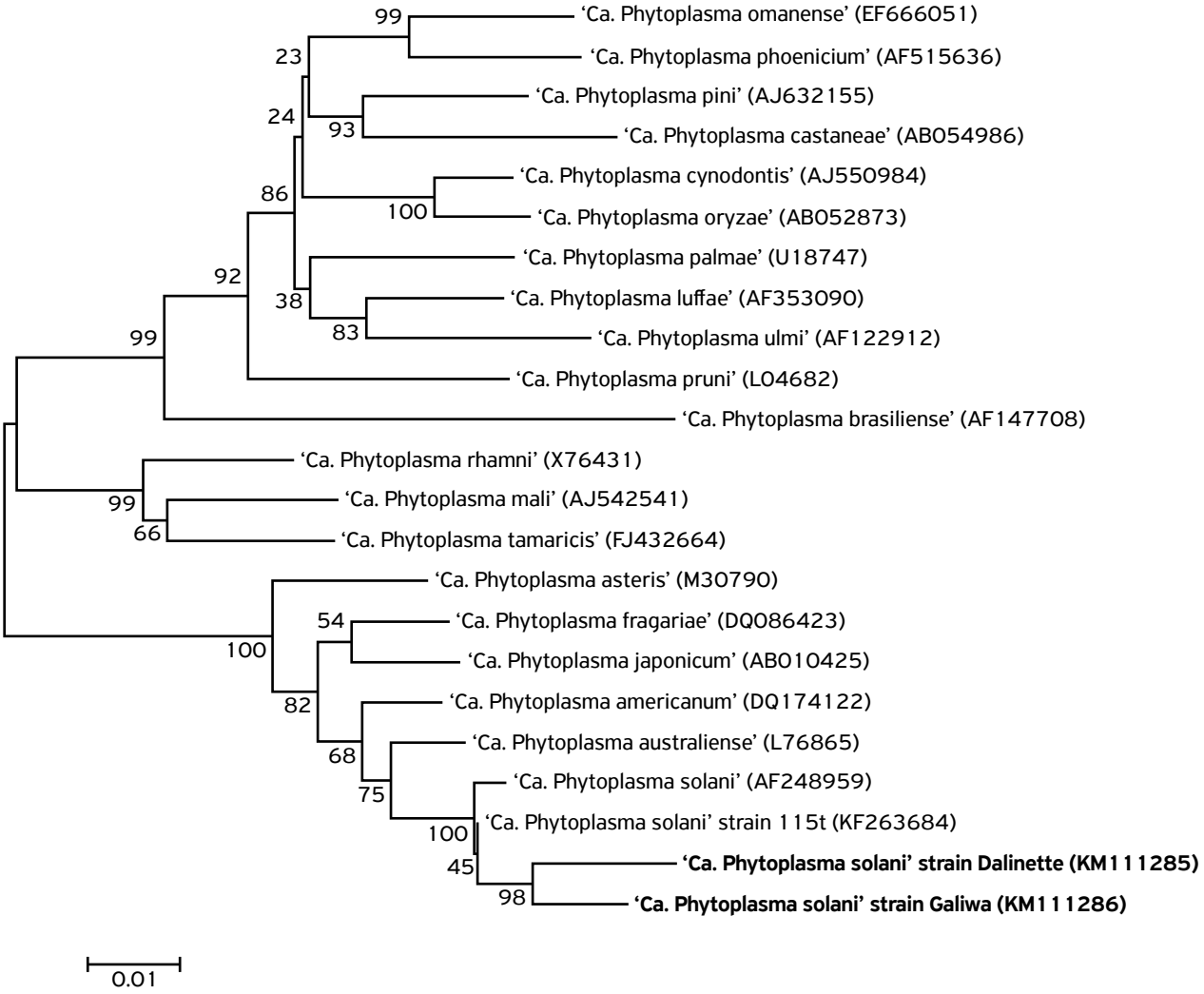

Figure 2. Phylogenetic tree inferred from 16S rDNA sequences using the neighbor-joining method. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1,000 replicates), is shown next to the branches.

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