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Mapping the spread of apricot chlorotic leaf roll (ACLR) in southern France and implication of *Cacopsylla pruni* as a vector of European stone fruit yellows (ESFY) phytoplasmas

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An epidemiological study on European stone fruit yellows (ESFY) phytoplasmas infecting *Prunus* fruit trees was carried out from 1994 to 2000 in Languedoc-Roussillon (southern France). The spread of the disease was monitored for 7 years by visual observation of symptoms and by PCR detection of the phytoplasma in an experimental orchard planted with apricot hybrid seedlings. This indicated that aerial vectors were responsible for disease spread, and that transmission rates were low at the beginning of the spread. Seventy thousand homopteran insects were captured within and in the surroundings of highly ESFY-infected apricot orchards, of which about 10 000 were used in PCR and nested-PCR assays with universal ribosomal and ESFY-specific nonribosomal primers to detect ESFY phytoplasmas. The other insects were confined in cages for trials of transmission to test plants. ESFY phytoplasmas could not be detected by PCR in any of the leafhopper species captured but could be detected in the psyllid *Cacopsylla pruni* caught on *Prunus domestica* and *Prunus cerasifera* rootstock suckers of apricot trees and on *Prunus spinosa*. Nested PCR revealed ESFY phytoplasmas in one individual of the deltocephalid *Synophropsis lauri* captured on an apricot tree. Transmission trials confirmed the role of *Cacopsylla pruni* as the ESFY phytoplasma vector in France. When apricot seedlings were used as bait plants from April to November during two consecutive years, no natural transmission could be demonstrated. However, one out of 50 apricot seedlings left for the whole year in the orchard became infected. An early spring ESFY infection is in agreement with both the natural transmission results and the life cycle of *Cacopsylla pruni*.

Keywords: apricot, Auchenorryhncha, bait-plant trials, PCR detection, Sternorrhyncha

Introduction

In southern Europe, several economically very important decline diseases of stone fruits are associated with genetically similar phytoplasmas (Jarausch *et al*., 1998) for which the name European stone fruit yellows (ESFY) phytoplasmas (Lorenz *et al*., 1994) has been adopted. The two most devastating diseases are apricot chlorotic leaf roll (ACLR) on apricots (Morvan, 1977) and plum leptonecrosis (PLN) on Japanese plums (Giunchedi *et al*., 1982). Based on 16S rDNA analyses, ESFY phytoplasmas belong to the apple proliferation (AP) cluster (Schneider *et al*., 1993; Seemüller *et al*., 1994) which comprises additional fruit tree phytoplasmas such as pear decline (PD) phytoplasmas. An ESFY-specific PCR assay has been developed to distinguish ESFY phytoplasmas from their relatives in the AP cluster (Jarausch *et al*., 1998). Members of the AP cluster are very distantly related to the phytoplasmas of the X-disease cluster which infect stone fruit trees in North America (Seemüller *et al*., 1994).

The present epidemiological study was mainly carried out in Roussillon, a region of southern France where about 80% of mortality and decline observed on apricots is due to ACLR (Cornaggia *et al*., 1994; Jarausch *et al*., 1998). Although ACLR symptoms were described in France as early as 1924 (Chabrolin, 1924), the epidemiology of the disease was poorly understood when the current study was started in 1994. Leafhoppers and psyllids are known to be vectors of phytoplasma diseases, but diseases are also spread by humans

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through infected budwood. All phytoplasma vectors identified to date are phloem-feeding members of the order Homoptera. Most of the vectors are found in the suborder Auchenorryhncha (e.g. plant- and leafhoppers), whilst some vectors are psyllids (Sternorrhyncha) (D'Arcy & Nault, 1982). At the start of this study, in 1994, the insect vector of ESFY was unknown, but later Carraro *et al*. (1998) showed that the psyllid *Cacopsylla pruni* was an efficient vector of ESFY phytoplasmas to Japanese plum in Italy. The present study aimed to identify one or more vector species of ESFY phytoplasmas in apricot orchards in southern France. Bait-plant trials were conducted to determine the infection period in the orchard, and the disease spread was studied in an experimental orchard planted with phytoplasma-free apricot seedlings. Partial preliminary results have been presented in Jarausch *et al*. (1999).

Materials and methods

Phytoplasma reference strains

The ACLR-isolate ECA-G32 maintained in *in vitro*-propagated *Prunus marianna* GF 8-1 (Jarausch *et al*., 1994a) was used as a reference strain for ESFY phytoplasmas. The other reference phytoplasma strains were maintained in periwinkle (*Catharanthus roseus*) plants by periodic graft-inoculation to healthy plants. These strains were: EAY (European aster yellows from France), STOL (stolbur from France), AYA (an aster yellows-like strain from Spain, formerly called ACLR-L), ULW (elm yellows from France) and AP (apple proliferation from Italy). Additional descriptions and references for the periwinkle-maintained isolates used in this work are given in Jarausch *et al*. (1994b); their classification has been published by Schneider *et al*. (1993).

PCR detection of phytoplasmas

Total DNA from crude phloem-enriched tissue fractions, prepared from branches of apricot or petioles of periwinkle, was extracted as described by Maixner *et al*. (1995) using a CTAB (cetyltrimethylammoniumbromide) extraction method. Total DNA of insects was extracted by the same procedure. Large insects were tested individually, small insects in groups of two to five individuals according to size.

PCR amplification of phytoplasma 16S rDNA was carried out with universal primers (Ahrens & Seemüller, 1992; Lorenz *et al*., 1995). The primer pair fU5/rU4 and the primer pair fU5/rU3 were used for plant material and insect DNA, respectively. Nonribosomal primers ECA1/ECA2 (Jarausch *et al*., 1998) were also used for ESFY-specific detection. With each primer pair, 40 cycles were performed 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: for ribosomal primers, 15 s at 95°C, 15 s at 55°C and 60 s at 72°C; for ECA1/ECA2 primers, 15 s at 95°C, 15 s at 55°C and 30 s at 72°C. Reaction mixtures of 20 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 of *Taq* polymerase (BRL-Life Technologies, Cergy Pontoise, France) in the reaction buffer supplied by the manufacturer.

Nested PCR (nPCR) was applied to insect DNA using either ribosomal or nonribosomal primers. For ribosomal nPCR, the first amplification was carried out with universal primers fD1 (Weisburg *et al*., 1989) and P7 (Schneider *et al*., 1995), and the second amplification was done with the internal, AP cluster-specific primers fO1/rO1(Lorenz *et al*., 1995). ESFY-specific nonribosomal nPCR was done with primers AP6 (Jarausch *et al*., 1995) and AP3 (Jarausch *et al*., 1994b) in the first amplification, and with internal primers ECA1/ECA2 in the second amplification. All first amplifications were for 40 cycles in the above reaction mixtures. One microlitre of the PCR mixture was subjected to 20 additional cycles with the nested primers. The following conditions were applied: for primer pair fD1/P7, 15 s at 95°C, 15 s at 55°C and 90 s at 72°C; for primer pair fO1/rO1, 15 s at 95°C, 15 s at 55°C and 60 s at 72°C; for primer pair AP6/AP3, 15 s at 95°C, 15 s at 54°C and 45 s at 72°C; for ECA1/ECA2, as above.

PCR amplification products (10 μL) were analysed by electrophoresis on 1 or 2% agarose gels. DNA was stained with ethidium bromide and visualized on a UV transilluminator.

RFLP analyses of ribosomal PCR products

RFLP analyses were carried out on the PCR products obtained with the primer pair fU5/rU4 from plant DNA and on the PCR products obtained with the primer pairs fU5/rU3 or fO1/rO1 from insect DNA. Fifteen microlitres of the PCR products were digested with *Alu*I or *Rsa*I according to the manufacturer's instructions (Eurogentec, Seraing, Belgium). Restriction enzyme digests were analysed by agarose gel electrophoresis using a mixture of 2% NuSieve GTG agarose (FMC, Rockland, ME, USA) and 1% agarose, and were visualized as above.

Bait-plant trials

In 1995 and 1996, batches of 25 periwinkle plants and 20–25 apricot seedlings (cv. Manicot) were exposed to natural transmission in four different highly ACLR-infected apricot orchards without insecticide treatments. In 1995, 14 sets were exposed bi-weekly from April to October, and in 1996, 11 sets were exposed bi-weekly from June to November. After exposure, periwinkle plants were kept under insect-proof conditions for 3 months and apricot seedlings for at least 12 months. Plants with symptoms and a random sample of symptomless plants of each set were tested by PCR.

In September 1996, a batch of 50 apricot seedlings was planted and left in the orchard until off-season growth symptoms appeared. Plants with symptoms were tested by PCR in 1997 and 1998.

As a positive control for the PCR assays, apricot seedlings (cv. Manicot) were inoculated by chip-budding in September 1996 with an ESFY phytoplasma isolate from a naturally ACLR-infected tree in Roussillon.

Insect captures

For PCR detection assays, captures of species of Auchenorrhyncha were made in apricot orchards without insecticide treatments and in different biotopes of the adjacent fallow land in six different locations of the Roussillon region. Insects were captured with a D-vac aspirator (Tecumseh Products Company, Grafton, USA) bi-weekly in each location from May to October in 1995, May to November in 1996, and June to September in 1998. For transmission trials, species of Auchenorryhncha were captured either by D-vac aspiration or by light traps as described by Labonne *et al*. (1998), in the same orchards as above, from the beginning of July to the end of September in 1997 and 1998.

Adults were observed after CO_2 anaesthesia under a stereobinocular microscope and identified at the family level according to Ribaut (1936, 1952) and Della Giustina (1989). In April 1999, and from February to April 2000, captures of psyllid species for transmission and PCR were made weekly. The psyllids were dislodged from trees by knocking branches with a large wooden pole and then collected with a sweep net as they dropped to the ground.

Transmission trials

Transmission of phytoplasmas to bait plants by field-collected insects

Insects belonging to a defined insect family (Auchenorryhncha species) were introduced into large screen-cages (6 m^3) containing five periwinkle plants and 40 apricot cv. Manicot seedlings. The test plants were incubated in an insect-proof screenhouse for 2 years for symptom expression.

Transmission by Cacopsylla pruni

Transmission trials with *Cacopsylla pruni* were conducted under controlled conditions (22°C, photoperiod 14 h) in pot-cages with healthy *Prunus* plants. Adults of *C. pruni* (135) were collected on *Prunus domestica* rootstock suckers growing from highly ACLR-infected apricot trees in Languedoc-Roussillon in April 1999. They were maintained on four healthy *P. cerasifera* plants for 20 days, and then batches of 10 insects were transferred onto three apricot seedlings (cv. Manicot) and four *P. insititia* seedlings (cv. St Julien) until death of the insect. The remaining psyllids were tested by PCR for the presence of the ESFY phytoplasma. Symptom expression of the test plants was recorded and the plants were tested by PCR for ESFY infection 8 months after inoculation.

Evaluation of Fieberiella florii *as a putative vector of EFSY phytoplasmas*

A population of the deltocephalid *Fieberiella florii*, kindly provided by G. Krczal (SLFA, Neustadt/W., Germany), was reared on healthy *Ligustrum* sp. plants under controlled conditions (25°C, photoperiod 16 h). Acquisition trials were performed with phytoplasma-free adults derived from eggs of reared females. For ESFY phytoplasma acquisition, 50, 200 and 70 individuals of *F. florii* were fed for 1, 2 and 4 months, respectively, on ESFY-infected *Prunus marianna* in pot-cages under the above conditions, and were then individually tested by PCR.

Field plot design for ACLR spread studies

The spread of ACLR was followed for 7 years in an experimental apricot orchard planted in 1990 with 1840 phytoplasma-free hybrid seedlings. The orchard was located in an area with a high incidence of ACLR-infected apricot orchards and was bordered on one side by an abandoned vineyard. The orchard was irrigated and managed as a normal orchard, with no undergrowth and three annual insecticide treatments from June to August. Off-season growth (January/February) and chlorotic leaf roll symptoms (September), characteristic of ACLR, were recorded each year from 1993 to 1997. Trees with clear or possible symptoms were tested by PCR.

Results

Disease spread in an experimental orchard planted with apricot hybrid seedlings

As only seedling material guarantees that planting material is phytoplasma-free, an experimental orchard planted in 1990 with 1840 apricot hybrid seedlings was chosen to follow the natural spread of ACLR. Symptoms were recorded twice a year and trees with symptoms were tested by ESFY-specific PCR. The first ACLR infections were observed 4 years after planting, on 0·5% of the trees. The disease started in various locations within the orchard, with no evidence of border effects. The number of infected trees slowly increased each year, reaching 5% of the trees in 1997, when the orchard was unfortunately uprooted. The distribution of the infected trees is shown in Fig. 1.

Attempts to determine the inoculation period of ESFY phytoplasmas in orchards by bait plant exposure

Periwinkle and apricot seedlings (cv. Manicot) were exposed as bait plants in four different, highly

ACLR-infected orchards during the growing season of two consecutive years. In 1995, 14 out of 50 periwinkle plants exposed the first 2 weeks of July developed symptoms, and two and one out of 50 developed symptoms when exposed at the end of July and begining of August, respectively. Similar results were obtained in 1996. All periwinkle plants showing symptoms tested positive by universal PCR for phytoplasmas, and subsequent RFLP analysis of the PCR products revealed that all plants were infected with the stolbur phytoplasma. As no infection with ESFY phytoplasmas could be demonstrated in the periwinkle plants with symptoms, 30% of the symptomless periwinkle plants exposed in the orchard were randomly tested. No phytoplasmas were detected by PCR in these plants.

None of the 1180 apricot seedlings exposed at 2-week intervals in the four orchards from April to November in 1995 and 1996 showed ACLR symptoms 1 year after exposure, whilst controls in which apricot seedlings were experimentally inoculated with ESFY phytoplasmas by chip-budding developed ACLR symptoms within 1 year and tested positive by ESFY-specific PCR. A random test on 30% of the symptomless apricot seedlings failed to detect any ESFY phytoplasmas.

In contrast, one out of 50 apricot seedlings exposed in one of these orchards from September 1996 onwards exhibited clear ACLR symptoms of off-season growth in January 1998 and tested positive for ESFY phytoplasmas by universal PCR-RFLP and ESFY-specific PCR. Later, during the summer, typical chlorotic leaf roll symptoms developed on this tree.

Survey for possible vectors of ESFY phytoplasmas in apricot orchards

In 1995, 1996 and 1998, 10 000 Auchenorrhyncha insects were identified at genus or species level and tested by PCR with universal, ribosomal primers. Table 1 shows the number of individuals tested for the 76 species collected as well as those identified at genus or family levels only. Six specimens of *Hyalesthes obsoletus,* captured in July on bindweed, were PCR-positive and RFLP analysis revealed that they were, as expected, infected by the stolbur phytoplasma (data not shown). One *Macrosteles* sp., collected in September from grass, was found to be infected with a phytoplasma of the aster yellows cluster (data not shown). No ESFY phytoplasma could be detected by PCR in the Auchenorryhncha species captured in 1995, 1996 and 1998. Therefore, 16S nested PCR was used on DNA

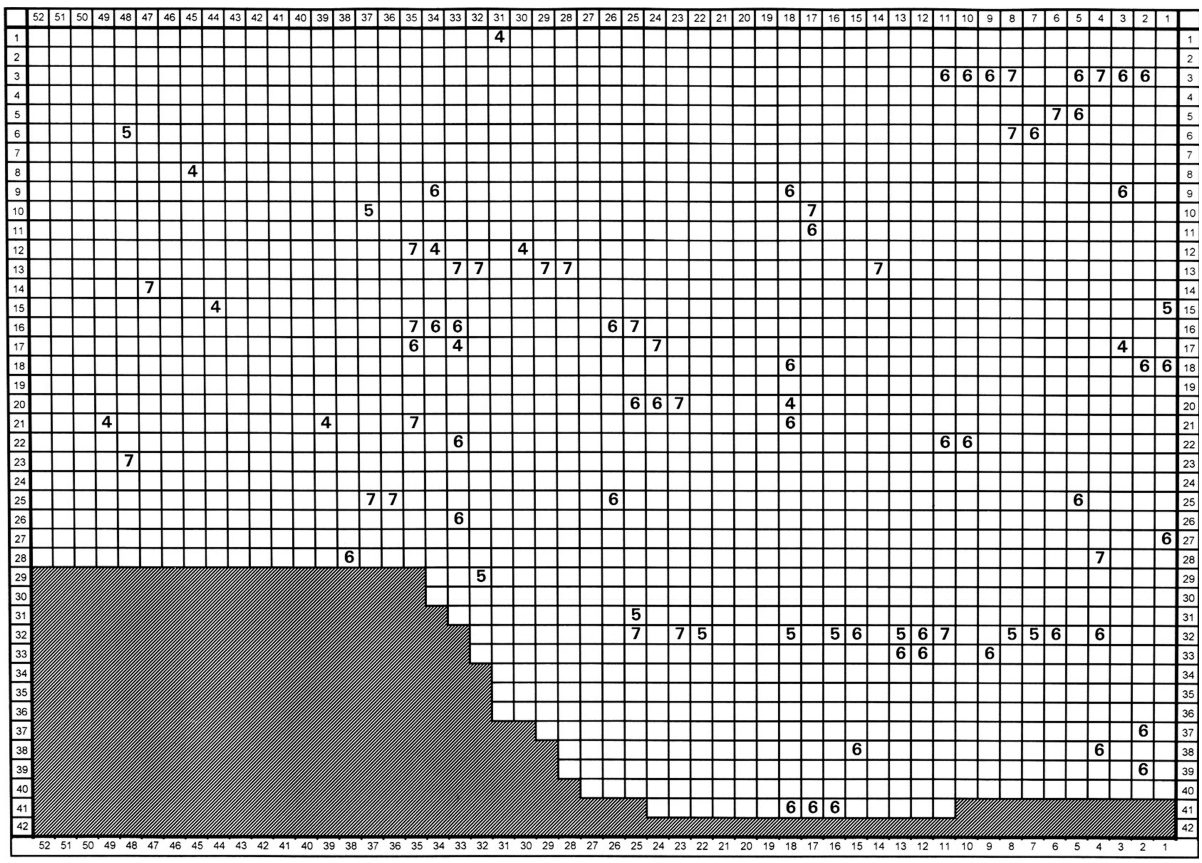

Figure 1 Spatial distribution of European stone fruit yellows (ESFY)-infected trees in an experimental orchard of 1840 apricot hybrid seedlings planted in 1990. Each box represents a tree, and infected trees are marked by a number which denotes the year of first symptom expression, i.e. 4 = 1994, 5 = 1995, 6 = 1996, 7 = 1997.

Table 1 List of Hemiptera captured in Languedoc-Roussillon by D-vac aspirator and tested for phytoplasmas by universal, single round and nested PCR in 1995, 1996 and 1999

Family/subfamily	Genus and species	PCR fU5/rU3[a]	Nested PCR[a]	Phytoplasma type
CIXIIDAE	*Cixius* sp.	0/6		
	Hyalesthes luteipes	0/713		
	Hyalesthes obsoletus	6/194		Stolbur
	Oliarus sp.	0/1	0/1	
	Reptalus sp.	0/5		
	Nondetermined species	0/4		
DELPHACIDAE	*Asiraca clavicornis*	0/40	0/13	
	Chloriona sp.	0/3		
	Laodelphax striatellus	0/70	0/21	
	Stenocranus major	0/30	0/1	
	Stenocranus minutus	0/2	0/2	
	Toya propinqua	0/33	0/18	
	Nondetermined species	0/322	0/144	
DICTYOPHARIDAE	*Dictyophara europaea*	0/105		
ISSIDAE	*Hysteropterum* sp.	0/90	0/9	
	Nondetermined species	0/21		
TROPIDUCHIDAE	*Trypetimorpha fenestrata*	0/186	0/5	
CERCOPIDAE	*Aphrophora alni*	0/22	0/17	
	Neophilaenus lineatus	0/17		
	Neophilaenus sp.	0/8	0/8	
	Philaenus spummarius	0/3		
	Cercopis sanguinolenta	0/1		
	Nondetermined species	0/105	0/42	
MEMBRACIDAE	*Gargara genistae*	0/2		
	Stictocephala bisonia	0/90	0/63	
CICADELLIDAE				
Agallinae	*Agallia consobrina*	0/293	0/66	
	Anaceratagallia laevis	0/182		
	Anaceratagallia ribauti	0/109		
	Anaceratagallia sp.	0/134	0/96	
	Austroagallia sinuata	0/39		
	Nondetermined species	0/9		
Aphrodinae	*Aphrodes bicinctus*	0/3		
	Aphrodes albifrons	0/4		
	Aphrodes sp.	0/4		
Cicadellinae	*Cicadella viridis*	0/5		
Deltocephalinae	*Adarrus taurus*	0/120		
	Adarrus sp.	0/15		
	Allygidius atomarius	0/12		
	Araldus propinquus	0/129		
	Arocephalus longiceps	0/358		
	Arocephalus sagittarius	0/9		
	Arthaldeus striifrons	0/260	0/3	
	Balclutha punctata	0/29		
	Balclutha saltuella	0/70		
	Balclutha sp.	0/24	0/1	
	Cicadula quadrinotata	0/3		
	Circulifer haematoceps	0/213		
	Chiasmus translucidus	0/5		
	Conosanus obsoletus	0/12		
	Euscelidius variegatus	0/259	0/11	
	Euscelis incisus	0/9		
	Euscelis lineolatus	0/158	0/27	
	Euscelis ulicis	0/1		
	Euscelis sp.	0/70	0/28	
	Exitianus capicola	0/23		
	Fieberiella florii	0/240	0/140	
	Goniagnathus brevis	0/4		
	Graphocraerus ventralis	0/1		

Table 1. *continued*

Family/subfamily	Genus and species	PCR fU5/rU3[a]	Nested PCR[a]	Phytoplasma type
	Macrosteles quadripunctulatus	0/7		
	Macrosteles sardus	0/105		
	Macrosteles sp.	1/83	0/8	AY
	Mocydia crocea	0/237	0/52	
	Mocydiopsis parvicauda	0/3		
	Neoaliterus fenestratus	0/114	0/67	
	Opsius stactogalus	0/34		
	Paralimnus phragmitis	0/5		
	Paralimnus pulchellus	0/7		
	Phlepsius ornatus	0/1		
	Phlepsius spinulosus	0/9		
	Phlepsius sp.	0/23		
	Phlogotettix cyclops	0/51	0/6	
	Platymetopius major	0/4	0/4	
	Psammotettix alienus	0/352		
	Psammotettix confinis	0/19		
	Psammotettix nodosus	0/21		
	Psammotettix sp.	0/301	0/35	
	Recilia coronifera	0/4		
	Recilia schmidtgeni	0/25		
	Recilia sp.	0/18		
	Rhopalopyx elongatus	0/73	0/5	
	Rhytidodus decimusquartus	0/129		
	Sardius argus	0/57		
	Scaphoideus titanus	0/61	0/1	
	Selenocephalus obsoletus	0/32	0/5	
	Synophropsis lauri	0/1058	1/6	ESFY
	Nondetermined species	0/4		
Dorycephalinae	*Eupelix cuspidata*	0/76		
	Paradorydium paradoxum	0/39		
Idiocerinae	*Idiocerus herrichii*	0/1		
	Idiocerus sp.	0/13		
Iassinae	*Iassus* sp.	0/18		
Macropsinae	*Macropsis fuscula*	0/52	0/24	
	Macropsis sp.	0/45	0/22	
Typhlocybinae	*Arboridia parvula*	0/57	0/57	
	Empoasca vitis	0/8		
	Empoasca sp.	0/65		
	Erythroneura flammigera	0/10		
	Eupterix sp.	0/9		
	Lyguropia juniperi	0/166	0/1	
	Zyginidia scutellaris	0/13		
	Nondetermined species	0/1468	0/721	
TOTAL		7/9746	1/1730	

[a]Ratio of individuals which tested positive to the total number of adults tested.

extracted from insect species which had been captured on apricot or wild *Prunus* trees. Only one leafhopper, *Synophropsis lauri*, captured on apricot in September 1998, reacted positively out of 1748 insects tested. RFLP analysis of the nested fO1/rO1 PCR product lead to a ESFY phytoplasma profile. The presence of the ESFY phytoplasma in the *S. lauri* individual was confirmed by nPCR with primers AP3/AP6 and ESFY-specific primers ECA1/ECA2 as shown in Fig. 2.

No *Cacopsylla pruni* specimens were captured during the experiments described above, in which insect trapping was done from May to November. Following the report that *C. pruni* was a vector of PLN in Italy (Carraro *et al*., 1998), early spring captures were performed in order to collect psyllid species in apricot orchards and on wild *Prunus* in 1999 and 2000. *C. pruni* could be captured from suckers of *Prunus domestica* and *P. cerasifera* rootstocks of apricot trees and from *P. spinosa*. One hundred and ninety-five adults captured on apricot rootstocks and 320 adults captured on *P. spinosa* were separated into batches of five individuals and tested by PCR with universal or ESFY-specific primers. Figure 3 shows some PCR results obtained with ESFY-specific primers ECA1/ECA2 on *C. pruni*.

Ten out of 39 insect groups collected from apricot

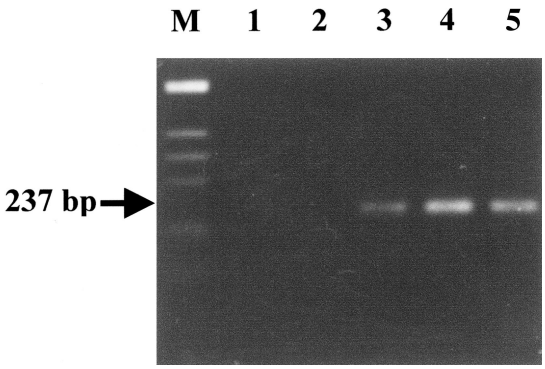

Figure 2 Agarose gel electrophoresis of nested PCR amplifications of a 237-bp chromosomal fragment of ESFY phytoplasmas obtained with primer pair AP3/AP6 in the first amplification and primer pair ECA1/ECA2 in the second amplification, and total DNA extracted from healthy *Synophropsis lauri* (lane 2), ESFY-infected *S. lauri* (lane 3), *Prunus marianna* infected with reference strain ECA-G32 (lane 4). Lane 5, positive control (ECA-G32) of the second amplification only; lane 1, water control; lane M, molecular size marker (1-kb ladder, Life Technologies, Cergy Pontoise, France).

rootstocks and 11 out of 74 insect groups collected from *P. spinosa* yielded an ESFY-specific amplification product. The proportion of infected individuals in these groups was calculated by its maximum-likelihood estimator, P, with a confidence interval calculated according to Monestier & Labonne (1981): $P = 1 - (1 - R/N)^{1/I}$, where R is the number of infected groups, N is the total number of tested groups and I is the number of insects per group (five in this case). This indicated that 5·75% of the psyllids captured from rootstocks and 3·1% of the psyllids captured from *P. spinosa* in the apricot orchards were infected by ESFY phytoplasmas. These percentages were not significantly different on a 95% confidence interval basis.

Transmission trials

Transmission of phytoplasmas to bait plants by field-collected insects

None of the apricot seedlings and only one periwinkle plant developed symptoms during the 2-year period following exposure to 57 000 individuals of various insect species in the Auchenorrhyncha family captured in 1997 and 1998. The periwinkle plant with symptoms was tested by PCR-RFLP and shown to be infected by an aster yellows phytoplasma.

Transmission by Cacopsylla pruni

One out of the four *P. insititia* rootstocks which were encaged with *Cacopsylla pruni* insects tested positive by ESFY-specfic PCR 8 months after insect inoculation, as shown in Fig. 3, lane 19, even though it showed no symptoms. None of the three apricot seedlings was positive at that time. The plants have been kept for further testing and observation for symptoms.

Evaluation of Fieberiella florii *as a putative vector of EFSY phytoplasmas*

The deltocephalid *Fieberiella florii*, known as a vector of the X-disease phytoplasma to stone fruits in North America (Gilmer & Blodgett, 1976) and a presumed vector of ACLR (Bonfils *et al.*, 1976), was tested for its ability to acquire ESFY phytoplasmas from highly infected *Prunus marianna*. After an acquisition period of 1, 2 or 4 months, none of the 320 individuals tested by PCR was found to be positive for ESFY phytoplasmas.

Discussion

Previous studies on the spread of ACLR in the orchard were hampered by the uncertain sanitary status of the planting material. The planting material for apricot orchards is usually produced through vegetative propagation, which favours the spread of the graft-transmissible ESFY phytoplasmas by contaminated rootstock or scion material. Thus, only seedling material guarantees the phytoplasma-free status of the planting material and enables an unequivocal analysis of the natural spread of ESFY phytoplasmas. The results of this study show that the disease was introduced by an aerial vector independently at different sites into the experimental orchard

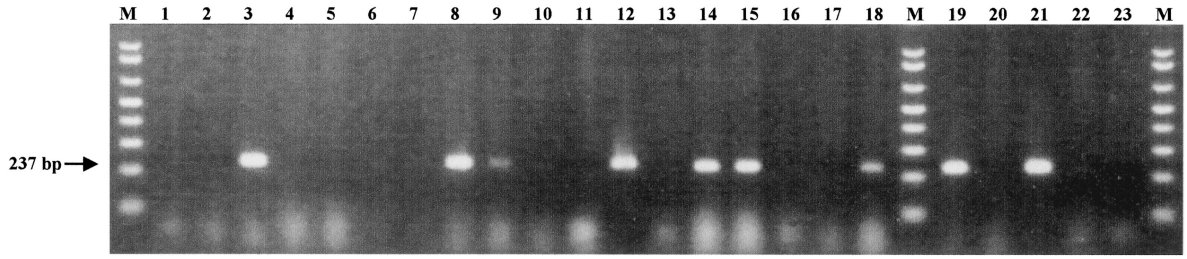

Figure 3 Agarose gel electrophoresis of PCR amplifications of a 237-bp chromosomal fragment of ESFY phytoplasmas obtained with primer pair ECA1/ECA2 and total DNA extracted from *Cacopsylla pruni* captured in 1999 and 2000 (lanes 1–18), *Prunus insititia* inoculated by infective *C. pruni* (lane 19), healthy *Prunus marianna* (lane 20), *P. marianna* infected with reference strain ECA-G32 (lane 21), healthy *P. marianna* (lane 22). Lane 23, water control; lane M, molecular size marker (1-kb ladder, Life Technologies, Cergy Pontoise, France). Specimens of *C. pruni* in lanes 3, 8, 9, 12, 14, 15 and 18 were ESFY-infected.

and that the disease spread was slow in the first years after infection.

The results presented in this paper demonstrate that, when starting from phytoplasma-free planting material, apricot orchards can become infected by the ESFY phytoplasma, indicating that it is a naturally vectored disease. Bait-plant trials and insect testings, carried out for several years from May to November, indicated that natural transmission of ESFY phytoplasmas to apricot seedlings did not occur during this period, as no bait plants and no insects were found to carry the ESFY phytoplasma. The presence of the ESFY phytoplasma in *Synophropsis lauri* was detected in only one individual insect and required nested PCR. It is thus likely that the ESFY phytoplasma was not multiplying in this insect and that the positive PCR result came from the presence of the phytoplasma in the digestive tract, especially since this insect was captured on apricots. Indeed, *S. lauri* was consistently found in the surroundings of ACLR-infected orchards but was found only occasionally on apricot, despite its presence in high numbers on laurel (*Laurus nobilis*) of adjacent hedges.

As was shown by Carraro *et al.* (1998) in Italy for PLN, the current studies indicate that *Cacopsylla pruni* can acquire ESFY phytoplasmas in southern France and transmission of the phytoplasma to one *Prunus* species has been demonstrated. The intensive testing of insects (67 000), with only negative results, suggests there is no important secondary vector of ESFY phytoplasmas in southern France. It is worth noting that *Fieberiella florii*, a presumed vector of ACLR (Bonfils *et al.*, 1976) and a reported vector of X-disease to *Prunus* in North America (Gilmer & Blodgett, 1976), was not shown to be a vector of the ESFY phytoplasmas in this study by experimental transmission or by testing 240 captured individuals. The role of *C. pruni* as a vector of the ESFY phytoplasma is in agreement with the epidemiological results. Indeed, the oligophagus *C. pruni* has a single generation per year emerging on *Prunus* in spring and migrating to shelter species like conifers or other perennial plants (Lauterer, 1999). This is why *C. pruni* could be easily captured on *Prunus* in early spring, but was absent from the captures carried out on apricots, rootstock suckers or wild *Prunus* from late spring to late autumn. Furthermore, apricot does not seem to be the preferred host of *C. pruni* as it was never collected directly from apricot but only from rootstock suckers and wild *Prunus*. Knowing that *C. pruni* is present on *Prunus* species only in early spring is relevant when devising appropriate insecticide treatments. However, this requires that the life cycle of *C. pruni* be studied in detail, as insecticides cannot be applied during the period of apricot pollination by honey bees.

The inoculum source responsible for the introduction of ESFY phytoplasmas into the apricot seedling orchard remains uncertain. Wild *Prunus* around the apricot orchards has rarely, but consistently, been found infected with ESFY phytoplasmas (Jarausch *et al.*, 1999), but it seems more likely that diseased apricot trees in the adjacent orchards served as inoculum sources. Indeed, Carraro *et al.* (1998) showed that *C. pruni* individuals hatching in spring on stone fruit trees can acquire the phytoplasma before leaving the tree, and this assumption is also supported by the polycyclic nature of the disease spread (Labonne *et al.*, 2000).

Finally, the current results and those of Carraro *et al.* (1998) demonstrate that efficient control strategies against ESFY infections on stone fruits have to include: (i) early eradication of infected trees to reduce inoculum sources; (ii) a strict control of the sanitary status of the planting material to prevent introduction of new inoculum sources; and (iii) the development of a suitable insecticide to control *C. pruni*.

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