***Wolbachia*-induced thelytoky in the rose gallwasp *Diplolepis spinosissimae* (Giraud) (Hymenoptera: Cynipidae), and its consequences on the genetic structure of its host**

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Cynipids are known to use various reproductive modes (arrhenotoky, thelytoky and strict cyclical parthenogenesis), but the mechanism remains unknown. We have studied the reproductive system of a rose gallwasp, *Diplolepis spinosissimae*, which was found to exhibit two different reproductive systems according to the population. In eight out of the ten populations studied, all along the Atlantic coast, *D. spinosissimae* is thelytokous. Males are extremely rare, and all females are homozygous at three microsatellite loci. 'Obligate homozygous parthenogenesis' was found to be strictly associated with the presence of the endosymbiotic bacterium *Wolbachia* sp. In the two remaining populations, deprived of *Wolbachia*, *D. spinosissimae* reproduced by arrhenotoky as indicated by the larger frequency of males and heterozygosity of females. Allelic diversity, although not zero, was highly reduced in the coastal populations, as a consequence of thelytoky and gamete duplication. We hypothesize that mating of uninfected males with infected females during the first generations after an infection event could explain the small but significant amount of polymorphism observed in those populations. The high level of differentiation indicates a low gene flow, even between geographically close coastal populations.

Keywords: gallwasp; *Wolbachia*; parthenogenesis; gamete duplication; microsatellite; heterozygosity

1. INTRODUCTION

In Hymenoptera, several types of parthenogenesis have been defined according to the sex of the offspring of a virgin female. Most species exhibit arrhenotokous parthenogenesis: unfertilized eggs produce haploid males, while fertilized eggs produce diploid females (haplodiploid sex-determination) (Crozier 1975). However, in a few groups, thelytokous parthenogenesis has been demonstrated, with virgin females producing only daughters. Various cytogenetic mechanisms for producing diploid females from unfertilized eggs have been described among insects (reviewed in White 1973). More recently, endosymbiotic bacteria of the *Wolbachia* genus (the alpha proteobacteria section), also known to be responsible for

cytoplasmic incompatibility in various arthropod species (Rousset *et al.* 1992a; Werren 1997), have been found to be associated with thelytoky in several hymenopteran parasitoids: *Trichogramma* spp. (Stouthamer *et al.* 1990), *Encarsia* spp. (Zchori-Fein *et al.* 1992), *Aphytis* spp. (Zchori-Fein *et al.* 1995), *Muscidifurax* spp. (Stouthamer *et al.* 1993), *Leptopilina* spp. (Werren *et al.* 1995) and *Diplolepis rosae* (van Meer *et al.* 1995), as well as one coleopteran species (*Naupactus tessellatus*; Werren *et al.* 1995). In these species (except the last two, for which the experiment has not been conducted), infected females produced males after antibiotic treatment which cures them from *Wolbachia* infection. The cytogenetic mechanism of oocyte diploidization involving the bacterium has been described in *Trichogramma* spp. (Stouthamer & Kazmer 1994). Oogenesis is normal and diploidy is restored in the unfertilized egg by the fusion of the mitotic products of the haploid female pronucleus. By such a process, also called 'gamete duplication', complete homozygosity (autozygosity) is achieved. However, Stouthamer & Kazmer (1994) have also shown that males can mate with infected females and, in this case, fertilization of the ovocyte occurs, precluding gamete duplication and resulting in an infected

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Figure 1. Locations of sampling sites of *Diplolepis spinosissimae* galls.

offspring with combinations of parental alleles. Thus, gene flow can occur between the arrhenotokous and thelytokous sections of the population. However, Zchori *et al.* (1992, 1995) have reported that sperm fail to fertilize eggs in *Encarsia* spp. and *Aphytis* spp.

Among Cynipidae, a family of phytophagous, mainly gall-inducing Hymenoptera, several species of the *Diplolepis* genus (Rhoditini tribe) are considered to be thelytokous because of male scarcity (Askew 1960; Eady & Quinlan 1963; Nieves-Aldrey 1980). According to the cytogenetic study performed by Stille & Dävring (1980) and Stille (1985a,b), *Diplolepis rosae* (L.) and *D. mayri* (Schlechtendal) reproduce by gamete duplication. Allozyme electrophoresis failed to detect heterozygous individuals in several Swedish populations of both species. All those observations suggested that *Wolbachia* could be found in some Cynipids, and these have been supported by a recent discovery in *D. rosae* by van Meer *et al.* (1995).

In the present study, we investigated the mode of reproduction of *Diplolepis spinosissimae* (Giraud). Twelve populations were separated into two groups with contrasting features, concerning (i) sex ratio; (ii) heterozygosity, using hypervariable genetic markers (microsatellite loci); and (iii) *Wolbachia* infection. Consequences of infection by *Wolbachia* on the genetic structure of the *D. spinosissimae* populations are discussed.

2. MATERIALS AND METHODS

(a) Biological material

Diplolepis spinosissimae is a Eurasiatic cynipid which has a patchy distribution due to its specific association with *Rosa pimpinellifolia* (Niblett 1951; Eady & Quinlan 1963). This plant is found in isolated populations on sand-dunes, the tops of calcareous cliffs and in heathlands, its occurrence being determined by its specific requirements for xeric and calciferous conditions (Des abbayes *et al.* 1971). Consequently, exhaustive mapping of rose populations and of its obligate gallwasp is possible. *Diplolepis spinosissimae* is a univoltine species, producing galls on rose leaves, stems or fruits (cynorhodons). Eighty per cent of the galls are unilocular, but the biggest ones can contain

up to ten larval cells (Plantard 1997). In the sites studied, females emerge from the galls in April or May and lay their eggs in the unopened rose buds. Galls grow during late spring and summer. Leaf galls fall on the ground in autumn, while stem galls remain on the bushes during winter. The larvae hibernate in the gall and pupate in March before chewing an exit hole to emerge.

Galls of *D. spinosissimae* have been collected in ten French populations (figure 1) for three consecutive years (1994, 1995 and 1996). Seven populations are from the Atlantic and Channel coasts. The three remaining populations (Argenton, Fontainebleau and Queyras) are continental, the first two being highly isolated from the nearest populations. All insects studied have been reared from galls collected in the field. Adults of *D. spinosissimae* were stored at -80°C prior to DNA extraction. In the case of plurilocular galls, only a single individual was used to avoid sampling siblings. High mortality rates (usually up to 80%, and mostly due to parasitism; Plantard 1997) resulted in a limited number of surviving adults.

(b) Microsatellite isolation and amplification

We have screened a partial genomic library of *D. spinosissimae* (using 500–700 bp Sau IIIA restriction fragments) to isolate microsatellite loci. Screening was performed as described in Estoup & Cornuet (1994), with (TG)₁₀, (TC)₁₀, (CAC)₅, (CCT)₅, (ATCT)₅ and (TGTA)₆ probes. Microsatellite loci were rare in *Diplolepis spinosissimae* and *D. rosae* (L.): only 18 positive clones were found out of 5150. Primers were designed for 16 loci, but only 3 gave clear and polymorphic amplification patterns (see table 1). Polymerase chain reactions (PCRs) were performed in a total volume of 12.5 μl . Products were labelled using $\gamma^{32}\text{P}$, separated electrophoretically in a denaturing polyacrylamide gel (7% urea), and the gels exposed to an autoradiographic film.

(c) Individual DNA extraction

Total DNA was prepared from single adults by SDS-proteinase K digestion and phenol-chloroform extraction according to Kocher *et al.* (1989). DNA was resuspended in 50 μl of ultrapure water. PCRs were performed with 1 μl of 1/10 dilution DNA using conditions described in table 1.

(d) Wolbachia-PCR assay

The presence of the endosymbiotic bacterium *Wolbachia* has been assessed by PCR using specific primers. We have used primers amplifying the 16S ribosomal DNA of *Wolbachia* designed by O'Neill *et al.* (1992) and Rousset & De Stordeur (1994) (positions 76–99 and 1012–994 of *E. coli* 16S rDNA) to check for the presence of *Wolbachia* in individual *D. spinosissimae*. PCR was performed in a total volume of 12.5 μl , containing 1.5 μl of 10 $\times$ buffer, 0.92 μl of MgCl_2 (50 mM), 0.125 μl of dNTP mix, 0.23 μl of BSA (10 mg ml^{-1}), 0.05 μl of each primer (200 mM), 0.05 μl of *Taq* DNA polymerase, and 1 μl of DNA solution. DNA amplification was done in a Biometra thermal cycler: 5 min at 93°C followed by 37 cycles of 1 min at 93°C (slope = 1%), 1 min at 47°C , 1 min at 72°C , and 5 min at 72°C . After PCR, 5 μl of amplification product were separated electrophoretically on a 1.4% agarose gel and visualized by ethidium bromide staining and UV fluorescence. When a first PCR assay gave no amplification, a second PCR was performed to confirm the absence of *Wolbachia*.

(e) Statistical analyses of genetic data

The Genepop (v. 1.2) software (Raymond & Rousset 1995) was used to compute the exact test for Hardy-Weinberg

Table 1. *Microsatellite loci, class of repeat sequences, primers used and PCR conditions (GenBank accession nos. AF034416–AF034418)*

locus	repeat	primer	PCR conditions	
			annealing T°	MgCl ₂ (mM)
DR 06	(TC) TT (TC) ₇ TA	5'-CTC ATC TCT TCT TCT TAT CTC AG-3'	52	1.0
DS 24	(TC) (T) ₂₆ (GA) ₄ (TAGA) ₇	5'-CCC AGG AGA GCA GAG G-3'	50	1.2
		5'-CAG AGC GTT GAT AAA CCG-3'		
DS 50	(TA) ₉	5'-CCT ATT CCA TTT TCC CAG-3'	52	1.6
		5'-CAA AGA AGA AAT AAG AAA GGA-3'		
		5'-CAA GGC AAA TAT GAA AAA TG-3'		

Table 2. *Frequency of males, of females heterozygous for at least one microsatellite loci and of females infected by Wolbachia in the different populations (n is sample size)*

population	frequency of males	n	frequency of heterozygous females	n	frequency of females infected by <i>Wolbachia</i>	n
Queyras	0.29	38	0.96	27	0.00	27
Fontainebleau	0.21	158	0.78	50	0.00	50
Argenton	0.00	66	0.05	40	0.95	40
Olonne	0.00	6	0.00	6	1.00	6
Saint-Pierre	0.02	43	0.00	22	1.00	22
Houat	0.00	10	0.00	8	1.00	8
Erdeven	0.01	91	0.00	29	0.93	29
Santec	0.00	17	0.00	13	1.00	13
Erquy	0.01	98	0.00	40	0.87	40
Saint-Coulomb	0.04	54	0.00	20	1.00	20

equilibrium, for genotypic disequilibrium among pairs of loci, and for population differentiation. Haploid males of the bisexual populations have been excluded from the analysis. Diploid females of the asexual populations were homozygous and considered as haploid data.

3. RESULTS

(a) *Male frequency in *Diplolepis spinosissimae* populations*

Male frequencies are very different in coastal and continental populations. In all the coastal populations, and in Argenton (ca. 120 km from the nearest coastal populations, and considered hereafter as a coastal population), males comprise about 1.3% of individuals (5 males out of 385 individuals; see table 2). The frequencies of males in these populations are not significantly different across years. A different situation is found in the two continental populations studied (Fontainebleau and Queyras), where males represent 21 and 29% of individuals, respectively.

(b) *Heterozygosity*

The same contrast is also observed between the continental and coastal populations for the frequency of heterozygous females. In the latter, all but 2 out of 178 females were homozygote for the three loci (see table 2). As several different alleles are found in each populations (see table 3), the absence of heterozygotes is not due to the fixation of a single allele. Conversely, in the Fontainebleau and Queyras populations, 78 and 96% of females are

heterozygote for at least one locus, respectively. All tested males ($n=15$) carried a single allele.

(c) *Detection of *Wolbachia**

Wolbachia-specific primers reveal the presence of the symbiotic bacteria in all *D. spinosissimae* populations where males are extremely rare (see table 2). Almost all females of the coastal populations and Argenton were infected by *Wolbachia*; only 5% of the females (9 out of 178, including one of the two heterozygous females) were *Wolbachia*-free. Conversely, none of the 77 females and the 15 males tested from the Fontainebleau or the Queyras population were infected by *Wolbachia*.

(d) *Genetic structure of *Diplolepis spinosissimae* populations*

Among the six possible exact tests between the two continental populations (2 populations $\times$ 3 locus), only one showed a heterozygote deficiency ($p=0.0372$ for locus 24 in the Fontainebleau population). In these two populations, loci 6–24, and 24–50 were in genotypic disequilibrium (respectively, $p=0.00078$ and 0.01298 for the Queyras population; $p=0.00296$ and 0.01046 for the Fontainebleau population). The differentiation test between these two populations is highly significant for two out of the three loci ($p=0.00001$ for loci 6 and 50; genic and genotypic differentiation).

In the coastal populations (including Argenton), all but two females exhibited a single microsatellite allele at each locus, and hence were treated as haploid data. For the two

Table 3. Observed allele frequency distributions by locus and population (number of individuals tested)

locus allele	continental populations		coastal populations (including Argenton)							
	Queyras (27)	Fontainebleau (50)	Argenton (40)	Olonne (6)	Saint-Pierre (22)	Houat (8)	Erdeven (29)	Santec (13)	Erquy (40)	Saint-Coulomb (20)
locus DR 06										
59	0.22	0.924	—	—	—	0.875	0.500	1.000	1.000	1.000
61	—	—	0.211	—	—	—	—	—	—	—
63	0.36	0.076	0.789	—	1.000	0.125	0.286	—	—	—
65	0.32	—	—	—	—	—	0.214	—	—	—
67	0.10	—	—	—	—	—	—	—	—	—
locus DS 24										
69	0.537	0.500	—	—	—	—	—	0.538	0.343	0.050
73	—	—	—	—	0.111	—	—	—	—	—
81	0.444	0.500	1.000	—	0.889	1.000	1.000	0.385	—	—
85	—	—	—	—	—	—	—	0.077	0.657	0.950
87	0.019	—	—	—	—	—	—	—	—	—
01	—	—	—	1.000	—	—	—	—	—	—
locus DS 50										
44	0.318	—	—	—	—	—	—	—	—	—
46	0.568	0.500	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
48	0.068	0.143	—	—	—	—	—	—	—	—
50	0.045	0.357	—	—	—	—	—	—	—	—

polymorphic loci (locus 50 was unvariant in all coastal populations), we observed significant differences in allelic frequency between populations in 33 out of the 36 possible tests (seven populations, two loci differentiation between populations taken pairwise). The large proportion of private alleles (four out of the eight alleles were found in only one population; see table 3) indicate a very small amount of gene flow among populations. The two populations on the Channel coast (Erquy and Saint-Coulomb) exhibited the same genotypes. The populations on the Atlantic coast (Erdeven, Saint-Pierre, Houat and Olonne) exhibit six different genotypes. The Santec population seems to be intermediate between the two sets, with one allele in common with Channel populations, the other ones with the Atlantic populations.

4. DISCUSSION

(a) Reproductive system of *Diplolepis spinosissimae*

The three data sets (frequency of males, frequency of females heterozygous at one or more locus, and the presence/absence of *Wolbachia*) allow distinction between two different types of populations (tables 2 and 3).

In two continental populations (Queyras and Fontainebleau), the percentage of males lies between 20 and 30%. Females are highly heterozygous at the three loci studied, while males present only one band (one allele). These populations are entirely *Wolbachia*-free. Consequently, they exhibit a standard haplodiploid system: hemizygous males are produced by arrhenotoky, and diploid females by syngamy; the genetic structure is in agreement with biparental, sexual reproduction.

In the remaining eight populations (i.e. all the coastal populations and the continental population of Argenton), males are very rare (one or two individuals in each sample). Females are always homozygous for the three

loci (except two females from Argenton) and 87–100% of them are infected by the endosymbiotic bacterium *Wolbachia*. In these populations, we consider that *D. spinosissimae* reproduces by *Wolbachia*-induced thelytoky, as indicated by the homozygosity of females (as expected in the case of *Wolbachia*-induced thelytoky; Stouthamer & Kazmer 1994). Although we have not formally proved the role of *Wolbachia* (we are currently trying to cure some females of the bacterium using antibiotic treatment, but being a phytophagous insect, this manipulation is technically difficult), we consider that the exact congruence of the three data sets (sex ratio, heterozygosity, and presence/absence of *Wolbachia*), showing strict and parallel variation in the different populations is a strong argument for concluding that *Wolbachia* is responsible for the observed thelytoky. Probably as in *Trichogramma* (Stouthamer & Kazmer 1994), diploidy is restored by gamete duplication and daughters are homozygous, sharing the same genotype as their mother.

A few individuals do not correspond to the situation described above. Rare males and uninfected females are found in the infected populations. We hypothesized that both are due to loss of *Wolbachia* by incomplete maternal transmission (Subbarao *et al.* 1974; Fine 1978; Barr 1980), environmental curing agents (Hoffman & Turelli 1988; Stevens 1989) or prolonged larval diapause (Perrot-Minot *et al.* 1996).

Two heterozygous females have also been found in the infected populations, one of them being infected by *Wolbachia*. If sperm of the rare *D. spinosissimae* males can effectively fertilize eggs (as in *Trichogramma* spp.; Stouthamer & Kazmer 1994), the infected heterozygous females could result from mating between an infected female and one of the rare males (fertilization of an infected egg), while the uninfected heterozygous female could be due to (i) the loss of *Wolbachia* in one daughter of

a similar mating; or (ii) the mating of an uninfected female with one of the rare males (fertilization of an uninfected egg). The other hypotheses to account for heterozygosity are mutation or immigration from an uninfected population. However, detection of a mutation at the heterozygous state in an infected female is highly unlikely, as this state can only be observed at the generation where mutation occurs (the offspring being homozygous as a consequence of *Wolbachia* infection). The immigration hypothesis is opposed to the observation that all populations in the area are infected, and by the identity of the alleles of those heterozygous that do not differ from those found in this population. Moreover, if gene flow occurs between infected and uninfected populations, modelling predicts rapid change from the uninfected to the infected state (see below).

If experimentally confirmed, the functionality of those rare males would be an original feature of *D. spinosissimae*, as the males (obtained by antibiotic treatment) of two species with highly infected populations have been found to be non-functional (Zchori-Fein *et al.* 1992, 1995). In *Trichogramma* spp., such males can effectively fertilize eggs, but in those species, infected individuals formed a minority among the uninfected individuals. However, the case of *D. spinosissimae* is not inconsistent with the hypothesis that male fertility is progressively lost in *Wolbachia*-infected species through accumulation of deleterious mutations (Zchori-Fein *et al.* 1992). Infection of *D. spinosissimae* by *Wolbachia* could be too recent to prevent males' fertility, but sufficiently ancient for the bacterium to spread to almost all individuals of the populations.

(b) Genetic structure of *Diplolepis spinosissimae*

Genetic markers showed that infected populations exhibit a dramatic loss in genetic variability compared to bisexual, uninfected populations (1–3 different genotypes in each coastal populations versus 46 and 26 different genotypes in the Queyras and Fontainebleau populations, respectively). The small genetic diversity observed in coastal populations can be explained as follows. First, all heterozygous genotypes are excluded by the gamete duplication. Moreover, thelytoky excludes any association of alleles from different loci that were not associated during the spread of *Wolbachia*. Finally, with males being too rare and too similar genetically to females to induce genetic flow, each infected genotype becomes an independent clonal line. In addition, genetic drift in isolated populations could have led to the loss of several of those genotypes, a process increased by a high mortality (usually more than 80%) due to parasitism by 13 different parasitoid species (Plantard 1997).

Despite most genotypes being homozygous, there is more than one infected genotype in the coastal populations (2.25 ± 0.707 per population, $n=8$). This diversity can be maintained or generated by several non-exclusive mechanisms. (1) Multiple infection: direct evidence for horizontal transmission appears not to have been reported, but such a process is likely to occur as phylogenetically distant hosts can share a common *Wolbachia* strain, while closely related hosts have been found to be infected by very distant *Wolbachia* strains (O'Neill *et al.* 1992; Rousset *et al.* 1992b; Stouthamer *et al.* 1993; Werren *et al.*

1995; Schilthuizen & Stouthamer 1997). However, infection events are usually considered to be rare (one infection over 1–2 million years for *Drosophila melanogaster* according to Rousset & Solignac (1995)). (2) Neomutation in thelytokous lines. However, such a process would not explain why numerous alleles are common both to infected and uninfected populations. (3) A unique infection event, but the first females infected produce as many homozygote genotypes as gamete types (no more than two per locus). This hypothesis can not explain the four and five different alleles found in locus 6 and 24, respectively. (4) As the infection spreads, mating of infected females with males could diversify the thelytokous genotypes (cf. *Trichogramma* spp.; Stouthamer & Kazmer 1994). Such a process is especially likely to occur during the initial generations following the infection event, as males are still numerous and genotypically diverse.

The strong differentiation observed among the populations suggests that gene flow between coastal populations is low. However, even a very low migration rate of females from infected to uninfected populations (perhaps a unique event?), could have been sufficient to make all those coastal populations definitively thelytokous. Because of the advantage of the thelytokous form (not 'wasting' eggs in producing males like the arrhenotokous form), infection should spread rapidly in the populations (Stouthamer 1993). Rather than environmental factors, the larger connectivity of coastal populations (compared to continental populations) due to the distribution of the host-plant (roughly linearly distributed, i.e. along the coastline) could be responsible for the infection of all those populations.

At a larger scale, gene flow from coastal to continental populations (at least in this direction) appears to be non-existent, otherwise the latter would probably be infected. Continental populations are more isolated, and would therefore be less exposed to invasion by infected females and also more prone to the random loss of *Wolbachia* infection. The large area without any *Rosa pimpinellifolia* bushes may constitute an important barrier for the dispersion of *D. spinosissimae* and gene flow among those two sets of populations.

Those new elements favour the role of *Wolbachia* in inducing thelytoky in a Cynipidae species, and provide new insights into parthenogenesis in this family. In particular, investigations of species with alternation of generations (Cynipini and Pediaspini), where *Wolbachia* could play an important role, is urgently needed.

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