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Comparing the spatial genetic structures of the Flavescence dorée phytoplasma and its leafhopper vector *Scaphoideus titanus*

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ABSTRACT

The Nearctic leafhopper *Scaphoideus titanus* Ball is the vector of "Flavescence dorée" phytoplasma (FDp) in European vineyards. We studied the genetic diversity and structure of *S. titanus* populations in France and of the FDp they carried. A total of 621 *S. titanus* individuals, sampled in 24 FDp-infected and uninfected vineyards, were genotyped using seven polymorphic microsatellite loci. The mean observed heterozygosity in *S. titanus* populations was between 0.364 and 0.548. There was evidence of only a low level of population genetic differentiation (mean F_{ST} = 0.027) suggesting that there is long-distance gene flow between *S. titanus* populations. This may be a consequence of the high migration capacity of the vector associated with large effective population size and, at least in part, of passive dispersion over long distances by the transport of grapevine-planting material carrying eggs. For each insect, FDp was detected and typed by nested-PCR followed by RFLP and sequencing of a 674 bp fragment of the FDp *map* gene. Twelve of the 24 populations were found to be infected by FDp, with the percentage of infected individuals varying from 3% to 29%. FDp isolates were classified into two FDp genetic clusters (FD1 and FD2), which differed by 12–13 SNPs. FD1 genotypes were detected in the insect populations at two sites and the FD2 genotypes in the other ten populations. Both FD1 and FD2 genotypes were found to be transmitted by the insect. No significant relationship was found between the genetic structure of these French *S. titanus* populations and the distribution of the various FDp strain types they carried. Nevertheless, overall genetic differentiation between FDp-infected and healthy *S. titanus* "subsamples" was found to be significantly higher than zero. These results suggest that FDp-infected *S. titanus* individuals are more philopatric (disperse less) than healthy *S. titanus*.

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1. Introduction

The epidemiology of vector-borne diseases can be strongly influenced by the genetic diversity and population structure of their insect vectors (Manguin et al., 2008). However, our knowledge of the genetic structure of both the pathogens and their vectors remains limited even for systems of medical or agronomical importance (Criscione et al., 2005). For the Plasmodium-mosquito interactions (malaria), information on the scale of mosquito vector dispersal is highly relevant to improving understanding of the epidemiology of the disease and consequently would be valuable for devising effective means of control. Two recent comparative genetic studies of the malaria parasite (*Plasmodium falciparum*) and the vector population structure (*Anopheles spp.*) revealed very little genetic differentiation between populations in this system, a finding that may explain the speed at which antimalarial drug resistance and insecticide resistance spread (Annan et al., 2007; Prugnolle et al., 2008). For agricultural pathosystems, comparative genetic studies between the population structure of plant pathogens and their insect vector were also carried out. For example in Uganda, the cassava mosaic geminiviruses (CMGs), which are transmitted by the whitefly *Bemisia tabaci*, cause major losses to cassava (*Manihot esculenta*) production. Two distinct genotype clusters of *B. tabaci* have been identified and associated with the geographical distribution of cassava mosaic disease epidemics (Legg et al., 2002). In another study, *B. tabaci* genotypes were found to be correlated to the geographical distribution of the cotton leaf curl virus in Pakistan (Simon et al., 2003).

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Flavescence dorée (FD) is a severe grapevine yellows which has been declared a quarantine disease in Europe (Boudon-Padieu, 2002). It is caused by the FD phytoplasma (FDp), a non-cultivatable wall-less bacterium recently suggested to be a *Candidatus* species (Firrao et al., 2004). This bacterium multiplies within the phloem cells of the host plant and is highly pathogenic for several important grapevine cultivars, triggering quick death of the vine stock (Boudon-Padieu, 1996; Osler et al., 2002; Pavan et al., 1997). FDp is transmitted from vine to vine by the phloem-feeding Nearctic leafhopper *Scaphoideus titanus* Ball (Homoptera: Deltocephalinae) (Schvester et al., 1963). Note, however, that there is no evidence of vertical transmission of FDp by trans-ovarial infection (Alma et al., 1997). FDp transmission can also occur in nurseries as a result of grafting of FDp-infected material. Reported for the first time in the late 50s in the vineyards of south-west France (Bonfils and Schvester, 1960), *S. titanus* was probably accidentally introduced into Europe from North America by importing grapevine canes carrying eggs under the bark (Caudwell, 1983). After its introduction, *S. titanus* spread from south-western France towards Italy and the Balkans and also towards Spain and Portugal. *S. titanus* has an univoltine biological cycle and all developmental stages occur on *Vitis vinifera*, the cultivated grape vine species in Europe (Vidano, 1964). Eggs are laid during the summer by mated females into the bark of grapevine wood and the overwintering eggs hatch during the following spring. *S. titanus* specifically transmits FDp with persistence and propagation of the phytoplasma in the insect body. In spite of compulsory protection regulations (large-scale insecticide treatments, eradication of FDp-infected grapevine plants and protection of mother plants in nurseries), FD disease is still spreading epidemically in the south-east and the west of Europe. This spread depends on the occurrence and diffusion of the leafhopper in vine-growing areas (Alma, 2002; Bressan et al., 2006).

Information about the genetic diversity and structure of both vector and phytoplasma populations would undoubtedly contribute to a better understanding of the role of host-mediated dispersal in FD epidemics. A first evaluation of the genetic variability and structure of *S. titanus* populations worldwide, using random amplified polymorphic (RAPD) DNA markers, has been reported (Bertin et al., 2007): European *S. titanus* populations are less diverse and structured than American populations. The low genetic variability in Europe was interpreted as a consequence of the recent introduction of *S. titanus*, whereas the lack of genetic structure was considered to result from the transport of grapevine canes and grafts carrying eggs to vineyards across Europe. multi locus sequence typing (MLST) has been used to study FDp isolates collected from French and Italian vineyards so as to describe the genetic diversity of phytoplasma strains and to trace their propagation in vineyards (Arnaud et al., 2007). The study involved analysing the sequences of house-keeping genes (*map, secY* and *uvrB*) in the genome of this non-cultivatable bacterium. These genes were selected because they display variability at the subspecies level. The study showed the existence of two genetic clusters of phytoplasma strains in the French vine host; these clusters present differences in molecular variability, prevalence and distribution at national scale (Arnaud et al., 2007). Strains from the cluster FD1 displayed some genetic variability and were responsible for 17% of the cases of the disease; they were preferentially found in south-west France. Strains of the cluster FD2 that showed no genetic variability were responsible for 83% of cases and were evenly distributed across vineyards in France.

Focusing on diffusion by the way of the insect, there are several possible explanations of the difference in geographical distribution of the FDp strains in French vineyards: first, coevolutionary interaction occurring between FDp and its insect vector could lead to a phenomenon of dispersion of phytoplasma strains by genetically and geographically differentiated insect populations. However, the association between this Nearctic leafhopper and the FDp pathogen has been demonstrated to be of recent origin. FD is indeed considered to be an emergent plant disease in Europe resulting from a recent association between a widely cultivated plant (*V. vinifera*), a local native phytopathogen and a newly introduced insect vector (Angelini et al., 2004; Arnaud et al., 2007). Therefore, a coevolutionary process between FDp and *S. titanus* seems unlikely, so two alternative possibilities need to be considered. First, assuming that *S. titanus* presents a spatial population genetic structure (that may be the result of a low migration rate, independent introductions into France, or adaptation to *V. vinifera* varieties), then the geographical distribution of different FDp strains in the French vineyards would be explained by the co-dispersion of the FDp by the genetically differentiated vector populations. Second, phytoplasma strains present significant differences of transmissibility (in the plant or in the insect); the limited spatial diffusion of FD1 strains in south-west France would then result from a lower transmission rate than that of FD2 strains. Were this the case, we would expect there to be no correlation between the genetic structure of FDp and that of *S. titanus*.

In order to determine the contribution of the leafhopper vector in shaping FDp population structure, we (1) assessed genetic diversity and structure of *S. titanus* populations in southern France, (2) characterised the prevalence and the genetic diversity of FDp strains found in *S. titanus* populations, (3) evaluated the ability of the insect to transmit the different FDp strains, and (4) evaluated the level of genetic differentiation between healthy and infected *S. titanus* populations. Using a large *S. titanus* sample ($N = 746$), we determined the prevalence of FDp in 24 vine plots in southern France. The FDp strains were characterised by RFLP and sequence typing of the *map* gene as previously described (Arnaud et al., 2007). A subsample of *S. titanus* individuals ($N = 621$) was further characterised using seven recently isolated co-dominant microsatellite markers that have proven to be highly polymorphic (Papura et al., 2006; Papura et al., 2007).

2. Materials and methods

2.1. Insect sampling

The *S. titanus* sample ($N = 746$) was collected from 24 vine plots in the south of France between 2004 and 2006 (as L5 larval stage in July and as adults in August) (Fig. 1, Table 1). Insects were randomly sampled from each vine plot using a vacuum insect net collector (D-vac). Samples collected from the same vine plots during the same period were considered as a single 'population'. For each population we tried to collect insects with the same development stage (larva or adult) and on the same number of symptomatic and non symptomatic plants. Ten of the 24 populations were sampled on plots declared to be FDp-infected by the regional plant protection services (SRPV): Casseuil, St. Sulpice et Cameyrac, St. Aulaye, La Force, Aire sur Adour, Lasseube, Pardaillan, Peyrière, Seyches and Auzeville. Forty percent *titanus* individuals collected from these FDp-infected populations were kept alive on vine leaves in insect-proof cages for subsequent transmission experiments.

2.2. Plant sampling

Plant samples were collected from FDp-infected vine plots by taking leaves with petioles of symptomatic vines from which insects had been aspired. Samples were kept for a maximum of one week at 4 °C until DNA extraction for phytoplasma genotyping.

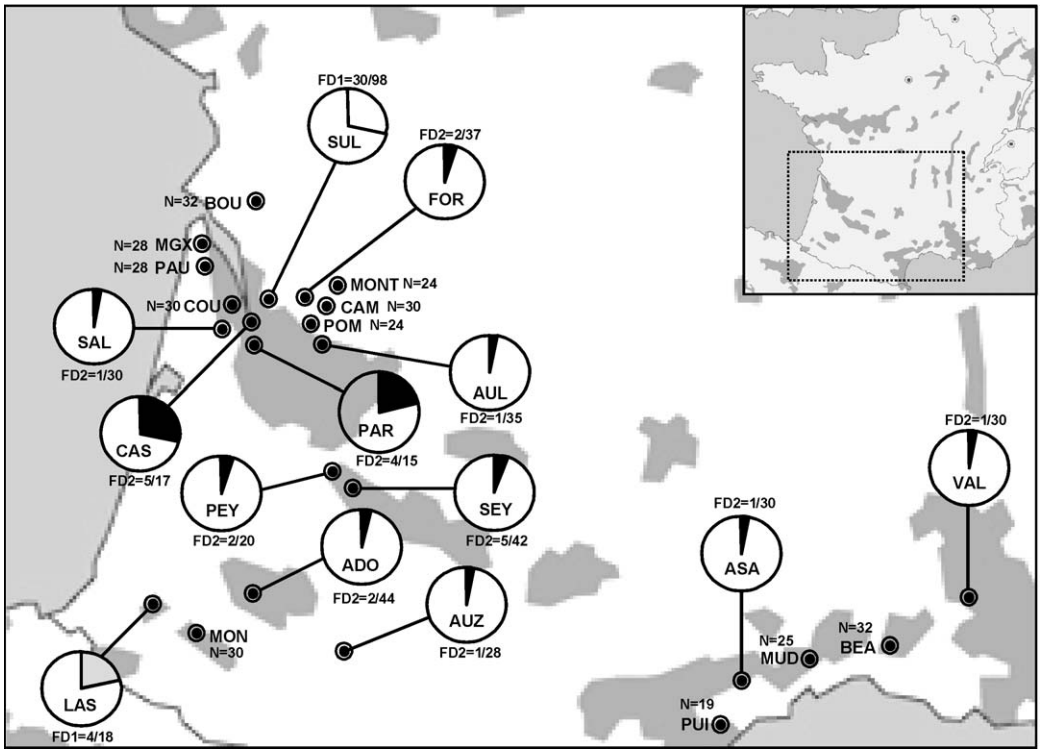

Fig. 1. Spatial distribution of the 24 vine plots sampled in the south of France and the prevalence of FD1 and FD2 strain types detected in *S. titanus*. *N*, total number of leafhoppers tested; FD1, FD2, number of *S. titanus* found positive for one of the two phytoplasma strains/*N*.

Table 1
Geographic location and genetic diversity of 24 French *S. titanus* populations.

GG	Location	Latitude	Longitude	Date	Population name	*N*	*A*	HO (SE)	HE (SE)	FIS multiloci
1	Couhins	44°45′17.07″N	0°34′09.85″O	16/06/2004	COU	30	6.8	0.416 (0.258)	0.632 (0.221)	0.364
	Margaux	45°02′33.70″N	0°40′32.88″O	05/07/2004	MGX	21	6.1	0.364 (0.224)	0.582 (0.221)	0.394
	Pauillac	45°11′49.25″N	0°44′54.51″O	11/06/2004	PAU	28	7	0.464 (0.265)	0.616 (0.236)	0.254
	Salles	44°33′10.11″O	0°52′02.33″N	04/06/2004	SAL	30	6.5	0.372 (0.281)	0.605 (0.270)	0.399
	Casseuil	44°35′8.11″N	0°6′56.95″W	10/08/2005	CAS	17	5.6	0.502 (0.259)	0.570 (0.211)	0.154
	St. Sulpice	44°54′49.27″N	0°23′38.57″W	01/07/2005	SUL	29	7.3	0.459 (0.262)	0.594 (0.227)	0.241
	Pomport	44°47′21.96″N	0°25′20.10″E	09/06/2004	POM	24	6.7	0.437 (0.280)	0.620 (0.233)	0.311
	Montazeau	44°53′33.63″N	0°08′15.40″E	08/06/2004	MON	30	7.1	0.369 (0.277)	0.638 (0.219)	0.44
	Campugnan	44°43′12.85″N	0°18′13.34″E	22/06/2004	CAM	30	6.7	0.450 (0.260)	0.659 (0.187)	0.328
	St. Aulaye	45°12′9.90″N	0°8′3.85″E	09/06/2005	AUL	15	5	0.419 (0.208)	0.582 (0.215)	0.314
	La Force	44°52′3.48″N	0°22′38.32″E	18/06/2006	FOR	21	5.3	0.452 (0.192)	0.589 (0.196)	0.243
	Boutiers	46°05′13.73″N	0°14′28.32″O	02/07/2004	BOU	32	6.8	0.385 (0.282)	0.592 (0.262)	0.361
	Pardaillan	44°39′48.86″N	0°16′48.40″E	11/08/2005	PAR	15	4.7	0.533 (0.282)	0.609 (0.195)	0.152
2	Aire sur Adour	43°42′16.67″N	0°15′41.71″W	18/06/2006	ADO	35	7.3	0.387 (0.250)	0.606 (0.257)	0.367
	Monein	343°18′39.45″N	0°35′28.99″O	11/06/2004	MON	28	6.7	0.443 (0.293)	0.616 (0.258)	0.296
	Lasseube	43°13′16.89″N	0°28′43.18″W	26/08/2005	LAS	18	5.1	0.340 (0.241)	0.552 (0.295)	0.404
	Auzeville	43°31′42.06″N	1°28′55.57″E	25/07/2006	AUZ	19	5.1	0.405 (0.266)	0.517 (0.258)	0.237
3	Peyriére	44°34′35.22″N	0°19′8.31″E	21/07/2005	PEY	20	6	0.512 (0.222)	0.640 (0.205)	0.219
	Seyches	44°33′6.39″N	0°18′17.23″E	21/07/2005	SEY	40	6.8	0.502 (0.169)	0.650 (0.200)	0.242
4	Assas	43°42′57.34″N	3°53′44.82″E	18/06/2004	ASA	30	6.7	0.508 (0.221)	0.652 (0.228)	0.237
	Puimisson	43°26′45.46″N	3°11′41.03″E	17/06/2004	PUI	19	5.4	0.492 (0.203)	0.585 (0.194)	0.183
	Mudaisson	43°37′53.57″N	4°02′17.39″E	18/06/2004	MUD	19	5.7	0.548 (0.299)	0.611 (0.277)	0.129
	Beaucaire	43°48′40.86″N	4°38′7.68″E	14/06/2004	BEAU	32	6	0.395 (0.218)	0.572 (0.247)	0.326
	Valreas	44°23′4.08″N	4°59′0.60″E	11/06/2004	VALR	31	6.1	0.448 (0.196)	0.641 (0.188)	0.321

GG, geographical groups; *N*, sample size (number of insects genotyped); *A*, mean number of alleles/locus; Ho and He, observed and expected heterozygosities; SE standard error; FIS, estimates of FIS values.

2.3. DNA extraction

Total DNA was extracted from each collected leafhopper using the salting-out method (Sunnucks and Hales, 1996). For each collected plant, petioles and midribs were separated from the leaves with a razorblade and pooled in a mixed sample of 1 g. Total DNA was extracted from each plant by the CTAB method according to a protocol described previously (Maixner et al., 1995). Resulting nucleic acid pellets were resuspended in 100 μl of 10 mM Tris–HCl, 1 mM EDTA, pH 7.8.

2.4. Microsatellite genotyping of insects and genetic data analysis

A subsample of 621 of the 746 *S. titanus* tested for FDp infection was genotyped for seven microsatellite loci (Sti6, Sti15, Sti34, Sti36, Sti38, Sti46 and Sti80). All insects that were found to be infected by FD1 or FD2 were included in this genetic characterization. Microsatellite primers and amplification conditions were as previously described (Papura et al., 2006). PCR products were sized on a Beckman Coulter Ceq8000 automated sequencer using the manufacturer's fragment detection chemistry.

Allele frequencies and unbiased heterozygosity (Nei, 1978) were calculated for each sample. Deviations from Hardy–Weinberg Equilibrium (HWE) expectations and unbiased estimates of F_{IS} (Weir and Cockerham, 1984) were estimated using FSTAT version 2.9.3.2. (Goudet et al., 1996), permuting alleles 1500 times among individuals. We estimated null allele frequencies for each locus and analysed populations following the Expectation Maximization (EM) algorithm of (Dempster et al., 1977) as described in the supplementary material of Chapuis and Estoup (2007). Global linkage disequilibrium between pairs of loci was tested using the exact tests in GENEPOP version 3.3 software (Raymond and Rousset, 1995).

Population structure inferred from microsatellite loci was assessed by calculating F_{ST} (Weir and Cockerham, 1984) between populations using GENEPOP 3.3. and genotypic differentiation was tested using a log-likelihood based exact test (Goudet et al., 1996). A hierarchical analysis of molecular variance (AMOVA) between the 24 French populations of *S. titanus* was performed using ARLEQUIN version 3.0 (Excoffier et al., 2005). The French populations were partitioned into groups according to: (i) geographical region (four groups): group 1 – Couhins, Margaux, Pauillac, Salles, Casseuil, St. Sulpice et Cameyrac, Pomport, Montazeau, Campugnan, St. Aulaye, La Force; group 2 – Pardaillan, Peyrière, Seyches; group 3 – Aire sur Adour, Monhein, Lasseube, Auzeville; and group 4 – Puimisson, Beaucaire, Mudaisson, Assas, Valreas; (ii) presence or absence of FDp in insects (three groups): group FD1 – *S. titanus* populations carrying FD1 (Lasseube, St. Sulpice et Cameyrac); group FD2 – *S. titanus* populations carrying FD2 (St. Aulaye, La Force, Pardaillan, Peyrière, Seyches, Aire sur Adour, Casseuil, Auzeville, Assas, Valreas); and group no-FD – *S. titanus* populations in which FDp was not detected (Couhins, Margaux, Pauillac, Salles, Pomport, Montazeau, Campugnan, Monhein, Boutiers, Puimisson, Beaucaire, Mudaisson). This method was used to partition the genotypic variance between groups, between populations, within groups and within populations. Levels of significance were determined by computing 1500 random permutation replicates. To test for correlation between genetic distance (pairwise $F_{ST}/(1 - F_{ST})$) according to (Rousset, 1997) and geographical distance (in km) among populations, the Mantel test was performed using GENEPOP 3.3. Significance was assessed with 1500 permutations.

The Bayesian clustering approach implemented in STRUCTURE version 2.1 (Pritchard et al., 2000) was used to infer the number of clusters (K) in the data set, with prior information of the healthy or FDp-infected status for each *S. titanus* individual. The software was run with the option of admixture, allowing for some mixed ancestry within individuals. Ten independent runs were done for each value of K ($K = 1$–8), with a burn-in period of 100,000 iterations and 100,000 replications. The method of (Evanno et al., 2005) was used to determine the most likely number of clusters. As a complementary approach, factorial correspondence analysis (FCA) was applied, using GENETIX v4.04 software (Belkhir et al., 2003) to visualize individuals in two-dimensional space according to allelic variation at the seven microsatellite loci with prior information concerning the healthy or FD1 and FD2-infected status.

Biased genetic structure between healthy and FDp-infected *S. titanus* was tested using a specific randomisation procedure (FSTAT 2.9.3.2.) Under the null hypothesis that uninfected and infected individuals disperse equally, overall population differentiation (θ, Weir and Cockerham's F_{ST} estimator, 1984) was calculated for each locus and for each sample of the two status (healthy/FDp-infected *S. titanus* individuals). The significance of global θ estimates was further evaluated with an exact G-test after 1000 randomizations of alleles among sites (Goudet et al., 1996). The infection status was assigned to each of the multilocus genotypes split into homogeneous subsamples. Finally, we used the test for biases in dispersal among the two defined groups of individuals based on infection status differences in F-statistics and assignment indices (Goudet et al., 2002). The infection status was randomly assigned 1500 times to each of the multilocus genotypes (keeping the individuals in their original population and the infected/healthy ratio in each sample constant).

2.5. FDp detection and typing

Each collected insect ($N = 746$) and plant ($N = 30$) was tested for FDp DNA by nested-PCR amplification of the *map* gene as described in (Arnaud et al., 2007). Routine FDp typing was performed by RFLP on one to seven positive insects from each infected population and on two to five positive plants sampled in each infected vineyard. Briefly, the *map* nested-PCR product was digested with both *Alu*I and *Eco*72I enzymes and the digestion products were analysed on 8% polyacrylamide gels by staining with ethidium bromide and visualization with UV transilluminator (Arnaud et al., 2007). Restriction profiles were compared to the profiles of reference strains from the FD1, FD2 and Italian FD3 clusters (Arnaud et al., 2007). Finer molecular typing was performed on one insect from each infected population by sequencing both strands of the *map* PCR product. Multiple-sequence alignments (674 bp long) were performed using the CLUSTAL W program (Thompson et al., 1994). Phylogenetic reconstructions by maximum parsimony were performed using MEGA3.1 (Kumar et al., 2004) with randomized bootstrapping evaluation (500 replicates) of branching validity. Phylogenetic analyses also included the *map* gene sequences of nine FDp reference strains from the FD1, FD2 and FD3 clusters described previously (Arnaud et al., 2007) and of *Candidatus* phytoplasma ulmi as an outgroup.

2.6. FDp transmission

Phytoplasma transmission to broad bean (*Vicia faba* cv. Agua dulce) was performed as described in (Caudwell et al., 1970). Briefly, *S. titanus* collected on symptomatic vines plants from FDp-infected vine plots were placed in groups of 10–30 on healthy broad beans. Insects were kept on the plants for one week to allow transmission and survivors were then transferred to new healthy broad beans for a further week. This was renewed until the death of every insect. All dead *S. titanus* were collected and tested for phytoplasma and microsatellite genotyping. Four to five weeks after transmission, broad beans were observed for the development of FD symptoms and tested by PCR to detect phytoplasma as described above.

3. Results

3.1. Genetic variability and structure of the S. titanus populations

Among the 621 leafhoppers genotyped, polymorphism of seven microsatellite loci ranged from three (Sti46 and Sti80) to 31 (Sti6) different alleles per locus, with a mean number of 13 alleles per locus (Table 1). The mean number of alleles per population for all loci was 7.

Table 2
Partition of genetic variability of *S. titanus* populations at the geographical scale and according to FDp phytoplasmas presence or absence in insects.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among geographical groups[a]	4	29.32	0.012	0.560
Among populations within groups	18	82.49	0.055	2.550
Within populations	1015	2150	2.119	96.89
Total	1037	2262	2.187	
Among healthy or FDp-infected groups[b]	2	4.973	0.000	0.090
Among populations within groups	21	44.45	0.020	1.880
Within populations	1206	1290	1.070	98.03
Total	1229	1340	1.091	

[a] Geographical groups: group 1 – Couhins, Margaux, Pauillac, Salles, Casseuil, St. Sulpice et Cameyrac, Pomport, Montazeau, Campugnan, St. Aulaye, La Force, Boutiers; group 2 – Pardaillan, Peyrière, Seyches, group 3 – Aire sur Adour, Monhein, Lasseube, Auzeville, and group 4 – Puimisson, Beaucaire, Mudaisson, Assas, Valreas).
[b] Healthy and FDp-infected groups: group FD1 – (Lasseube, St. Sulpice et Cameyrac), group FD2 – (St. Aulaye, La Force, Pardaillan, Peyrière, Seyches, Aire sur Adour, Casseuil, Auzeville, Assas, Valreas), group no-FD – (Couhins, Margaux, Pauillac, Salles, Pomport, Montazeau, Campugnan, Monhein, Boutiers, Puimisson, Beaucaire, Mudaisson).

Analysis of the genetic disequilibrium between each pair of loci in each population revealed significant linkage disequilibrium (LD): of the 21 pairwise comparisons performed for each population ($P < 0.03$), LD was found for three pairs of loci in two populations, and for one pair of loci in nine populations. Across all populations, three of the 21 pairs of loci were in significant LD (Sti36&Sti15, Sti36&Sti34 and Sti36&Sti80; $P < 0.01$).

Observed (H_O) and expected heterozygosity (H_E) calculated for the seven microsatellite loci were between 0.364 and 0.548, and 0.517 and 0.659, respectively (Table 1). Significant deviation from Hardy–Weinberg equilibrium was observed for two of the seven microsatellite loci analysed in all sampling localities, except for four populations that were in Hardy–Weinberg equilibrium (Puimisson, Casseuil, Pardaillan and La Force). The deviations from Hardy–Weinberg equilibrium were due to a deficit of heterozygotes at only two loci (Sti36, Sti38) of the seven, these findings are consistent with the existence of null alleles at these two loci. Indeed, null alleles were estimated to represent more than 10% for these two loci: 13.1% of null alleles for Sti36 and 18% for Sti38. Therefore, assuming the presence of null alleles at these loci, we also analysed the data set after having removed them: most of the French *S. titanus* populations were in Hardy–Weinberg equilibrium after the removal of Sti36 and Sti38. Only four of the 24 samples showed significant excess of homozygotes ($P < 0.05$): Couhins and Aire sur Adour at Sti80, Montazeau at Sti6, Sti46 and Sti80 and Boutiers at Sti6.

The global estimate of F_{ST} for the 24 populations and the seven microsatellites loci was significantly different from zero ($F_{ST} = 0.027$, exact test for genotypic differentiation, $P < 0.0065$ for each locus) indicating slight but significant overall our samples for these markers: 196 of the 276 pairwise F_{ST} values were significantly different from zero. F_{ST} values ranged from 0 (−0.010 between Peyrière and Seyches, populations at 2.9 km apart) to 0.111 (Auzeville and Assas, populations at 124 km apart) (see supplementary material). F_{ST} pairwise values between populations from the same geographical groups ranged from 0 to 0.056. Limiting estimates of genetic differentiation to the five loci that conformed to Hardy–Weinberg expectations (Sti6, Sti15, Sti34, Sti46 and Sti80) did not significantly change these results.

Partitioning of molecular variability was assessed by AMOVA: 97.74% of the genetic variability was within populations, 1.35% between populations from the same geographical areas and only 0.91% between the four geographical groups (Table 2). This virtual absence of regional population structure indicates substantial gene flow at this geographical scale.

Genetic distances (F_{ST}) between pairs of populations were slightly but significantly correlated with geographical distance between sample sites (Mantel test, $r = 0.175$, $P = 0.007$) when all seven microsatellite loci were used for the analysis. By contrast, no significant correlation was found when the two loci showing null allele excess (Sti36 and Sti38) were eliminated from analysis ($r = 0.093$, $P = 0.07$, Fig. 2).

3.2. Detection and genetic variability of FDp

Of the 746 insects analysed, 59 were found to be FDp-positive by nested-PCR analysis of the *map* gene. FDp-positive insects were

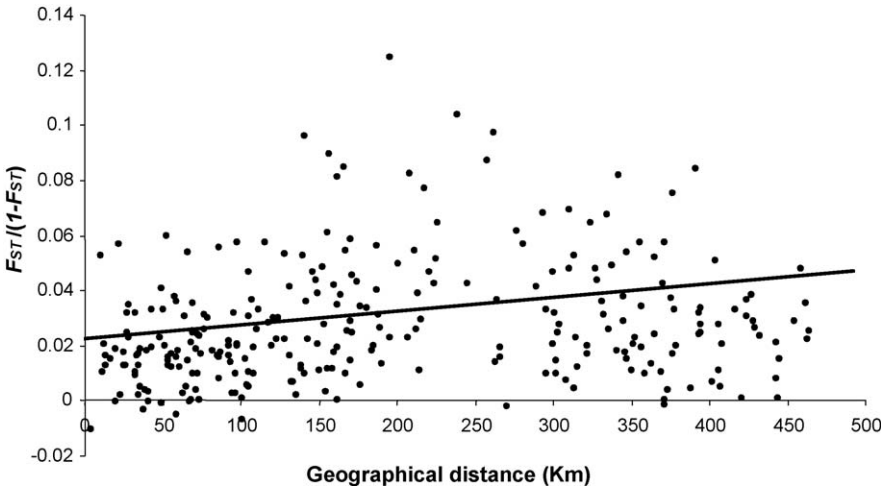

Fig. 2. Pairwise F_{ST} values between populations of *S. titanus* plotted against geographical distances using the seven microsatellite loci.

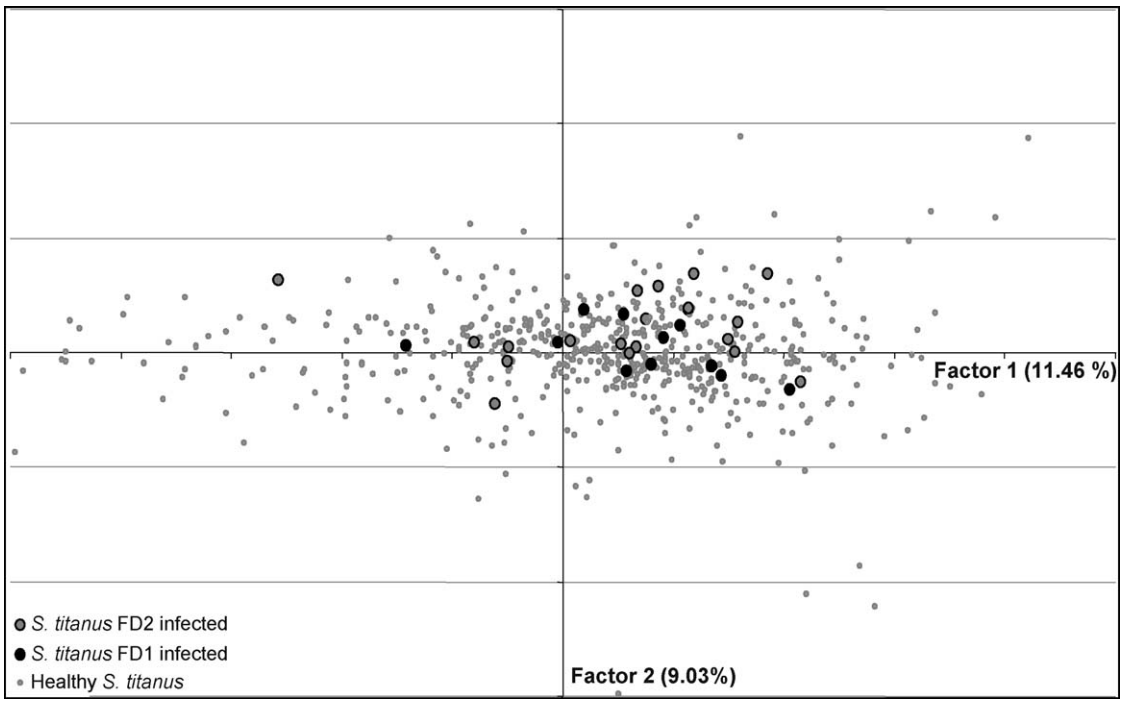

Fig. 3. Plot of the first two axes of a factorial correspondence analysis (FCA) performed on the total data set of 621 *S. titanus* individuals, with prior information of the healthy or FD1 and FD2-infected status.

found on all 12 vine plots (Fig. 1). The percentage of infected *S. titanus* varied from 3% (St. Aulaye Assas and Valreas) to 30% (St. Sulpice et Cameyrac) (Fig. 1). All symptomatic plants ($N = 30$) collected in these vine plots were found to be FDp-positive by nested-PCR (note that no plants were collected from the Assas and Valreas vine plots). RFLP of the *map* gene was used for molecular typing of positive insects and plants. Two different restriction profiles were evidenced (Fig. 4). Identical FDp profiles were found for all the insects and plants isolates from any one vine plot. The restriction profiles of FDp isolates found in St. Sulpice et Cameyrac and Lasseube vine plots (Fig. 4, lane 1 and 2, respectively) were identical to that of the FD1 reference strain FD70 (lane 9). The restriction profiles of FDp isolates found in the eight other vine plots (lanes 3–8) and for Assas and Valreas populations (not shown) were identical to that of the FD2 reference strain FD92 (lane 10). None of the infected samples presented the restriction profile of the Italian FD3 reference strain VI04-C28.

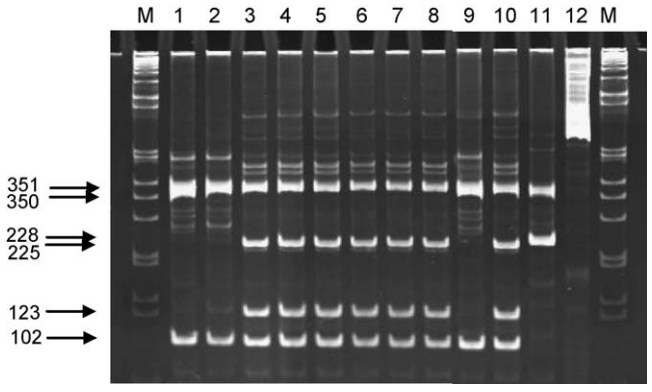

Fig. 4. Typing of the FDp in insects by nested-PCR RFLP for the *map* gene. Migration profiles on 8% polyacrylamide gel of the PCR products digested with *Alu*I-*Eco*72I. Lanes 1–8: *S. titanus* samples from CAM, LAS, PAR, SEY, PEY, CAS AUL and FOR populations, respectively; lane 9: FD1 cluster reference strain (FD 70); lane 10: FD2 cluster reference strain (FD 92); lane 11: FD3 cluster reference strain (VI04-C28) and lane 12: undigested PCR product. Lane M: 1 kb ladder from Invitrogen.

The *map* gene from one FDp-positive insect from each of the 12 infected populations was sequenced. The evolutionary relationships between the *map* genetic loci for the 12 FDp isolates, including sequences of nine FDp reference strains, were inferred using the Maximum Parsimony method. One of the 230 most parsimonious trees is shown Fig. 5. The 674 bp analysed did not contain any gap or missing data. Consistent with a previous report (Arnaud et al., 2007), three genetic clusters of FDp isolates with high bootstrap branching validity (> 80%) could be distinguished. They were named FD1, FD2 and FD3. The Lasseube and St. Sulpice isolates presented the same sequence as the V03-9-2 reference strain (accession number AM384888) and as the V02-101 reference strain (AM384887) (Arnaud et al., 2007), respectively. They differed from each other by one single-nucleotide polymorphism (SNP) and were each collected in the same department as their homologous reference strain (Pyrénées Atlantiques and Gironde). They belong to the FD1 cluster. The sequences of the other isolates were all identical to the reference strain V00-SP5 from Gironde (AM384886): they belong to the FD2 cluster. The FD1 and FD2 clusters differed by 12–13 SNPs. All of the sequences differed from that of the reference strains of the FD3 cluster which were not detected in the insects collected.

To test for the ability of *S. titanus* to transmit both FD1 and FD2 strain types, transmission assays were performed with insects collected from vine plots in Saint Sulpice et Cameyrac (91 insects, FD1) and Peyrière (20 insects, FD2). The insects were placed on nine and two broad bean plants respectively. Five weeks after the contact with FDp-positive insects, five of the nine broad bean plants exposed to FD1 and one of the two exposed to FD2 presented dwarfism and bending of the upper leaves with generalised yellowing; these plants also scored positive in nested-PCR tests for FDp. Typing by sequencing confirmed that phytoplasmas detected in broad beans were the same as those found in the insects to which they were exposed. Broad beans exposed to FDp-negative insects did not show any symptoms and were FDp-negative. Thus, isolates of both strain types FD1 and FD2 were efficiently transmitted by *S. titanus*.

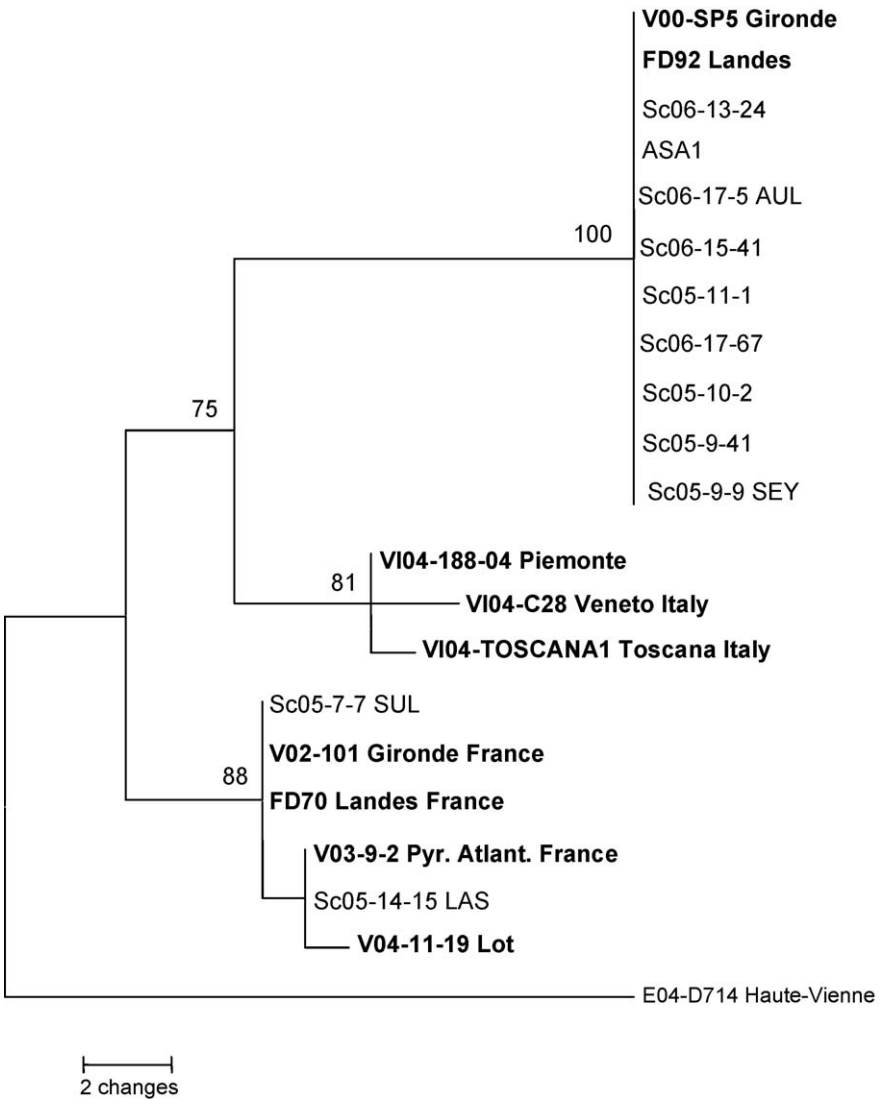

Fig. 5. Phylogenetic tree constructed by parsimony analysis of the *map* gene sequence (674 bp) from FDp isolates found in *S. titanus*. "*Ca*. Phytoplasma ulmi" reference strain (E04-D714) is included as the out-group. Branch lengths are proportional to the number of inferred character state transformations. Bootstrap values for 500 replicates are shown on the branches. FDp isolates found in *S. titanus* and the name of their insect population of origin are indicated in light characters. Grapevine FDp reference strains from each FDp cluster (FD1, FD2 and FD3) and their geographical origin are indicated in bold characters. The FDp clusters identified are shown to the right of the tree.

3.3. Comparative genetic structure of healthy and infected (FD1 or FD2) S. titanus populations

The partitioning of molecular variability achieved with the seven microsatellites loci was assessed by AMOVA: only 0.09% of the genetic variability was between the three groups of healthy and FDp-infected *S. titanus* populations (group no-FD, group FD1 and group FD2), 1.88% was between populations of the same group and 98.03% was within populations (Table 2). These results suggest that the population structure of the vector *S. titanus* does not correlate with FDp strain type. Thus, no relationship could be established between the genetic structure of *S. titanus* populations and the distribution of the FD1 and FD2 strain type they carry.

We used a Bayesian clustering method (part of the Structure software) to detect possible cryptic substructure in our data set (using prior information concerning the healthy, FD1 and FD2-infected status for each *S. titanus* individual). The ΔK statistic, described by Evanno et al. (2005), indicated that the sample included a single group of individuals (the highest ΔK was obtained with $K = 1$). Results from the Bayesian analysis thus show that the healthy, FD1 and FD2-infected *S. titanus* are genetically unstructured. These findings are consistent with the results of factorial correspondence analysis in which individuals of healthy or FD1 and FD2-infected status do not form separate groups (Fig. 3).

In addition, we tested whether the dispersion of healthy or infected insect individuals differed on the spatial scale of a vine plot. For this analysis, we used the five *S. titanus* populations for which we found more than four FDp-infected insects: Casseuil, St. Sulpice et Cameyrac, Lasseube, Pardaillan and Seyches. The gene diversity (H_E) and the allelic richness (number of alleles corrected for sample size) did not differ significantly between healthy and FDp-infected *S. titanus* subsamples. Nevertheless, the genetic differentiation was found to be significantly different from zero and higher in FDp-infected ($\theta = 0.049$, $P = 0.0025$) than in healthy leafhoppers ($\theta = 0.009$, $P < 8.10^{-4}$). To assess and compare dispersal abilities between healthy and FDp-infected *S. titanus* leafhoppers, the genetic relatedness was calculated between individuals in subsamples. For each population, this analysis revealed significantly ($P = 0.04$) higher values for FDp-infected (relatedness = 0.0778) than for healthy (relatedness = 0.0024) *S. titanus*. These results indicate that FDp-infected individuals may be

more philopatric (less dispersing) than healthy *S. titanus* individuals. However, it must be noted that splitting the data greatly increased the sampling variance of θ, especially for the FDp-infected sub-sample (only 33 insects).

4. Discussion

Here, we analysed the genetic structure of the leafhopper *S. titanus* and that of the phytoplasma strains they transmit which are responsible for the FD disease on grapevine. By examining genetic differentiation of *S. titanus* insects according to their infection status (healthy and infected with different strains of the phytoplasma), we contribute to describing the role of the leafhopper genetic structure in shaping the FDp population.

4.1. Genetic structure of S. titanus populations

Our results illustrate the great value of microsatellite markers for characterising the genetic diversity and structure of French *S. titanus* populations at a regional scale. F_{ST} estimates revealed slight but significant overall genetic differentiation with no significant correlation with geographical distances between populations. Gene flow among French *S. titanus* populations therefore appears to be high enough to maintain extensive genetic homogeneity on a large geographical scale. This suggests that *S. titanus* has substantial dispersal abilities or a large effective population size or both. Note that, as previously suggested by Bertin et al. (2007), the low genetic differentiation between *S. titanus* populations that we describe could also be indicative of a passive dispersion over long distances by human transportation of grapevine canes and grafts carrying eggs. Disentangling the effects of active or passive dispersion could be difficult because we lack direct information about the scale of migration by this leafhopper species. Nevertheless, investigations of the genetic structuring of geographically distant Brazilian populations of the leafhopper *Dalbulus maidis*, the main vector of *Spiroplasma kunkelii*, revealed relatively high rates of gene flow between central and southern populations. The authors suggested the occurrence of substantial migration within the corresponding region of Brazil (De Oliveira et al., 2007). The lack of genetic differentiation over a large geographical area has been documented for other insect pests including aphids like *Sitobion avenae*, (Llewellyn et al., 2003); *Rhopalosiphum padi*, (Delmotte et al., 2002) and Lepidoptera pests including *Plutella xylostella*, (Endersby et al., 2006) and *Cyda pomonella*, (Franck et al., 2007). The authors of these various studies also support the view that weak population differentiation results from the highly migratory behaviour of these insect species.

A surprising result of our study is the absence of significant correlation between genetic and geographical distance, as assessed by analysis of the loci conforming to Hardy–Weinberg expectations. The lack of isolation by distance (IBD) could be due to population size reduction resulting from insecticide treatments within vine plots. Six of the *S. titanus* populations (Valreas, Saint Sulpice, Seyches, Assas, Puimisson and Mudaisson) were indeed sampled from grapevine areas that had been under pyrethroid insecticide control for more than three years. Insecticide application causes a reduction of the insect population size or even its local extinction. Small population sizes and founder effects following insecticide treatments increase genetic drift and generate significant genetic structure that is not correlated to the geographical distance between insect populations. However, we found no IBD when the populations under pyrethroid insecticide control were eliminated from the analysis and this suggests that another process is blurring the IBD pattern. For *S. titanus*, the lack of IBD may also be a consequence of the passive (and sometimes long-distance) dispersal of vectors due to commercial exchange. Indeed, passive dissemination of vectors by transport across Europe of grapevine canes and grafts carrying eggs has been described (Maixner, 2005).

4.2. Prevalence and diversity of FDp in the leafhopper vector

In this study we report the prevalence and the genetic characterization of a phytoplasma within its vector insect over a large geographical range. In the case of FD disease, sampling is complicated by the fact that it is difficult to find leafhoppers carrying FDp that are not under insecticide control management. Sequence analysis of the FDp *map* gene showed the presence of two genetically distinct strain clusters in the insect populations. The cluster FD2 showed no molecular variability, it was predominant (80% of the insects tested) and present in every wine-growing area studied. FD1 was composed of two different haplotypes, it was present as a minority (20%) and was localized in the western vineyards. The relative proportion and geographic distribution of FD1 and FD2 types found in *S. titanus* is in agreement with previous findings for those from plants in infected French vineyards (Arnaud et al., 2007). This shows that insects can acquire and carry both phytoplasma strains. Furthermore, our results also demonstrate that FD1 and FD2 strains are both transmitted by the insect. So, the difference in geographic distribution between strains is unlikely to be related to the transmission capacity of *S. titanus*. However, the limited diffusion of FD1 strains may be a consequence of weaker multiplication in both plants and insects. Indeed, it has been suggested (Bressan et al., 2005a,b) that the transmission of FDp from vine to vine is less efficient if the concentration of phytoplasma on the plant source is lower. This should be evaluated by suing quantitative methods (quantitative real-time PCR) to compare the multiplication kinetics of FD1 and FD2 in both plants and insects.

4.3. S. titanus genetic differentiation and FDp-infection status

By comparing the genetic structure of French *S. titanus* and FDp strains, we showed that there was no genetic differentiation between healthy and infected leafhopper populations. Moreover, no relationship was found between the distribution of the two different strain types (FD1 and FD2) in southern France and the genetic structure of the leafhopper populations. These observations suggest the absence of natural co-dispersion involving particular *S. titanus* subpopulations and FD phytoplasma strains. The difference of geographic distribution and prevalence of phytoplasma strains may be the consequence of extensive diffusion of the phytoplasma through commercial exchange of infected grapevine material. Indeed, only the clonal and widely spread FD2 type was found in infected nurseries in the 1990s and 2000 (Boudon-Padieu, unpublished).

The most striking result of our study is the difference of genetic structure between healthy and infected individuals within wine plots, which can be considered as a difference of dispersal at this fine spatial scale. Phytoplasma invasion and multiplication in *S. titanus* reduces both the vector's life span and its fecundity (Bressan et al., 2005a,b). Therefore, the presence of FDp may limit the potential rate of spread (diffusion) of the insect by reducing its fitness (Bressan et al., 2005a,b). Tests for biases in dispersal among the FDp-infected and healthy groups of insects using *F*-statistics and assignment indices have been used by several authors to detect dispersal differences between sexes (Knight et al., 1999; Mossman and Waser, 1999). Using the same type of reasoning, if individuals do not disperse far from the natal site (philopatric), they would be expected to be more related than individuals taken at random from the whole population; higher between subpopulations genetic differentiation would therefore be expected.

Although these tests are mostly used for sex-biased differences in dispersal, they can be generalised for any individual status (Goudet et al., 2002). In our case of FDp-infected and healthy *S. titanus*, we favour the hypothesis that the pattern observed for FDp-infected insects is due to limited active dispersal. This phenomenon has also been described for *Ixodes ricinus*, the tick vector of *Borrelia afzelii* (Lyme disease agent). According to the migration-infection survival hypothesis (MISH), survival is severely compromised for migrating ticks when infected by *B. afzelii* (de Meeus et al., 2004). This genetic differentiation of *S. titanus* populations carrying FDp clusters from non FDp carrying clusters will have to be confirmed by analysis of a larger number of populations and by temporal analysis in the same sites. We are currently using genetic parentage analysis in combination with capture recapture methods to study the dispersion of *S. titanus* winged adults at local geographical scale.

Conflict of interest

The authors declare that they have no conflict of interest and certified that experiments comply with the French current laws.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2009.05.009.

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