*Molecular and Cellular Probes* (2000) **14**, 171–179
doi:10.1006/mcpr.2000.0304, available online at http://www.idealibrary.com on IDEAL®

# PCR-RFLP and sequence analysis of a non-ribosomal fragment for genetic characterization of European stone fruit yellows phytoplasmas infecting various *Prunus* species

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*(Received 8 February 2000, Accepted 11 April 2000)*

A 927 bp non-ribosomal fragment was used to assess the genetic variability of the European stone fruit yellows (ESFY) phytoplasma infecting 14 different *Prunus* species. For this, 175 isolates originating from four different Mediterranean countries were tested by PCR-RFLP analysis with seven restriction enzymes. No polymorphism among the ESFY phytoplasma could be observed but 12 out of 18 restriction sites differed between the homologous fragments of ESFY and apple proliferation (AP) phytoplasmas. An 846 bp fragment of a French ESFY isolate was sequenced, it included the 3′-end of a putative nitroreductase gene, an intergenic region and a truncated open reading frame. This ESFY phytoplasma sequence showed 89·7% identity with the equivalent AP phytoplasma nucleotide sequence (83·9% identity at the amino acid level). The G+C content of the entire sequence was extremely low (15·4%) and A+T-rich codons were highly preferred in codon usage. In this paper, we report the presence of the ESFY phytoplasma for the first time in Turkey and in five *Prunus* hosts never reported previously. Our results also indicate that the ESFY phytoplasma isolates affecting various *Prunus* species are genetically homogenous but can be distinguished from the AP phytoplasma. Therefore, they are likely to represent different taxons.
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***KEYWORDS:*** phytoplasma, taxonomy, apple proliferation, apricot chlorotic leaf roll, *Mollicutes*, nitroreductase gene.

## INTRODUCTION

Severe decline of European stone fruits was reported as early as 1924 on apricots in France[1] and, then, on Japanese plums in Italy,[2] but it was only in 1973 that the phytoplasma etiology of the diseases was demonstrated.[3] At that time phytoplasmas were called mycoplasma-like organisms (MLOs). As different *Prunus* species were affected, and showed somewhat different symptoms, several disease names were given: apricot chlorotic leaf roll (ACLR) on apricot trees,[4] plum leptonecrosis (PLN) on Japanese plum trees,[5] 'Molières disease' on sweet cherry trees,[6] peach yellows,[7] peach rosette[8] and peach vein clearing (PVC)[9] on peach trees and several other decline diseases on European plum trees,[10,11] almond and flowering cherry trees.[12] *Prunus* rootstocks are also severely affected by similar disorders.[10–13] Common symptoms were yellowing and leaf roll in summer, off-season growth in winter, die-back and a more or

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0890–8508/00/030171+09 $35.00/0
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less rapid decline. Up to now these diseases have been restricted to southern Europe with a northern border in Germany.[12] In the past decades they have been of increasing economic importance. ACLR and PLN are especially devastating for apricot and Japanese plum trees.

PCR-RFLP and sequence analysis of the phytoplasmas 16 S rRNA gene revealed that the phytoplasmas infecting stone fruits in Europe form a unique cluster together with the AP and pear decline (PD) phytoplasmas.[14,15] This cluster has been named AP phytoplasma group or subclade.[16]

Southern hybridization studies revealed that the European stone fruit phytoplasmas were genetically similar but clearly different from phytoplasmas infecting *Prunus* species in North America.[17] The name European stone fruit yellows (ESFY) phytoplasma was proposed for these phytoplasmas.[18]

Despite considerable progress in phylogenetic classification of phytoplasmas, little work has been done so far to assess the genetic variability of natural populations of a given phytoplasma. Conserved phylogenetic markers such as ribosomal genes are insufficiently variable for this purpose. Therefore, in 1994, Jarausch *et al.*,[19] using the sequence of a cloned 1·8 kbp chromosomal fragment of the AP phytoplasma, defined primers for PCR and RFLP analysis of fruit tree phytoplasmas. The fragment included the gene for a putative nitroreductase, an intergenic region and a truncated open reading frame of unknown function. PCR showed that the AP phytoplasmas could be separated into three subtypes and that some of the selected PCR primers also amplified ESFY phytoplasma DNA. Amplification of a fragment homologous to that of the AP phytoplasma was thus obtained. Furthermore, preliminary results seemed to indicate that no polymorphism was present among nine ESFY phytoplasma isolates tested.

In this paper, we analyse 175 isolates from four southern Europe countries and from different prunus hosts, using primers defined on the 1·8 kbp fragment.[20] RFLP analysis, cloning and sequencing of some of the PCR products is also carried out to determine the genetic diversity of ESFY phytoplasmas.

## MATERIAL AND METHODS

### Sources of phytoplasmas

Samples from naturally infected *Prunus* species were collected from all major stone fruit growing regions in France as well as from three other Mediterranean countries (Table 1). A total of 175 isolates from 14 different *Prunus* species and four different interspecific hybrids were analysed (Table 1). Several ESFY phytoplasma reference strains maintained *in vitro* on micropropagated *Prunus marianna* GF 8-1[21] were included in the study. These strains were ECA-G32 from *Prunus armeniaca*, DBT from *P. marianna* GF 8-1 and RCL from *Prunus domestica* Reine-Claude. Isolates FOURM from *P. domestica* and B22 from *Prunus persica* were maintained in the nursery by regular graft transmission.[10] PVC isolates LA8, LA9 and M17 were kindly provided by J. C. Desvignes (CTIFL, La Force, France).

### Nucleic acid extraction

Crude phloem-enriched tissue fractions were prepared from branches of field-collected samples or from shoots of diseased *in vitro* propagated plants. DNA extraction was from 1·0 g of freshly prepared plant material according to the protocol of Maixner *et al.*[22]

### PCR amplification

PCR amplification of ESFY phytoplasma DNA was carried out with cross-reacting AP primers derived from the sequence of a 1·8 kbp chromosomal DNA fragment of AP phytoplasma: AP3, 5′-GAA ACA TGT CCT ATT GGT GG-3′; AP10, 5′-TTT TCA CAA CGT ATT CCG CC-3′;[19] AP6, 5′-TTT ATG TTG TCG ACT TTT CCA G-3′.[23] ESFY phytoplasma-specific primers ECA1 (5′-AAT AAT CAA GAA CAA GAA GT-3′) and ECA2 (5′-GTT TAT AAA AAT TAA TGA CTC-3′)[20] were also used. The location of the primer pairs on the 1·8 kbp fragment and the sizes of the amplified fragments are shown on Fig. 1. All PCR were of 40 cycles in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, USA), preceded by a 1-min denaturation step at 95°C and followed by an elongation step for 4 min at 72°C. Cycle conditions were as follows: primer pair ECA1/ECA2, 15 s at 95°C, 15 s at 55°C and 30 s at 72°C; primer pair AP3/AP6, 15 s at 95°C, 15 s at 58°C and 45 s at 72°C; primer pairs AP3/AP10 and ECA1/AP10, 15 s at 95°C, 15 s at 55°C and 60 s at 72°C. Reaction mixtures of 40 µl contained 10–100 ng total DNA, 0·5 µM of each primer, 125 µM dNTP, 1·5 mM $MgCl_2$, and 0·5 U *Taq* polymerase (BRL-Life Technologies, Cergy Pontoise, France) in the reaction buffer supplied by the manufacturer. PCR amplification products (10 µl) were analysed by 1 or 2% (w/v) agarose gel electrophoresis. DNA was stained with ethidium bromide and visualized on a U.V. transilluminator.

**Table 1.** Geographic and host plant origin of ESFY phytoplasma isolates examined

| *Prunus* species | French department code[a] | | | | | | | | | | Italy | Spain | Turkey | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2B | 06 | 26 | 30 | 66 | 84 | 33 | 47 | 49 | 82 | | | | |
| *P. armeniaca* (apricot) | | 1[b] | | 7 | 66 | 23 | 1 | | | | 3 | 1 | | 102 |
| *P. salicina* (Japanese plum) | 1 | | 3 | | | 2 | 1 | 6 | 3 | 9 | 2 | | 1 | 28 |
| *P. domestica* (European plum) | | | | | 2 | | 2 | | | 1 | | | | 5 |
| *P. persica* (peach) | | | | | 2 | | 4 | | | | | | | 6 |
| *P. marianna* (rootstock GF 8-1) | | | | | 6 | 5 | 5 | | | | | | | 16 |
| *P. cerasifera* (Myrobalan) | | | | | 1 | | 2 | | | | | | | 3 |
| *P. bokhariensis* | | | | | | | 1 | | | | | | | 1 |
| *P. brigantina* | | | | | | 1 | | | | | | | | 1 |
| *P. cocomilia* | | | | | | | 1 | | | | | | | 1 |
| *P.* Hollywood | | | | | | | 1 | | | | | | | 1 |
| *P. orthosepal* | | | | | | | 1 | | | | | | | 1 |
| *P. simonii* | | | | | | | 1 | | | | | | | 1 |
| *P. spinosa* | | | | | | | 1 | | | | | | | 1 |
| *P. subcordata* | | | | | | | 1 | | | | | | | 1 |
| Rootstock hybrids | | | | | | | | | | | | | | |
| *P. salicina* × *P. armeniaca* | | | | | | | 2 | | | | | | | 2 |
| *P. salicina* × *P. cerasifera* | | | | | | | 1 | 1 | | | | | | 2 |
| *P. besseyi* × *P. hortulana* | | | | | | | 2 | | | | | | | 2 |
| *P. persica* × *P. cerasifera* | | | | | | | 1 | | | | | | | 1 |
| Total | 1 | 1 | 3 | 7 | 77 | 31 | 28 | 7 | 3 | 10 | 5 | 1 | 1 | 175 |

[a] French departments are: Haute Corse (2B), Alpes-Maritimes (06), Drôme (26), Gard (30), Pyrénées-Orientales (66), Vaucluse (84), Gironde (33), Lot-et-Garonne (47), Maine-et-Loire (49) and Tarn-et-Garonne (82).
[b] The number of isolates tested.

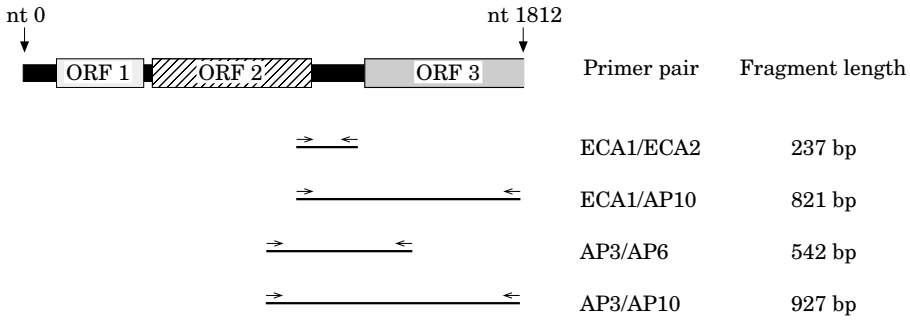


**Fig. 1.** Schematic representation of the 1·8 chromosomal fragment of AP phytoplasma isolate AT, and positions of the primer pairs used for PCR amplification. The predicted size of each PCR product is given. Filled boxes represent three putative ORFs.

## PCR-RFLP analysis

RFLP analysis was done on PCR products obtained with primer pairs AP3/AP6, AP3/AP10, ECA1/ECA2 and ECA1/AP10. Fifteen microlitres of PCR product were digested with *Bcl*I, *Hin*cII, *Hin*fI, *Rca*I, *Rsa*I, *Ssp*I or *Vsp*I according to the manufacturer's instructions (BRL-Life Technologies, Cergy Pontoise, France; Eurogentec, Seraing, Belgium). Restriction enzyme digests were analysed by agarose gel electrophoresis using a mixture of 2% (w/v) NuSieve GTG agarose (FMC, Rockland, Maine, USA) with 1% agarose. After ethi-

dium bromide staining, the DNA fragments were visualized and photographed on a U.V. transilluminator.

### Cloning and sequencing

An 821 bp fragment of ESFY phytoplasma amplified with primer pair ECA1/AP10 from DNA extracted from a French ACLR-affected apricot tree (isolate ECA-G32) was purified by phenol extraction and ligated in pTAg vector (R&D Systems, Abingdon, UK) according to the manufacturer's instruction. An aliquot of the ligation mixture was used to transform *Escherichia coli*, DH5 and transformants were screened by PCR with ESFY phytoplasma-specific primers ECA1/ECA2 under the same conditions as above. Three independent clones were sequenced.

Primers AP3 and AP6 were used to amplify a 542 bp DNA fragment of ESFY phytoplasma from total DNA extracted from three ACLR-affected apricot trees: one infected with the isolate ECA-G32 and two naturally infected in the Roussillon area. These PCR products were purified by WIZARD PCR-Prep columns (Promega, Charbonnières, France) and sequenced with primers AP3, AP6, ECA1 or ECA2 using the *fmol* DNA sequencing system (Promega, Charbonnières, France).

The nucleotide sequence of 846 bp reported here for the ESFY phytoplasma (isolate ECA-G32) has been submitted to the GenBank database and has been assigned the accession number AF195780. The ESFY phytoplasma sequence was aligned to the homologous sequence of AP phytoplasma deposited in the GenBank data library under the accession number L22217.

## RESULTS

### Geographic distribution and host plants of ESFY phytoplasmas as determined by PCR amplification of ESFY phytoplasma DNA with specific primers ECA1/ECA2

A total of 175 ESFY phytoplasma isolates sampled in four different Mediterranean countries on 14 different *Prunus* species and four interspecific rootstock hybrids were analysed. The majority of the isolates were from ten different French departments covering most of the stone fruit growing areas (Table 1). One isolate was sampled at Izmir (Turkey), others came from Spain and Italy (Table 1). A band of 237 bp could be obtained with primers ECA1/ECA2 for all isolates, including those from Turkey (results not shown), showing that the trees were infected by ESFY phytoplasmas. In addition, five *Prunus* species from the botanical collection of INRA, Bordeaux, *Prunus bokhariensis*, *P. Hollywood*, *P. orthosepal*, *P. subcordata* and the interspecific rootstock hybrid *Prunus besseyi* × *Prunus hortulana*, showing off-season growth, were also tested. All gave a positive PCR, even though summer symptoms were not characteristic and ranged from leaf chlorosis to die-back of branches and general decline of the tree.

### PCR-RFLP analysis of the various isolates

For RFLP analysis, primer pairs AP3/AP6, AP3/AP10 and ECA1/AP10 were used as they yielded longer PCR products of 542 bp, 927 bp and 821 bp, respectively, than the primer pair ECA1/ECA2 (Fig. 1). The primer combination ECA1/AP10 is specific for ESFY while the two others can also amplify AP phytoplasma DNA. In addition, the 237 bp PCR product amplified with primer pair ECA1/ECA2 was used to confirm specific restriction sites.

Restriction endonucleases *Bcl*I, *Hin*cII, *Hin*fI, *Rca*I, *Rsa*I, *Ssp*I and *Vsp*I were used to digest the different PCR products. Figs 2 and 3 show examples of the restriction patterns obtained with AP3/AP6 and ECA1/AP10 PCR products, respectively. No polymorphism could be observed among the 175 ESFY phytoplasma isolates including the reference strains ECA-G32, DBT, RCL, FOURM and B22. The three PVC isolates (LA8, LA9, M17) tested gave also the same pattern.

However, comparison of the RFLP results with those deduced from the known sequence of the AP phytoplasma subtype AT-1[19] showed that only six out of 18 restriction sites were common to both ESFY and AP phytoplasmas.

### Sequence analysis of ESFY phytoplasma amplified DNA

As no RFLP could be found among the different ESFY phytoplasma isolates, the AP3/AP6 amplified products of three French ACLR isolates were sequenced for comparison. This provided sequence information over 542 bp corresponding to the 3′-terminal part of the putative nitroreductase gene (ORF2), a 189 bp intergenic region and the 5′-terminal part of an open reading frame (ORF3) coding for a protein of unknown function, as previously determined for the AP phytoplasma. The three ESFY phytoplasma sequences were identical.

The ESFY phytoplasma sequence (isolate ECA-G32) of 846 bp was aligned to the homologous sequence

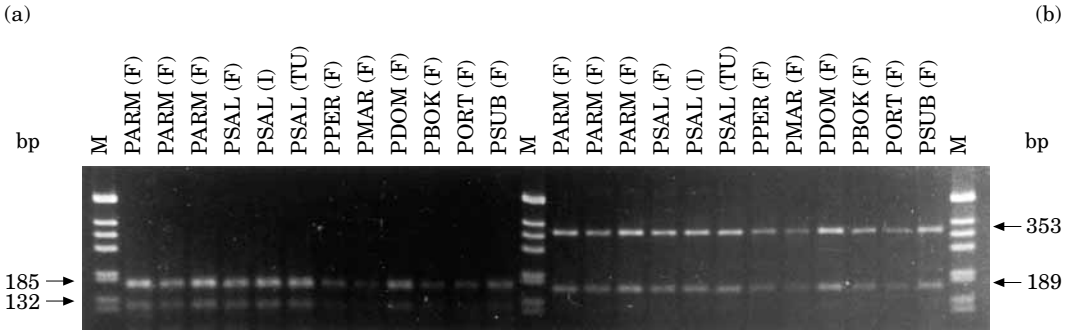


**Fig. 2.** RFLP analysis with restriction enzymes (a) *Vsp*I and (b) *Hin*cII of AP3/AP6-PCR products of representative European stone fruit yellows phytoplasma isolates. PARM, *Prunus armeniaca*; PSAL, *P. salicina*; PPER, *P. persica*; PMAR, *P. marianna*; PDOM, *P. domestica*; PBOK, *P. bokhariensis*; PORT, *P. orthosepal*; PSUB, *P. subcordata*. The geographic origin is indicated in parenthesis: F, France; I, Italy; TU, Turkey. M: molecular mass marker (1-kb ladder, Life Technologies, Cergy Pontoise, France). The sizes of the RFLP fragments are deduced from the sequence.

of AP phytoplasma subtype AT-1 (Fig. 4). Thirteen base insertions and four deletions were found in the ESFY phytoplasma sequence compared to the AP phytoplasma sequence. The similarity of the aligned sequences was 92·7% in ORF3, 88·2% in the intergenic region and 82·2% in the 3′-end of the nitroreductase gene. The overall identity was 89·7%. It is interesting that the deduced amino acid sequences were less homologous than the nucleotide sequence. The hypothetical proteins of ORF3 showed 90·3% amino acid identity and the C-terminal part of the putative nitroreductase had only 64·2% amino acid identity with its counterpart in the AP phytoplasma. The G + C content of the ESFY phytoplasma sequence was extremely low (15·4%), even lower than that of the AP phytoplasma (16·1%). Accordingly, codon usage was strongly shifted to A + T-rich codons. Fifty-six percent of the codons in the ESFY and AP phytoplasma sequences are pure A + T-codons and codons in which the third (wobble) positions are A or T represent 90·8% and 88·0%, respectively. Putative regulatory elements previously described in the intergenic region such as the rho-independent transcriptional terminator of ORF2, the −35 and −10 regions of ORF3 promoter, as well as the ribosome binding site, were conserved among ESFY and AP phytoplasmas (Fig. 4).

## DISCUSSION

All cultivated *Prunus* fruit trees in Europe suffer from phytoplasma-associated declines which have received various names. Previous reports indicated that the phytoplasmas associated with these declines belong to the same phylogenetic group called European stone fruit yellows phytoplasma.[18–20] Our PCR results and those of others[18–20] indicate the association of ESFY phytoplasma with at least 18 different *Prunus* species in Europe. Our work demonstrates the infection of *Prunus cocomilia*, *P. brigantina*, *P. simonii* and *P. spinosa* with the ESFY phytoplasma as suggested by Morvan[4] and natural infections on new host plants such as *P. bokhariensis*, *P. Hollywood*, *P. orthosepal*, *P. subcordata* and *P. besseyi* × *P. hortulana*. Although natural Molières disease infections on *Prunus avium* could not be verified,[20] experimental inoculation of *P. avium* by the ESFY phytoplasma has been obtained.[24] As of today, no resistance to ESFY phytoplasma in the genus *Prunus* has been reported.

To our knowledge, this is the first report of an ESFY infection in Turkey and confirmation that ACLR on apricot trees in Valencia (Spain), Napoli (southern Italy) and Bologna (northern Italy) are indeed due to ESFY phytoplasma infections. These results also show that ESFY phytoplasma is associated with PLN of Japanese plum trees near Bologna (Italy).

The aim of our study was to analyse the nature and genetic variability of ESFY phytoplasmas on a large scale and to compare them with phytoplasmas infecting other fruit trees in the same phylogenetic cluster. Indeed, some isolates have been reported to induce different symptoms on the same host plant species.[9] Our results show that the various ESFY phytoplasma isolates present in different *Prunus* species of the area surveyed could not be distinguished. Thus further work is required to determine which plant- and/or pathogen-derived factors are responsible for symptom variations. This genetic homogeneity of ESFY phytoplasmas was surprising as genetic variations within the same chromosomal region had been found for AP phytoplasma isolates infecting *Malus* species in the same geographical area.[19]

As phytoplasmas cannot be cultured, their phylogenetic classification is based on 16 S rRNA gene

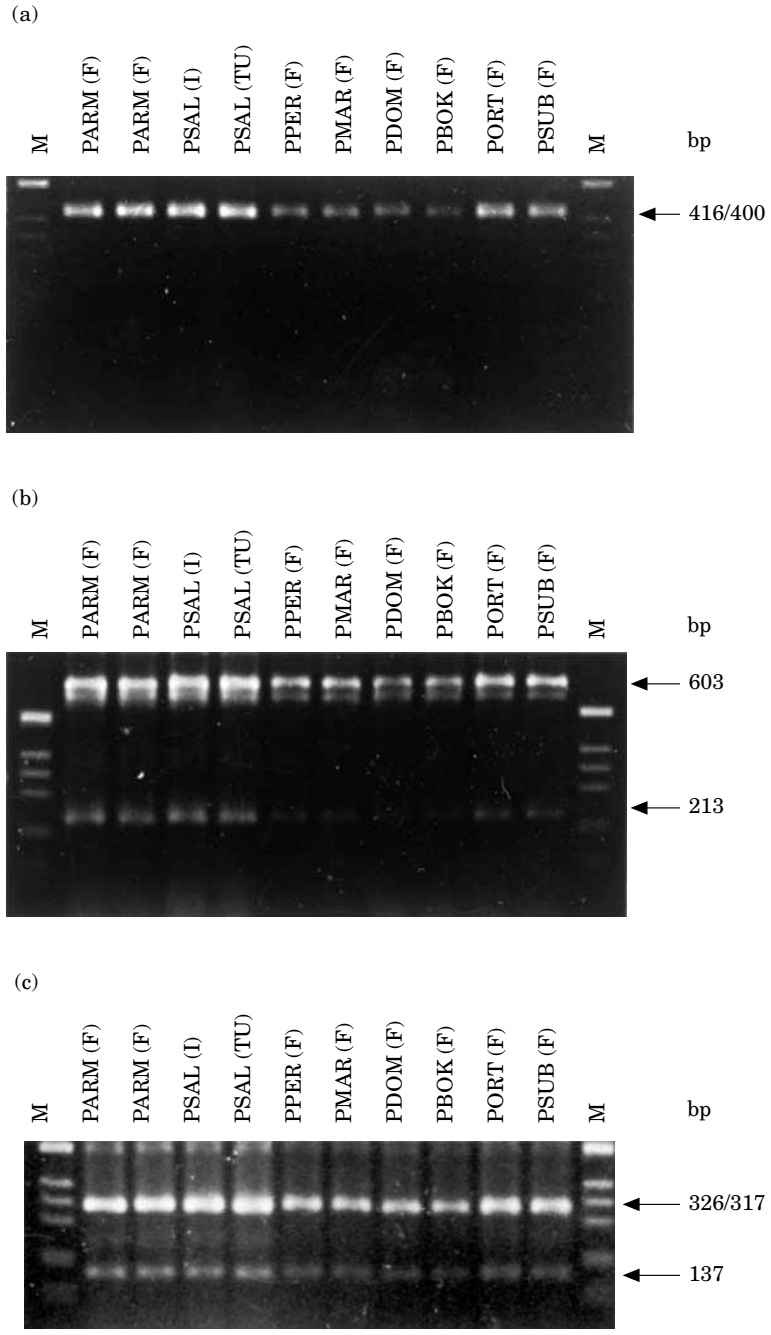


**Fig. 3.** RFLP analysis with restriction enzymes (a) *Rsa*I, (b) *Hin*fI and (c) *Ssp*I of ECA1/AP10-PCR products of representative European stone fruit yellows phytoplasma isolates (see Fig. 2). M: molecular mass marker (1 kb ladder, Life Technologies, Cergy Pontoise, France). The sizes of the RFLP fragments are deduced from the sequence.

sequences (ICSB Subcommittee on the Taxonomy of *Mollicutes*).[25] Each of the 20 major phylogenetic subclades defined so far represent potential *Candidatus* species,[16] but only a limited number have been fully described.[26–28] The ESFY, AP and PD phytoplasmas belong to the same phylogenetic cluster with 16 S rRNA homologies higher than 98%.[15] The results presented here show that clear genomic differences can be evidenced on chromosomal DNA of AP and ESFY isolates and strengthened previous proposal for subdivision of this phytoplasma cluster.[19–21] As shown in the present study the ESFY phytoplasma is able to infect all *Prunus* species tested whereas AP and PD phytoplasmas have only been found on *Malus* or *Pyrus* species, respectively. Hence, these phytoplasmas can be considered as pathovars. Different


**Fig. 4.** Nucleotide sequences and deduced amino acid sequences of homologous chromosomal fragments of European stone fruit yellows (ESFY) and apple proliferation (AP) phytoplasmas. Nucleotides and amino acid residues which differ in the aligned sequences are marked with bold letters. Base numbers correspond to the published sequence of AP phytoplasmas (accession number L22217). Restriction sites are underlined and putative transcriptional regulatory elements are indicated. The first coding region corresponds to the 3′-terminal end of ORF2 which codes for a putative nitroreductase. Stars indicate the stop codon. The second coding region corresponds to the 5′-end of ORF3.

insect vectors are responsible for the transmission of these three phytoplasmas and can explain their different host range. Indeed, two different psyllid species have been identified as vectors of PD and ESFY phytoplasmas[29,30] while the leafhopper *Fieberiella florii* has been found to transmit the AP phytoplasma.[31]

Whether these three phytoplasmas correspond to different species or subspecies, it will require comparison of additional genes.

## ACKNOWLEDGMENTS

The authors thank J. C. Desvignes, L. Giunchedi and INRA Avignon for providing ESFY-infected samples. The excellent technical assistance of S. Bonnefille is gratefully acknowledged. We thank P. Duthil for preparing the photos.

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