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Oosterhout *et al.* 2004). These results indicate that all nine novel markers are highly polymorphic and powerful enough for our future kinship analysis.

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Nine polymorphic microsatellite loci from the psyllid *Cacopsylla pruni* (Scopoli), the vector of European stone fruit yellows

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Abstract

Cacopsylla pruni is the vector of European stone fruit yellows, a quarantine disease of *Prunus* trees. Nine polymorphic microsatellite markers were developed from enriched DNA libraries. Allelic variability was assessed in a collection of 149 females obtained from five localities covering a large geographical area in France. The number of detected alleles ranged from 8 to 37. Within the localities, observed and expected heterozygosities averaged

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across loci ranged from 0.39 to 0.55, and from 0.68 to 0.81, respectively. A heterozygote deficiency was detected for almost all loci, possibly due to a high null allele frequency. Other possible causes of the homozygote excess (mode of reproduction, inbreeding, assortative mating or Wahlund effect) are discussed. These variable microsatellite loci can provide tools to assess overall genetic variation in this important vector species. They will be used to search for population structure and migration patterns of *C. pruni*.

Keywords: blackthorn, disease, epidemiology, microsatellite, phytoplasma, psyllid

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The psyllid *Cacopsylla pruni* (Scopoli) is a phloem-feeding insect, which is widespread in Europe (from Finland to Portugal), Caucasus and Siberia (Ossiannilsson 1992). This psyllid is univoltine (having one generation per year), develops on several species of *Prunus* (mainly on blackthorn *P. spinosa*), and overwinters on conifers. In France, *C. pruni* is widespread and common either on wild or cultivated *Prunus*. It is the vector of '*Candidatus* Phytoplasma prunorum', which causes European stone fruit yellows and damages mainly apricot (*P. armeniaca*) and Japanese plum (*P. salicina*). The phytoplasma is mostly transmitted by insects infected the previous year, on their return from overwintering (Thébaud *et al.* 2009). To understand the processes underlying disease spread, it is important to better estimate distance and direction of the psyllid migratory flights. As these cannot be directly followed, we aim at characterizing them in an indirect way by analysing the genetic differentiation among *C. pruni* populations. Microsatellites are usually regarded as the most appropriate tool to study neutral intraspecific genetic variation because of their high mutation rate and hence high levels of polymorphism. Ten microsatellite loci have been previously characterized in the related species *C. melanoneura* (Malagnini *et al.* 2007), but cross-priming tests in our laboratory with *C. pruni* showed that eight were monomorphic and two showed unclear multiband patterns. As a result, none of them was suitable for genetic studies in *C. pruni*, and other markers had to be found for this purpose. Here we report the isolation and characterization of nine polymorphic microsatellite loci from *C. pruni*.

We performed microsatellite screening using Estoup & Cornuet's method (1994). Genomic DNA was isolated using a cetyltrimethyl ammonium bromide protocol (Doyle & Doyle 1987) from 10 pooled *C. pruni* females, collected on *P. spinosa* near Sauclières (France) on 5 December 2004. Total genomic DNA (~7 μg) was digested using *Rsa*I restriction enzyme. A 400–900 bp fraction of the digested DNA was selected, purified and ligated to *Rsa* linkers.

The enrichment procedure followed the protocol from Kijas *et al.* (1994) based on streptavidin-coated magnetic particules (Magnesphere, Promega), with slight modifications. The following 5′-biotinylated $(CT)_{10}$, $(GT)_{10}$, $(AAG)_{10}$, $(AAT)_{10}$, $(TGTA)_{10}$, and $(ATCT)_{10}$ oligonucleotides were used as probes. We amplified the enriched single-stranded DNA using one of the *Rsa* linkers as a primer to obtain more enriched product and to recover double-stranded DNA. Polymerase chain reaction (PCR) products were purified and ligated into pGEM-T Easy vector (Promega), and the plasmid transformed into *Escherichia coli* super-competent cell (XL1 blue, Stratagene). In total (all motifs included), 1153 recombinant clones were cultivated onto LB agar/ampicillin Petri dishes at 37 °C overnight. Each *E. coli* colony was separately transferred on positively charged Hybond-N nylon membranes (Amersham). DNA was fixed on the membrane by heating at 80 °C for an hour. Positive colonies were identified by hybridization with $(CT)_{10}$, $(GT)_{10}$, $(AAG)_{10}$, $(AAT)_{10}$, $(TGTA)_{10}$, and $(ATCT)_{10}$ probes labelled with digoxigenin using the DIG Oligonucleotide Tailing Kit (Roche Applied Science). A total of 253 positives clones were picked and stored in plates containing glycerol at –80 °C. All of them were sequenced and 225 sequences were obtained, of which 218 contained repeated units. Redundant clones were eliminated, leading to a set of 140 unique sequences. For 42 sequences, several primer pairs were successfully designed using the software Primer 3 (Rozen & Skaletsky 2000). New primers were first tested using a single PCR per locus. Among the 42 loci tested, 12 provided good quality amplification products and were retained for multiplex PCRs. The forward primer of each locus was 5′-end labelled with a fluorescent dye (FAM, HEX or DragonFly). Because of the high allele size variability, no more than three loci were simultaneously analysed per run. For each run, a single PCR was performed on a T-1 Thermoblock (Biometra) using the Multiplex PCR Kit (QIAGEN). The multiplex PCRs were conducted in 10-μL reaction volume containing the QIAGEN Multiplex PCR Master Mix (1×) (including HotStar Taq, dNTPs and 3 mm of $MgCl_2$ as final concentration), 0.2 μm of each primer, about 50 ng of genomic DNA, and RNAse-free water. All multiplex PCRs started with an initial activation step at 95 °C for 15 min, followed by 40 cycles with denaturation at 94 °C for 30 s, annealing at 57 °C (multiplex sets 1, 2 and 4) or 60 °C (multiplex set 3) for 90 s, extension at 72 °C for 60 s and final extension of 60 °C for 30 min. The PCR products were sized on a MegaBace 1000 automated sequencer (GE Healthcare). Screening for polymorphism and allele

Table 1 Characteristics of nine microsatellite loci in *Cacopsylla pruni* genotyped on individuals of five localities in France

Locus	Accession no.	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	N_A	H_O	H_E
PCR multiplex set 1 (T_a: 57 °C)							
Cp6–115	FM200775	F-AATTGAGGGAGGACAGAGTC CGTAGCGAATAGGCACC	$(TTC)_9$	123–150	9	0.384	0.699
Cp5–45	FM200776	D-CTCCCTATTCACAAGTTATG GGACGAGGCAAACATTC	$(TG)_6$	77–131	11	0.407	0.622
Cp6–144	FM200777	H-AACTCATAATGTTCATGTAAAC CCGATAGGAGAGGAACC	$(TTC)_{14}GTC(TTC)_{68}$	72–171	25	0.222	0.759
PCR multiplex set 2 (T_a: 57 °C)							
Cp6–15	FM200778	F-GCGTGGATCTAAGCATTC CCAGCTACTGACAGTCAGG	$(GAA)_5AAA(GAA)_{29}$	70–206	37	0.764	0.917
Cp6–129	FM200779	D-AGGTGCCTATTCGCTAC ACGAGACGAGACTCTTC	$(TTC)_4CTC(TTC)_1$	181–195	8	0.270	0.461
Cp4–108	FM200780	H-GCACCACTCTAATATAATAG TATGATTAGAGTTTAGACGC	$(TAGA)_{10}$	113–213	27	0.432	0.829
PCR multiplex set 3 (T_a: 60 °C)							
Cp4–127	FM200781	F-GAACCACCAACCTCCAGATC CGGACTCGTAATCTGGAGAG	$(GATA)_{11}$	226–394	27	0.652	0.889
Cp5–43	FM200782	D-ATGTGATGGTTACACTTATC CTTAGACTTTGAGATAAGCG	$(TATC)_{13}$	95–254	31	0.529	0.859
PCR multiplex set 4 (T_a: 57 °C)							
Cp4–105	FM200783	D-GGCCTCAACTGGTGTTC CTAACACCTAGCAAGTGG	$(ATCT)_6$	159–240	23	0.683	0.778

Given are locus name and GenBank Accession no., primer sequences, repeated motif in cloned allele, and annealing temperature (T_a). The fluorescent-labelled primers are indicated by F (FAM), D (DragonFly) or H (HEX) at the 5′-end. For each locus, the allelic size range, number of amplified alleles (N_A), and observed and expected heterozygosities (H_O, H_E) are given over all localities.

scoring was performed using the Genetic Profiler software version 1.5. Among 12 loci tested, one gave inconsistent amplification, and two showed unclear multiband patterns. Finally, nine loci giving clear amplifications were chosen for population analysis (Table 1).

We tested these nine markers on 149 females of *C. pruni*, sampled in five localities covering a large geographical area in France: Torreilles (TOR, 42°45′18.35″N, 2°59′33.93″E), Prades-le-Lez (PRA, 43°42′18.38″N, 3°51′12.41″E), La Tieule (TIE, 44°23′12.57″N, 3°7′26.00″E), Coursegoules (COU, 43°47′45.12″N, 7°2′54.56″E), and Montgamé (MON, 46°44′3.89″N, 0°30′35.52″E). Thirty females per locality were genotyped except for TOR and MON where 27 and 32 females, respectively, had been sampled. All the psyllids (overwintered adults) were collected in spring (March–April) between 2002 and 2007, from a same site on different *P. spinosa*. Genetic diversity within each locality was quantified by the number of alleles per locus, and the observed and expected heterozygosities (H_O, H_E). These statistics were calculated using Genetix 4.05 (Belkhir *et al.* 1996–2004). For each locus and each locality, deviation from Hardy–Weinberg equilibrium (HWE) was tested by first using the 'exact HW test', and then using the score (or U) test for testing alternative hypothesis 'heterozygote deficiency' with GenePop 4.0 (Rousset 2007). The same software was also used to test for genetic linkage disequilibrium.

All nine microsatellites showed high to very high polymorphism (Table 1) with 8–37 different alleles per locus. H_O, averaged across the five localities, varied from 0.222 for the least variable locus to 0.764 for the most variable, all values being slightly to very strongly lower than the H_E levels. In the five localities (Table 2), allele numbers ranged from four to 21 (for the least variable locality, COU) and from three to 24 (for the most variable locality, MON), with averages in the range of 10.8 and 13.6. Averaged H_O and H_E ranged from 0.39 to 0.55, and from 0.68 to 0.81, respectively, with all H_O values lower than the H_E levels. Exact tests performed within each locality showed significant deviation from HWE, and more particularly a heterozygote deficiency, for almost all loci (Table 2). Micro-Checker (Van Oosterhout *et al.* 2004) detected no evidence for scoring error due to stuttering or short allele dominance, but 'true' null alleles were detected ($P < 0.05$) in all localities for almost all loci, with the estimates of null alleles frequency ranging between 0.05 and 0.40. Only females have been genotyped in this study, and thus, a particular mode of reproduction like arrhenotokous parthenogenesis cannot explain the excess of homozygotes. We cannot entirely dismiss the

Table 2 Summary statistics for microsatellite loci from *Cacopsylla pruni*, genotyped on 27–32 individuals of five localities in France (TOR, Torreilles; PRA, Prades-le-Lez; TIE, La Tieule; COU, Coursegoules; MON, Montagmé.; *N*, number of individuals analysed)

	TOR (*N* = 27)			PRA (*N* = 30)			TIE (*N* = 30)			COU (*N* = 30)			MON (*N* = 32)		
Locus	n_A	H_O	H_E	n_A	H_O	H_E	n_A	H_O	H_E	n_A	H_O	H_E	n_A	H_O	H_E
Cp6–115	7	0.32***	0.68	8	0.30***	0.79	8	0.46**	0.79	7	0.40***	0.77	4	0.44*	0.46
Cp5–45	8	0.32***	0.73	7	0.56**	0.77	7	0.54^{ns}	0.62	9	0.50***	0.80	3	0.12*	0.18
Cp6–144	11	0.17***	0.77	14	0.37***	0.78	13	0.32***	0.74	11	0.17***	0.71	11	0.08***	0.79
Cp6–15	21	0.69***	0.94	19	0.79***	0.89	16	0.86*	0.88	21	0.79***	0.94	24	0.68***	0.94
Cp6–129	5	0.27**	0.41	6	0.29**	0.56	2	0.33^{ns}	0.28	4	0.20***	0.50	4	0.25**	0.56
Cp4–108	15	0.46***	0.89	15	0.46***	0.86	7	0.27***	0.71	15	0.52***	0.80	17	0.46***	0.88
Cp4–127	14	0.67***	0.89	18	0.76***	0.91	16	0.78**	0.90	19	0.78***	0.91	13	0.28***	0.83
Cp5–43	17	0.62***	0.89	21	0.63***	0.93	15	0.59***	0.90	15	0.26***	0.89	8	0.54**	0.67
Cp4–105	17	0.74*	0.86	14	0.78^{ns}	0.78	13	0.66^{ns}	0.72	12	0.55*	0.72	14	0.69*	0.81
	12.8	0.47	0.78	13.6	0.55	0.81	10.8	0.53	0.73	12.6	0.46	0.78	10.9	0.39	0.68

For each locus, the number of allele (n_A), observed and expected heterozygosities (H_O, H_E) are given per population The mean number are given in the last row. '*' denotes significant deviation of Hardy–Weinberg equilibrium, with the alternative hypothesis 'heterozygote deficiency'. ns, not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

possibilities that the deviations from HWE could be caused by inbreeding, assortative mating or Wahlund effect, but our sampling regime, the mobility of the adults (psyllids were caught after a long-distance migration from conifers), and the ability of females to mate repeatedly would make this unlikely. For most pairs of loci (31 out of 36), no significant linkage disequilibrium was found after applying sequential Bonferroni corrections for multiple tests.

The nine polymorphic microsatellite markers described in this study represent a useful and sensitive tool to investigate population structure of *C. pruni* at a large scale. Understanding the factors influencing the genetic diversity of this psyllid would provide key information about the scale of its movements (and therefore of phytoplasma spread). It could also help in determining whether *Candidatus* Phytoplasma prunorum vectoring is influenced by underlying genetic differences among *C. pruni* populations.

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