Variability Within the Seychelles Cytoplasmic Incompatibility System in *Drosophila simulans*

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Manuscript received April 5, 1995
Accepted for publication July 22, 1995

ABSTRACT

In *Drosophila simulans*, we described a cytoplasmic incompatibility (CI) system (Seychelles) restricted to insular populations that harbor the mitochondrial type SiI. Since then, these populations have been shown to be heterogeneous, some being infected by one Wolbachia genetic variant only (wHa), while others are infected simultaneously by wHa and by another variant (wNo) always found in association with wHa. We have experimentally obtained two *D. simulans* strains only infected by the wNo variant. This variant determines its own cytoplasmic incompatibility type. In particular, the cross between wNo-bearing flies and wHa-bearing ones is bidirectionally incompatible. The Seychelles CI type, *stricto sensu*, is distinguished by being determined by the simultaneous presence of two Wolbachia variants that we found to be mutually incompatible. In addition, we observed incomplete maternal transmission of the Wolbachia.

WOLBACHIA are endosymbiotic bacteria of arthropods, present in the germ cells of both sexes. They are maternally inherited and responsible for a variety of alterations of sexuality and reproduction, *e.g.*, feminization, thelytokous parthenogenesis and cytoplasmic incompatibility (reviewed in SOLIGNAC and ROUSSET 1993). Molecularly, they are currently characterized through sequence variations of their 16S rRNA gene (WEISBURG *et al.* 1991; BREEUWER *et al.* 1992; O'NEILL *et al.* 1992; ROUSSET *et al.* 1992; STOUTHAMER *et al.* 1993) and more recently using DNA sequences from *ftsZ*, a rapidly evolving bacterial cell-cycle gene (HOLDEN *et al.* 1993; WERREN *et al.* 1995).

In *Drosophila simulans*, four Wolbachia variants are known, related to different cytoplasmic incompatibility (CI) types (Table 1). Typically, incompatibility occurs when infected males are crossed with uninfected females or when females are infected by a different bacterial variant. In an incompatible cross, mating and oviposition occur normally, but a large proportion of eggs fails to hatch. The Wolbachia are not present in mature sperm, but they somehow render the sperm incapable of a successful fertilization after entry into an incompatible cytoplasm (O'NEILL and KARR 1990).

The CI type R, first described in a Californian population (Riverside) by HOFFMANN *et al.* (1986), corresponds to the presence of a single Wolbachia variant (wRi). This CI type as well as the uninfected type (ϕ) are widespread across the world in *D. simulans* populations that possess the SiII mtDNA type (HOFFMANN and TURELLI 1988; MONTCHAMP-MOREAU *et al.* 1991). Studies of sequence variations within the SiII mtDNA type have shown that all infected flies (type R) possess the same mtDNA allele, whereas uninfected flies are polymorphic (HALE and HOFFMANN 1990; TURELLI *et al.* 1992).

The CI type M is related to the presence of a rare Wolbachia variant called wMa and was only observed in Mont-d'Ambre (NIGRO 1991), a strain from Madagascar, whose mtDNA type is SiIII. The incompatibility relationships of this type are still under debate (see Table 1).

We described the CI type S in SiI mtDNA-type populations of *D. simulans* from Indo-Pacific islands (MONTCHAMP-MOREAU *et al.* 1991). From some single flies belonging to this CI type S, ROUSSET and SOLIGNAC (1995) found that PCR products of the bacterial 16S rRNA gene showed heterogeneity detectable by directed sequencing and confirmed by cloning two different sequences previously described (ROUSSET *et al.* 1992). They attributed this heterogeneity to a double infection by two different Wolbachia genetic variants (wHa and wNo). Indeed, the strains that we previously classified into the CI type S are of different possible configurations. In the present work, we found only bi-infected (wHa + wNo) flies in the reference strain (Seychelles) for this CI type. Nevertheless, from four isofemelle lines, ROUSSET and SOLIGNAC (1995) observed two bi-infected lines and two monoinfected by wHa. In the Nouméa strain (from New Caledonia), this polymorphism is recovered within mass breed (the present work) as well as between isofemale lines (ROUSSET and SOLIGNAC 1995). Last, strains from Hawaii and French Polynesia exhibit only flies monoinfected by wHa

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TABLE 1

Cytoplasmic incompatibility types and related Wolbachia variants

Cytoplasmic incompatibility type	Geographical distribution	MtDNA type[a]	Wolbachia variant[b,c]	Relationship of incompatibility					
				Males	Females				
					ϕ	R	M	H	S
Uninfected strains (ϕ)	Widespread	SiII	none	ϕ	C	C	C	C	C
Riverside (R)[d]	Widespread	SiII	wRi	R	I	C	±C or I	I	I
Mont d'Ambre (M)[e]	Madagascar	SiIII	wMa	M	I or C	±C or C	C	C	C
Hawaii (H)[f]	Polynesia	SiI	wHa	H	I	I	I	C	C
Seychelles (S)[g]	Seychelles; Nouméa[h]	SiI	(wHa + wNo)	S	I	I	I	±C	C

C, compatible cross (unhatched eggs < 20%); I, incompatible cross (unhatched eggs > 20%); ±C, 20% < unhatched eggs < 80%.

[a] BABA-AÏSSA *et al.* (1988).

[b] ROUSSET *et al.* (1992).

[c] ROUSSET and SOLIGNAC (1995).

[d] HOFFMANN *et al.* (1986).

[e] NIGRO (1991) describes the males infected by wMa as incompatible with uninfected females, whereas ROUSSET and SOLIGNAC (1995) found the cross compatible. Their results are also in discrepancy about the relationship between the M and R CI types.

[f] O'NEILL and KARR (1990).

[g] MONTCHAMP-MOREAU *et al.* (1991).

[h] The isofemale line from Nouméa described by ROUSSET *et al.* (1992) as infected by wNo was also infected by wHa (ROUSSET 1993).

(O'NEILL *et al.* 1992; ROUSSET *et al.* 1992; ROUSSET and SOLIGNAC 1995; S. BASMACIOGULLARI, personal communication). We consider that the wHa-monoinfected strains, which show a partial unidirectional incompatibility with the bi-infected strain Seychelles (MONTCHAMP-MOREAU *et al.* 1991), define a fourth CI type (type H) initially described in a *D. simulans* strain from Hawaii (O'NEILL and KARR 1990). No fly infected only by the bacterial variant wNo has ever been observed.

In the present work, through a backcross experiment, we isolated the Wolbachia genetic variant wNo from a bi-infected strain (Nouméa). This confirmed the presence of two Wolbachia as assumed by ROUSSET and SOLIGNAC (1995). We demonstrated that wNo is bidirectionally incompatible with the wHa variant. Therefore, flies belonging to the Seychelles CI type (*stricto sensu*) are infected by two Wolbachia variants (wHa and wNo) that are mutually incompatible.

MATERIALS AND METHODS

Strains: The strains used are listed in Table 2. We should emphasize that at the beginning of our work (April 1991) both Seychelles and Nouméa strains were thought to be monoinfected. Consequently the relative proportion of mono- and bi-infected flies in the Nouméa strain was unknown.

Backcrosses: To compare the gradual change of infection in different crossing designs, bi-infected Nouméa females were backcrossed following three different crossing schemes, leading to three backcross series (with two replicates, A and B, each). They are detailed in Figure 1 and correspond to generations F_1 to F_n. The first one (BC1) was the infected control backcross with males from the Nouméa strain. The second (BC2) was a uninfected control backcross with uninfected males (Nouméa-TC), but without nuclear replacement. In the third backcross series (BC3), the infected Nouméa strain was subjected to chromosome replacement by means of successive backcrosses with males from a naturally uninfected strain, Nasr'allah. This procedure was described in MERÇOT *et al.* (1995).

Intraline crosses: From each replicate of the different backcross series, intraline crosses were set up with males and females from the 11th generation of backcross series. These were used to setup lines designated as R1A, R1B, R2A, R2B, R3A and R3B (Figure 1).

Incompatibility tests: Tests were performed at 25°. Thirty 4-or 5-day-old virgin females were allowed to mate for 8 hr with 40 3-day-old virgin males in a bottle with standard axenic medium. Flies were transferred for oviposition on fresh axenic medium supplemented with animal charcoal powder. After 24 hr, the adults were discarded. Eggs (hatched and unhatched) were counted ≥24 hr after removal of the adults. Crosses with >80% unhatched eggs were considered as incompatible and those with <20% unhatched eggs as compatible. Incompatibility status of the BC series and R lines was followed by means of two crosses carried out simultaneously at various generations during the experiment: male incompatibility was tested in crosses with standard uninfected females, while female compatibility was tested with regard to the bi-infected males from the Seychelles strain.

Male infection level: After fixation and DNA staining by incubation in 4′,6 diamidine-2-phenylindole dihydrochloride (DAPI, Sigma), testes were observed on an Olympus microscope equipped for epifluorescence with a ×100 Zeiss objective as described by BRESSAC and ROUSSET (1993). The infection level, defined as the frequency of sperm cysts that are infected in the testes of 2-day-old males, was determined from 10 cysts per male. The observations were made between the 24th and 28th generations of BC series and the 13th and 17th generations of the R lines.

Egg infection: The presence of Wolbachia was detected by direct epifluorescence observation of DAPI-stained fixed eggs according to the protocol established by KARR and ALBERTS (1986). Observations were made on 10 unfertilized eggs.

PCR conditions: Total DNA was extracted from the ovaries of individual females according to the protocol of KOCHER *et*

TABLE 2

Description of strains

Strains	Origin	Collection year	n^a	mtDNA type	Type of infection detected	Characteristics
Seychelles	Seychelles	1981	30	SiI	(wHa+wNo)	Reference for CI type S
Nouméa	New-Caledonia	1989	28	SiI	(wHa+wNo); (wHa)[b]	Compatible with Seychelles
Nouméa-Ha	Laboratory	—	—	SiI	(wHa)	A replicate of the Nouméa strain that lost the bi-infected flies
Nouméa-TC	Laboratory	—	—	SiI	Uninfected	From Nouméa, after treatment with tetracycline[c]
Nasr'allah	Tunisia	1983	50	SiII	Uninfected	Our standard uninfected strain
Riverside	California	1984	30	SiII	(wRi)	Reference for CI type R
Hawaii 1	Hawaii	1985	1	SiI	(wHa)	Reference for CI type H

[a] Number of founder females.
[b] The initial proportion of bi-infected and monoinfected flies was unknown in this strain.
[c] The Nouméa strain was treated during three generations with tetracycline (an antibiotic that kills *Wolbachia*) following the procedure in HOFFMANN *et al.* (1986).

al. (1989). The 16S ribosomal subunit DNA sequence was amplified as described in ROUSSET *et al.* (1992). The specific primers for Wolbachia used in the PCR were as follows: 5'TTGTAGCCTGCTATGGTATAACT3' (76–99) and 5'GAATAGGTATGATTTTCATGT3' (1012–994).

Restriction fragment length polymorphism: The restriction fragment length polymorphisms (RFLPs) of PCR-amplified 16S rDNA fragments were used to determine the nature of the infection. The wNo sequence contains an *Asn*I restriction site absent in the wHa sequence (ROUSSET *et al.* 1992). The PCR product was digested by the *Vsp*I enzyme (an isoschizomer of *Asn*I) at 37° during 3 hr. The PCR product from wNo yields two fragments of 516 and 361 pb, while the PCR product from wHa gives a fragment of 878 pb.

RESULTS

For each backcross series and R lines, we successively present: the incompatibility behavior over 60 generations (BC series) or 49 generations (R lines), data on infection levels, obtained by DAPI staining, and PCR/RFLP typing results, which permitted us identification of Wolbachia types present by the end of the experiment.

Infected control backcross: In BC1A and BC1B series, as well as in R1A and R1B lines, males remained incompatible with uninfected standard females and females remained compatible with bi-infected Seychelles males (Figure 2). This was in accordance with the DAPI-staining results, which revealed a high level of infection in males from the two BC series and the two R lines (Figure 5). Wolbachia PCR/RFLP typing showed that in these four cases a large majority of flies were bi-infected, a few being infected only by wHa (Table 3).

Uninfected control backcross: The BC2A and BC2B series showed a divergent evolution of their incompatibility characteristics (Figure 3): in the BC2B series, males became compatible with uninfected females, in conjunction with the decrease of the female compatibility with Seychelles males. This can be explained by a decrease of the infection level in this series, because the frequency of infected cysts observed in males between the 24th and the 28th generations was moderate (Figure 5) until its complete disappearance at the end of the experiment (Table 3). In the BC2A series, the male incompatibility level fluctuated between 35 and 80%, whereas the female compatibility with Seychelles males steadily decreased and disappeared (Figure 3). DAPI staining showed that between the 24th and the 28th generation, the infection level was still high (Figure 5). The PCR/RFLP results indicated the reason for this discrepancy between male and female incompatibility (Table 3). In fact, in this series only uninfected or wHa monoinfected flies were observed by the last generations; this implies that both types of females were incompatible with bi-infected Seychelles males whereas the fraction of wHa monoinfected males was probably responsible for the intermediate levels of male incompatibility observed in crosses with uninfected females.

The progressive infection loss may result from the selection of uninfected cytoplasmic lineages present at low frequency at the beginning of the experiment and/or from incomplete maternal transmission of Wolbachia. To check the possibility of incomplete maternal transmission, three extra backcross series were established (BC2C, BC2D and BC2E). The protocol was as for the BC2A and BC2B series (Figure 1) except that the initial cross involved a single infected Nouméa-Ha female (the infection of which by wHa was confirmed by PCR/RFLP) and two Nouméa-TC males (*i.e.*, uninfected). During four generations, 40 females of each series were backcrossed with 50 Nouméa-TC males. At the fifth generation, 118 isofemale lines were founded from 39 BC2C females, 40 BC2D females and 39 BC2E females, each crossed with a Nouméa-TC male. The presence or absence of Wolbachia was tested by PCR on the 118 foundresses. When the PCR was negative, the absence of infection in an isofemale line was further tested in three ways: (i) PCR on a sample of four daugh-

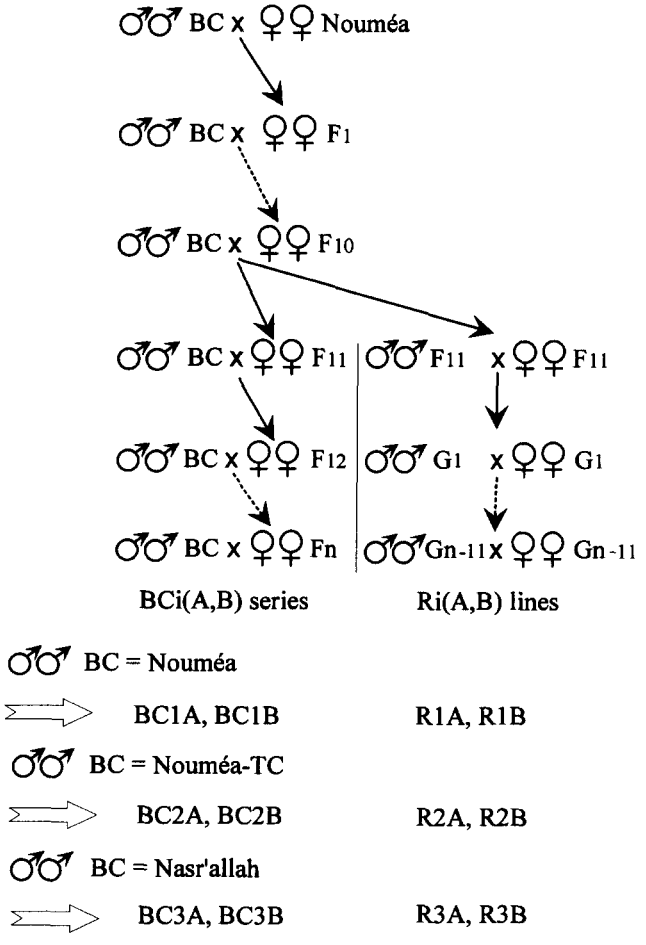

FIGURE 1.—Protocol of the backcross experiment. Three series of backcrosses (generations F_1 to F_n) were performed starting with Nouméa females. Males used in backcrosses were from Nouméa (control backcross: BC1), from Nouméa-TC (backcross with uninfected males without genome replacement: BC2) or from Nasr'allah (backcross with uninfected males and genome replacement: BC3). Two replicates (A and B) were performed in each backcross series. Intraline crosses were set up with males and females from the 11th generation of backcross replicate series to give the R lines (generations G1 to Gn-11). For all crosses, 40 virgin females (4- or 5-day-old) were allowed to mate, for 36hr, with 50 virgin males (3- or 4-day-old) and to lay eggs in a bottle with standard axenic medium (DAVID 1962). The experiment was performed at 25°.

ters, (ii) direct epifluorescence observation of DAPI-stained eggs laid by females from the first generation of isofemale lines, and (iii) test of cytoplasmic incompatibility between males from the second or third generation of isofemale lines and standard uninfected females. Results showed that among the 118 lines tested, 23 were devoid of Wolbachia: two isofemale lines from BC2C, nine from BC2D and 12 from BC2E gave negative PCR and DAPI-staining results, as well as <20% unhatched eggs in the cytoplasmic incompatibility test (from 2.5 to 19.5%, with a mean of 7.9%). In contrast, male incompatibility test performed on 12 lines among the PCR-positive ones gave between 45 and 99.5% of unhatched eggs, with a mean of 82.9%. Five successive

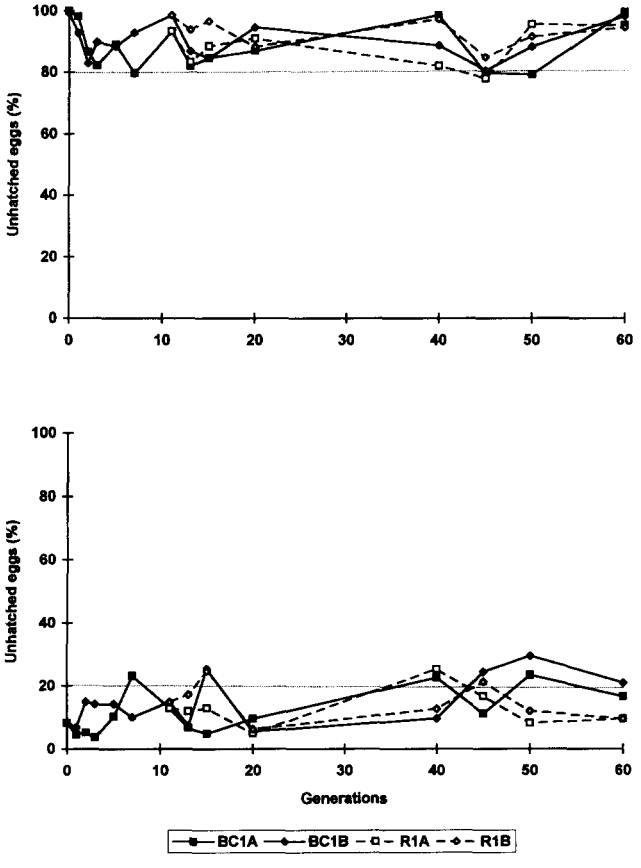

FIGURE 2.—Infected control backcross. Behavior of (top) male incompatibility with uninfected females and (bottom) female compatibility with bi-infected Seychelles males in the two infected control backcross series (BC1A, BC1B) and the two intraline cross lines (R1A, R1B). The number of generations corresponds to the generations of backcrossing.

backcrosses from infected Nouméa females with uninfected Nouméa males therefore produced 19.5% of uninfected flies, indicating incomplete maternal transmission of Wolbachia.

The intraline crosses, carried out at the 11th generation of backcrossing (Figure 1), was supposed to restore selection in favor of infected eggs, leading to an increase in the infection level if Wolbachia are still present. In fact, in R2A and R2B lines, the initial incompatibility status of males and females was restored (Figure 3), suggesting that bi-infected flies were still present in both lines after 11 backcross generations. This was confirmed by PCR/RFLP analysis (Table 3).

Nuclear replacement backcross: In BC3A and BC3B series, a fast decrease of male incompatibility with uninfected females was observed (30 and 25%, respectively, of embryo lethality by generation 11), which correlated with a decreasing compatibility of females with Seychelles males (Figure 4). This suggests a decrease of the infection level and is in accordance with the low percentage of infected cysts observed in males between the 24th and the 28th generations (Figure 5) and with the negative results of PCR by the last generations (Table 3).

The intraline crosses performed from generation 11

TABLE 3

Type of infection detected in the BC series, R lines and reference strains at the end of the experiment

Series, lines and strains[a]	Nuclear genome	mtDNA type	Wolbachia variant detected (frequency)				
			n	None	wHa	wNo	(wHa+wNo)
BC1A	Nouméa	SiI	15	—	—	—	1.00
BC1B	Nouméa	SiI	16	—	0.25	—	0.75
R1A	Nouméa	SiI	16	—	—	—	1.00
R1B	Nouméa	SiI	16	—	0.38	—	0.62
BC2A	Nouméa	SiI	24	0.67	0.33	—	—
BC2B	Nouméa	SiI	15	1.00	—	—	—
R2A	Nouméa	SiI	15	—	—	—	1.00
R2B	Nouméa	SiI	15	0.07	—	—	0.93
BC3A	Nasr'allah	SiI	15	1.00	—	—	—
BC3B	Nasr'allah	SiI	15	1.00	—	—	—
R3A	Nasr'allah	SiI	20	—	—	1.00	—
R3B	Nasr'allah	SiI	20	0.15	—	0.85	—
Nouméa[b]	Nouméa	SiI	16	0.06	0.94	—	—
Nouméa-TC	Nouméa	SiI	10	1.00	—	—	—
Nasr'allah	Nasr'allah	SiII	12	1.00	—	—	—
Seychelles	Seychelles	SiI	15	—	—	—	1.00

n, number of individual females assayed, from the 58th to 61st generations for the BC series and from the 47th to the 50th generations for the R lines.

[a] We have analyzed the experimental stocks used for the backcrosses (Nouméa, Nouméa-TC and Nasr'allah) or for the CI test (Seychelles). The analysis was performed at the same time as for the BC series and R lines.

[b] This replicate of the Nouméa strain has lost the bi-infected flies. Its monoinfection by wHa was later confirmed (D. POINSOT, personal communication). This replicate is the origin of the Nouméa-Ha strain.

restore a strong male incompatibility with uninfected females, with >80% of eggs unhatched at generation 49 (Figure 4). This appears to result from an increase in infection level because the percentages of infected cysts observed in R3A (50%) and R3B (38%) was higher than those observed in BC3A (18%) and BC3B (24%). Nevertheless the difference was only significant between R3A and BC3A (Wilcoxon's test: $\epsilon_w = 1.971$; $P < 0.05$). Unexpectedly, in spite of infection in these lines, R3A and R3B females remained incompatible with Seychelles males. PCR typing revealed the presence of only the wNo variant (Table 3) by the last generations. These results show that wNo-bearing males are able to cause cytoplasmic incompatibility in crosses with uninfected females whereas wNo-bearing females are incompatible with males of the Seychelles strain which are infected by both wNo and wHa.

Relationship between infection and male incompatibility levels: Figure 5 shows the percentages of unhatched eggs obtained in male incompatibility tests against the frequencies of infected spermatocysts for males of each BC series and R line. We observed a significant correlation between the two traits (Spearman's test: $r = 0.839$; 10 d.f.; $P < 0.01$). Despite between and within line differences in the nature of infection (wHa, wNo, or wHa + wNo), as well as in the nuclear genome (Nouméa or Nasr'allah), the incompatibility level appears related to the frequency of infected cysts.

Characterization of the CI type N related to the wNo Wolbachia: To establish the incompatibility relationships between flies bearing the wNo bacteria and the different CI types already described in *D. simulans*, we performed incompatibility tests (Table 4). Both R3A and R3B males were incompatible with uninfected females as well as with females from the reference strains for infection by wHa (Hawaii) and wRi (Riverside). R3A and R3B females were incompatible with males respectively infected by wHa (Hawaii) and wRi (Riverside). wNo is therefore responsible for a CI type, which we called N, that is bidirectionally incompatible with the CI types H and R. With respect to the incompatibility of monoinfected males with monoinfected and uninfected females (Table 4), the average percentage of unhatched eggs produced by wNo-bearing males (R3A + R3B: 80.0%) was significantly lower than those obtained with the wHa-bearing males (Nouméa-Ha + Hawaii: 93.6%, $\chi^2 = 193.7$; 1 d.f.; $P < 0.001$) and the wRi-bearing males (Riverside: 96.1%, $\chi^2 = 199.3$; 1 d.f.; $P < 0.001$). Considering these incompatibility relationships between the bi-infected flies and the monoinfected flies, the fact that only wNo flies were recovered from R3A and R3B series strongly suggests that bi-infected and wHa-bearing flies had disappeared from BC3A and BC3B by the 11th generation of backcross (unlike in BC2A and BC2B series).

In other respects, we checked that the Nouméa-Ha strain, monoinfected by wHa, was bidirectionally compatible with Hawaii, the reference strain for the CI type H (Table 4). This indicates that the wHa variant harbored by both the bi-infected strains of the CI type S

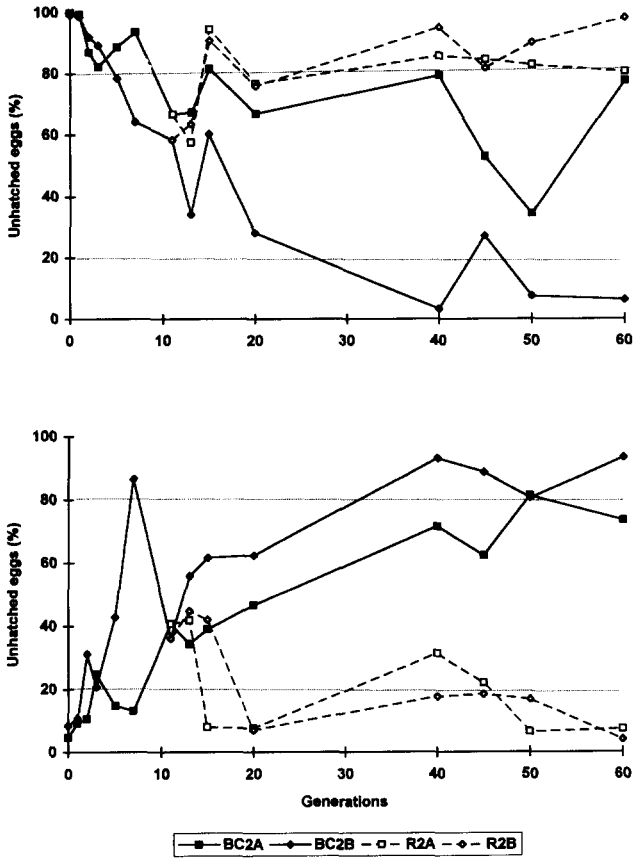

FIGURE 3.—Uninfected control backcross. Same as Figure 2 for the two uninfected control backcross series without genome replacement (BC2A, BC2B) and the two related intraline cross lines (R2A, R2B).

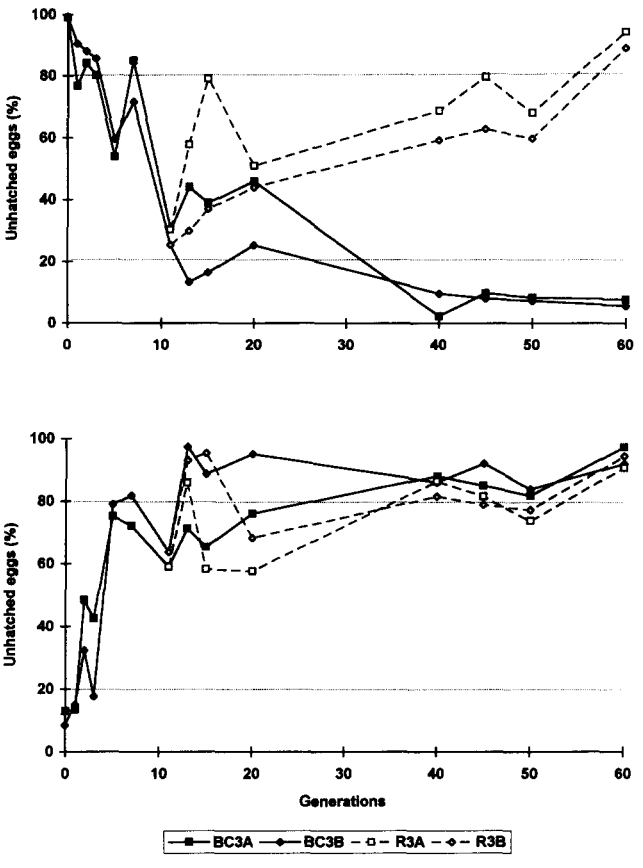

FIGURE 4.—Nuclear replacement backcross. Same as Figure 2 for the two backcross series with nuclear replacement (BC3A, BC3B) and the two related intraline cross lines (R3A, R3B).

and the monoinfected CI type H, and which are identical in the sequence for the 16S rRNA gene, are also identical with respect to the CI type they induce. Likewise, the Nouméa-Ha strain is bidirectionally incompatible with the R3A and R3B lines as well as with Riverside.

In contrast to the usually low percentages of unhatched eggs recovered in crosses between uninfected males and females from naturally infected strains (5.3, 1.7 and 4.0% with Seychelles, Nouméa and Riverside females, respectively), the percentage of unhatched eggs reached 37 and 15.7% in crosses between Nasr'allah males and respectively R3A and R3B females (Table 4). Similarly, intraline crosses as well as between-line crosses of R3A and R3B resulted in >25% of unhatched eggs. These observations suggested that the association of the wNo Wolbachia with the R3A and R3B genome might induce physiological defects in the flies. To test this hypothesis, we treated both R3A and R3B lines with tetracycline and compared the percentages of unhatched eggs laid by infected and treated females in crosses with the uninfected Nasr'allah males. These percentages (from samples of 300 eggs) were the following: 19.2% (R3A), 23.7% (R3ATC), 19.7% (R3B), 25.7% (R3BTC), and no difference was found between them ($\chi^2 = 5.005$; 3 d.f.; NS) Therefore, wNo was not responsible for the high level of unhatched eggs within these lines.

Incompatibility relationships between mono- and bi-infected strains: Bi-infected (wHa + wNo) Seychelles males appeared completely incompatible with wNo monoinfected females from lines R3A and R3B because the percentages of unhatched eggs observed in these crosses (92.7–93.3%) were similar to those obtained in crosses with uninfected females or with females infected by wRi (Table 4). In contrast, these males exhibited a significantly lower incompatibility level (68.4 and 63% of unhatched eggs) in crosses with wHa monoinfected females from the Nouméa-Ha and Hawaii strains. The same results were obtained by using bi-infected R1A males instead of Seychelles males. On the other hand, bi-infected Seychelles and R1A females were compatible with males infected by wHa as well as with males infected by wNo. These results show that the incompatibilities induced by wHa and wNo are independently expressed in bi-infected flies. In both cases the wNo-induced male incompatibility appeared lower than that of wHa.

DISCUSSION

Incomplete vertical transmission of the symbionts: The first argument for incomplete maternal transmission of Wolbachia is the gradual decrease of infection

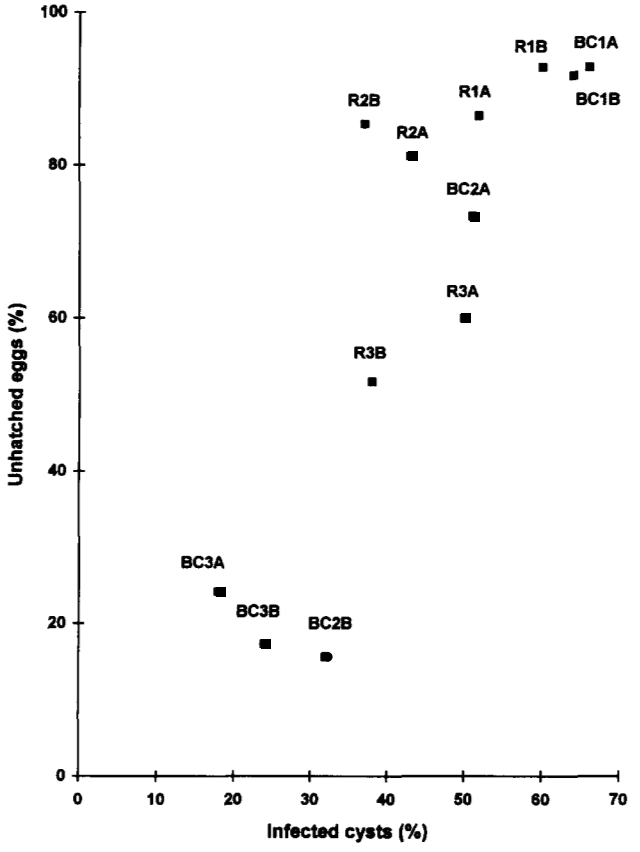

FIGURE 5.—Relationship between percentage of infected cysts in the BC series and R lines males and percentage of unhatched eggs in crosses between uninfected females and males from the 20th generation of BC series and ninth generation of R lines.

in BC2 and BC3 series. In BC1 series, females were crossed at each generation with males from the Nouméa infected stock. If some of the eggs produced by BC1 females were not infected, most of them must have been killed by sperm from infected males, which tended to maintain the infection through successive generations, as actually observed. By contrast, in BC2 and BC3 series, females were crossed with uninfected males. If uninfected eggs were produced, they therefore survived and the frequency of infected individuals was expected to decrease over generations. The percentage of unhatched eggs obtained in male incompatibility tests correlated with the infection level measured as the percentage of infected cysts. This is consistent with DAPI staining data from BRESSAC and ROUSSET (1993) who observed that the decrease of incompatibility level in ageing infected males is related to a decrease in the proportion of infected cysts. An alternative hypothesis could be that the loss of infection results only from selection of uninfected Nouméa females present at a low proportion in the beginning of the backcross experiment. Two observations indicate that this is not the case. First, in crosses with uninfected males, the fecundity of females from bi-infected (R1A) or monoinfected (Nouméa-Ha; R3A) strains is the same than that of their related cured females (mean ratio = 0.98; D. POINSOT, personal communication) and they produce, at least, as many adult offsprings (D. POINSOT in BROOKFIELD 1995). Second, the complement BC2C, BC2D and BC2E series started with females whose infection status had been checked and produced an average of 19.5% of uninfected flies after five generations of backcross with uninfected males (Nouméa-TC). It can therefore be ascertained that the maternal transmission of the infection is incomplete in the Nouméa strain. Such result is in agreement with the observations of HOFFMANN *et al.* (1990) who recovered 1–3% uninfected progeny from field collected females infected by the wRi variant. Another, not exclusive, hypothesis for infection loss is that paternal factors are necessary for its maintenance via the maternal lineage. Wolbachia are eliminated in the waste bag during spermatogenesis (BRESSAC and ROUSSET 1993) and are rarely (HOFFMANN and TURELLI 1988) if not at all (MONTCHAMP-MOREAU *et al.* 1991) paternally transmitted. However, the mature sperm has been modified by the symbionts because it "kills" uninfected embryos. In addition to this deleterious effect

TABLE 4

Characterization of the CI type N related to the wNo Wolbachia

	Females							
Males	Seychelles (wHa+wNo)	R1A (wHa+wNo)	Nouméa-Ha (wHa)	Hawaii (wHa)	R3A (wNo)	R3B (wNo)	Riverside (wRi)	Nasr'allah (ϕ)
Seychelles	22.6[a]	20.7	68.4	63.0	92.7	93.3	96.3	99.3
R1A	27.1	7.3	66.4	62.7	97.7	91.5	98.3	99.0
Nouméa-Ha	7.9	8.7	11.0	10.0	93.1	92.7	78.3	96.3
Hawaii	11.0	24.5	9.0	12.7	99.3	91.0	100.0	98.3
R3A	14.0	10.0	75.0	74.7	27.7	41.2	79.0	84.0
R3B	9.0	4.3	80.3	84.3	44.3	20.0	78.0	78.3
Riverside	99.0	99.3	97.3	99.3	86.3	98.4	15.7	99.0
Nasr'allah	5.3	6.3	1.7	5.7	37.0	15.7	4.0	2.7

The types of infection are in brackets; (ϕ), Uninfected strain.

[a] Incompatibility is measured as the percentage of unhatched eggs (from samples of 300 eggs).

on uninfected eggs, sperm from infected males might improve the multiplication of symbionts already present in infected eggs or somehow act on their transmission to the adult germ-line.

The double infection: First observations from *Nasonia* species have suggested that double infection by Wolbachia could occurred in a same individual (BREEUWER *et al.* 1992). Since then strong molecular probes have confirmed this hypothesis (WERREN *et al.* 1995) and extended such occurrence to some *species* of other *genus* such as Aedes, Ephestia, Spalangia (WERREN *et al.* 1995) and Drosophila (ROUSSET and SOLIGNAC 1995). But our work is the first case where two Wolbachia variants involved in double infection have been separated in two independant cytoplasmic lineages. The isolation of the wNo symbiont in the R3A and R3B lines demonstrated that the two PCR/RFLP products obtained with some Nouméa flies correspond to two different symbionts and not to a duplication of the 16S rRNA gene. This isolation, never observed in the wild, has been obtained from both replicates of a backcross with nuclear replacement. But the data available do not allow statistical analysis, which would permit us to distinguish between stochastic variations and an effect of the host genome. As no fly bearing the wNo variant only has ever been observed in the Nouméa strain, this result allows us to postulate that wNo was isolated from initially bi-infected cytoplasmic lineages through Wolbachia segregation.

The CI type N related to wNo: The wNo variant determines its own CI type in *D. simulans*, which is bidirectionally incompatible with the CI types due to wHa and wRi. But wNo, whether this bacterium is alone or associated with wHa, induces significantly less embryonic mortality than wHa (Table 4). This could explain why wNo monoinfected flies were never observed in the wild where competition with wHa monoinfected flies or with wHa + wNo bi-infected flies probably occurs.

Another peculiar feature of the R3A and R3B lines was the high percentage of unhatched eggs recovered in crosses within wNo-bearing lines as well as in crosses between wNo-bearing females and uninfected males (Table 4). But this did not result from a deleterious effect of the wNo variant in these lines as tetracycline treatment did not reduce the percentage of unhatched eggs in the cured lines. One possible explanation may be a deleterious interaction between the Nasr'allah nuclear genome (associated in the wild with the siII mitochondrion variant) and the siI mitochondrion type.

The Seychelles CI type revisited: The remarkable aspect of the Seychelles CI type is that it is determined by the simultaneous presence of two Wolbachia variants (ROUSSET and SOLIGNAC 1995) that we found to be mutually incompatible. The incompatibilities induced by wNo and wHa are independently expressed by bi-infected flies. This accounts for previous observations of partial unidirectional incompatibility between some strains at first classified into the CI type S (Table 5). In the light of molecular data on Wolbachia, and after lab maintenance of infected strains over 6 years, we distinguish three classes of strains: strains from Seychelles, in which the bi-infection (wHa + wNo) appears complete and stable in mass-breed [nevertheless, from this strain, ROUSSET and SOLIGNAC (1995) observed two isofemales lines only infected by wHa]; strains from French Polynesia and Hawaii in which only the wHa monoinfection is observed; and the New-Caledonian Nouméa strain, which harbors both wHa monoinfected flies and bi-infected flies. In some of our laboratory experimental strains from Nouméa, bi- and monoinfected flies coexist, but this polymorphism may as well disappear, with only wHa infected flies being found. Of the four other new-Caledonian strains described earlier (MONTCHAMP-MOREAU *et al.* 1991), three have been shown to be completely (Amieu and Roussette) or almost completely (Monirange) compatible with the Seychelles strain (Table 5). It follows that these three strains were partly or totally bi-infected. When it comes to the Karaka strain, its high level of incompatibility with Seychelles males might be explained by the absence of bi-infected individuals, but also by a low infection level because the cross between Karaka males and uninfected females had given only 41% of unhatched

TABLE 5

Characteristics of strains previously described in the Seychelles cytoplasmic incompatibility system

Strains[a]	Wolbachia variant[b]	CI test[c]
Seychelles (55.4 E)		
Seychelles-81	(wHa+wNo)[e]	17.5
Seychelles-85	Unknown	15.0
New-caledonia (165.3 E)		
Amieu	Unknown	6.5
Karaka	Unknown	70.2
Monirange	Unknown	30.8
Nouméa	(wHa+wNo) or wHa[d,e,f]	14.7
Roussette	Unknown	15.4
Hawaii (158.0 W)		
Hawaii 1	wHa[f]	54.9
Hawaii 4	wHa[f]	68.4
French Polynesia (149.4 W)		
Morea 1	wHa[f]	66.0
Morea 8	wHa[f]	52.4
Marau bas	wHa[g]	67.0
Marau haut	wHa[g]	59.0
Papeete	wHa[g]	65.5

[a] Longitude of countries are in parentheses.
[b] Determinated by PCR/RFLP.
[c] Percentage of unhatched eggs from crosses between tested females and Seychelles-81 males (MONTCHAMP-MOREAU *et al.* 1991, except MARAU and PAPEETE, new data).
[d] ROUSSET and SOLIGNAC (1995).
[e] The present work.
[f] ROUSSET (1993).
[g] S. BASMACIOGULLARI, personal communication.

eggs (MONTCHAMP-MOREAU *et al.* 1991). Regarding the origin of these infection differences among the siI-type *D. simulans* populations, there are two alternative hypotheses: the ancestral SiI cytoplasm was originally infected by wHa, and some populations secondarily acquired wNo, and the ancestral SiI cytoplasm was originally bi-infected, and some populations secondarily lost wNo. Arguments supporting the second hypothesis are suggested by research on the sibling species, *D. sechellia*, endemic to the Seychelles archipelago (TSACAS and BACHLI 1980; LOUIS and DAVID 1986; CARIOU 1987). The mitochondrial type of *D. sechellia* is closely related to the SiI mitochondrial type of *D. simulans* (SOLIGNAC and MONNEROT 1986) and some cytoplasmic lines of *D. sechellia* harbor three Wolbachia variants, two very closed to wNo, the third to wHa (ROUSSET and SOLIGNAC 1995). ROUSSET and SOLIGNAC (1995) suggest that the bi-infection was already present in the common ancestor of the *D. simulans* SiI geographic variant and of *D. sechellia*, the origin of latter species being probably older than 0.5 myr (HEY and KLIMAN 1993). During the expansion of the *D. simulans* SiI geographic variant to the east, probably following human migrations, the bi-infection either has been maintained (New Caledonia) or has led to wHa monoinfection (Polynesia) by the loss of wNo. Combined effects of incomplete maternal transmission, unequal strength of the cytoplasmic incompatibility induced by the two variants and founder effect may account for this situation.

We are grateful to FRANÇOIS ROUSSET and MICHEL SOLIGNAC for determinant informations and valuable discussions. We thank CHRISTOPHE BRESSAC for sharing his competence in DAPI-staining method. We thank also DOMINIQUE VAUTRIN and MARION DELATTRE for guidance and advice with PCR. We are grateful to JERRY COYNE and to anonymous reviewers for useful criticisms and constructive comments of the manuscript and to DENIS POINSOT for helping with the text.

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Communicating editor: A. R. HOFFMANN