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Evolution

Evolution of single and double *Wolbachia* symbioses during speciation in the *Drosophila simulans* complex

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ABSTRACT **Maternally inherited bacteria of the genus *Wolbachia* are responsible for the early death of embryos in crosses between uninfected females and infected males in several insect species. This phenomenon, known as cytoplasmic incompatibility, also occurs between strains infected by different symbionts in some species, including *Drosophila simulans*. *Wolbachia* was found in two species closely related to *D. simulans*, *Drosophila mauritiana*, and *Drosophila sechellia*, and shown to cause incompatibility in the latter species but not in *D. mauritiana*. Comparison of bacterial and mtDNA history clarifies the origins of bacterial and incompatibility polymorphisms in *D. simulans*. Infection in *D. mauritiana* is probably the result of introgression of an infected *D. simulans* cytoplasm. Some *D. simulans* and *D. sechellia* cytoplasmic lineages harbor two bacteria as a consequence of a double infection which probably occurred in a common ancestor. The descendant symbionts in each species are associated with similar incompatibility relationships, which suggests that little variation of incompatibility types has occurred within maternal lineages beyond that related to the density of symbionts in their hosts.**

Wolbachia symbionts are responsible for different modifications of sexual reproduction in insects and isopods, including parthenogenesis (1–3), feminization (2, 4), and cytoplasmic incompatibility (2, 5–11). The latter phenomenon is characterized by anomalies in the first mitoses of the zygote (12–15), generally a loss of paternal chromosomes, which results in death of offspring from crosses between uninfected females and infected males or all male progenies in haplodiploid species. The death of offspring of uninfected females favors the spread and maintenance of the infection in natural populations. Incompatibility also occurs in certain crosses between infected individuals harboring different bacterial strains (16–19). In this case, different incompatibility types are distinguished on the basis of incompatibility relationships.

Two cases of incompatibility-type polymorphism have been the subject of detailed studies: the large polymorphism observed in the mosquito *Culex pipiens* (20–22) and the more limited one described in *Drosophila simulans* (15, 19, 23, 24). In the latter species, uninfected flies (type W) and three infected types (R, S, and M) have been distinguished from a large geographical survey. These types are associated with different mitochondrial types, as expected if both mtDNA and symbionts are maternally transmitted. However, there was heritable variation of incompatibility properties of S strains (23). Incompatibility-type polymorphism can have many causes, including variation of symbionts themselves or variation of symbiont density in their hosts (11, 23, 25, 26). In *Drosophila*, a simple method to assess density effects in males is to measure the frequency of infected sperm cysts during spermatogenesis (27), while variation of symbionts can be detected by crosses and experimental transfer of symbionts (19, 23).

Identification of *D. simulans* symbionts by partial 16S and 23S ribosomal DNA sequencing has revealed a strikingly high molecular differentiation (2, 9). The time of divergence of symbionts is 25–50 million years ago according to estimated 16S rDNA rates (28, 29), whereas mtDNA of their hosts is estimated to have diverged only 1–2 million years ago (9, 30), and close relatives of each of these symbionts are present in other orders of insects (2). This suggests that at least two separate infection events occurred in ancestors of *D. simulans*. To understand the origin of this polymorphism and its relationship to incompatibility-type polymorphism, we have investigated the presence and nature of *Wolbachia* symbionts in the two island endemics, *Drosophila sechellia* and *Drosophila mauritiana*, closely related to *D. simulans*, and their associated incompatibility types.‡

MATERIALS AND METHODS

Nomenclature of Molecular Types. Mitochondrial types representing the major subdivisions of mitochondrial polymorphism are symbolized by two letters for the species, followed by roman numbers: three mitochondrial types have been distinguished in *D. simulans* (*si*I, *si*II, and *si*III), two in *D. mauritiana* (*ma*I and *ma*II), and one (*se*) in *D. sechellia* (31). *Drosophila* strains are identified by two letters for species, followed by letters for geographical origin or numbers for year of collection—e.g., *si*No or *se*81. Symbiont rDNA sequences are identified as *w*No, *w*Ha, *w*Ri, or *w*Ma in reference to a *D. simulans* strain in which they were found or as *w*Sh, *w*Sn1 or *w*Sn2 for *D. sechellia*. The host strain is sometimes recalled as follows: *w*Ma(*si*Ma) is symbiont *w*Ma from strain *si*Ma.

Hosts Strains. Strains were reared at 20°C. Their mtDNA types were identified through their *Hpa* II or *Hin*dIII restriction profiles (32). Isofemale *D. simulans* lines were *si*Wa (Watsonville, CA; *si*II), *si*Ri (Riverside, CA; *si*II), *si*Ha (Hawaii; *si*I), *si*Ma (Mont d'Ambre, Madagascar; *si*III) (6, 19, 23), and *si*No (Nouméa, New Caledonia; *si*I). These strains were used for cytoplasm injections or crosses. The previously studied *si*Ma strain (2, 9) was found to be polymorphic for presence/absence of infection at the time of the present experiments and an infected subline was selected. Eight additional *si*I strains from the Seychelles, Hawaii, or New Caledonia were investigated for the presence of *w*Ha and *w*No sequences by *Vsp* I digestion (9) and partial sequencing of PCR products, including nt positions 561–646.

D. mauritiana lines were *ma*79 and *ma*85 (*ma*I, collected in Mauritius in 1979 and 1985), and 23 other maI and 3 maII lines collected in 1985. *D. sechellia* lines were *se*81, *se*85, and *se*89 from the Seychelles, collected in 1981, 1985, and 1989, respec-

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X80977–X80979).

tively. The aposymbiotic *se*85TC strain was obtained by tetracycline treatment of *se*85 as in ref. 27.

Experimentally infected strains were obtained by transferring cytoplasm from infected eggs into uninfected *si*Wa eggs. Females that developed from such eggs were individually crossed with *si*Wa males as described (19).

Infection Level of Males. Infection level—i.e., the frequency of infected sperm cysts in 2-day-old males—was evaluated by 4′,6-diamidino-2-phenylindole (DAPI) staining (27).

PCR and DNA Sequencing. We amplified a fragment of the 16S ribosomal DNA homologous to nt positions 83–928 of the *Escherichia coli* sequence by using previously described *Wolbachia* primers (8, 19). PCR products were directly sequenced as described (2). PCR products from *si*No, *se*85, and *se*89 were cloned by using pBluescript KS(+) (Stratagene) and sequenced according to methods reported in ref. 33. For each host strain, only sequence variation present in at least two clones was taken into account. Previously unreported *Wolbachia* sequences *w*Sh, *w*Sn1, and *w*Sn2 from *D. sechellia* strains have received accession numbers X80977–X80979 in the GenBank data base.

Incompatibility. Incompatibility relationships were determined by measuring the frequency of unhatched eggs produced by 2- to 4-day-old females mated to 2-day-old males. *D. simulans* and *D. mauritiana* females were reared individually after mating, but *D. sechellia* females laid very few eggs in such conditions and were reared in groups of six. The crossing experiments with the experimentally infected strain were performed simultaneously to those described for similarly obtained strains (19) by using the same methods.

RESULTS

***Wolbachia* Presence and rDNA Polymorphism.** Symbionts have been found in both *D. mauritiana* and *D. sechellia*. In *D. mauritiana*, infection was detected by PCR in *ma*79 but not *ma*85, in 12 of 23 other *ma*I strains, and in none of three *ma*II strains. Infection was found in *D. sechellia se*81, *se*85, and *se*89 but not in the tetracycline-treated *se*85TC strain.

Four sequences have been described in *D. simulans* strains (8, 9), here referred to as *w*Ri, *w*Ha, *w*Ma, and *w*No. Sequencing the same 803-bp fragment for one *D. mauritiana* strain and three *D. sechellia* strains (Table 1) revealed three different sequences in *D. sechellia* and one sequence identical to *w*Ma in *D. mauritiana*. The differences were repeatedly observed by direct sequencing of PCR products and are therefore not due to cloning artifacts.

Table 1. *Wolbachia* and mitochondrial variation in the *D. simulans* complex

	Variable positions	
	1155555666666678	
	86667889124488964	
Wolbachia types	45918793405623303	mtDNA
Cluster I		
*w*Ma	taCTctgagtatgaaA-	*si*III/*ma*I
*w*No	taCCctgagtatgaaG-	*si*I
*w*Sn1	taCCctgagtatgaaA-	*se*
*w*Sn2	taTCctgagtatgaaA-	*se*
Cluster II		
*w*Sh	TgctTgagacgcagggt	*se*
*w*Ri	TgctCgagacgcagggt	*si*II
*w*Ha	CgctTgagacgcagggt	*si*I

Variable positions are those found within an 803-bp fragment of symbiont 16S rDNA. Nucleotide positions (read vertically) are defined as in ref. 2. Variable nucleotides within each of the two main clusters are in capitals. Associated mitochondrial types are given in parallel, combining results presented in text and those in refs. 8, 9, 23, and 34. Absence of symbionts is found among *si*II, *si*III, *ma*I, and *ma*II strains.

Initially, *Wolbachia* PCR products amplified from individual flies were sequenced directly. In some instances, heterogeneity was detected (bands could be observed in two nucleotide lanes at some positions of the sequence), and in those cases PCR products were cloned. In *D. simulans*, heterogeneity was found in the *si*No strain, which harbors *w*Ha and *w*No sequences. Among the 10 *D. simulans si*I strains studied, the *w*Ha sequence is always present, whereas the *w*No sequence is present in three strains (*si*No, but none of three other strains from Nouméa, New Caledonia, and two from Hawaii; *w*No is found in two of four strains from the Seychelles). In *D. sechellia*, *w*Sh has been directly sequenced from *se*81, cloned from *se*89, and detected, together with *w*Sn1 or *w*Sn2 by direct sequencing in strains *se*85 and *se*89, respectively. *w*Sn1 was also sequenced after cloning of PCR products from *se*85, as was *w*Sn2 from *se*89. Thus, the three strains harbor the *w*Sh sequence, and two of them additionally possess one of the two closely related sequences *w*Sn1 or *w*Sn2.

Polymorphism of DNA sequences within individual hosts can be due to the presence of at least two different ribosomal operons in the same symbiont or to the presence of two different symbionts (7). In the present case, this issue was settled by injection experiments. We have transferred symbionts from *D. simulans si*No eggs harboring *w*Ha and *w*No sequences by cytoplasmic injection into eggs of the uninfected *si*Wa strain. Among the seven lines derived from one female that developed from a recipient egg and was crossed to *si*Wa males, two were infected by *w*Ha only (no symbiont was found in the others). This result demonstrates the presence of two different symbionts in the donor strain. The *w*Sn1 sequence was similarly isolated (details not shown). Thus, different symbiont types can be defined by the different sequences, and the sequence nomenclature can and will be used to distinguish symbionts. The properties of strains used for crosses are summarized in Table 2.

Cytoplasmic Incompatibilities. Crosses were performed to establish the incompatibility properties of the different strains in relation to the previously identified crossing types and to characterize the relationships between symbiont types and crossing types.

Crosses between *si*Wa, *si*Ri, and *si*Ha replicated earlier results with the same strains (6, 19, 23): *si*Ri and *si*Ha were bidirectionally incompatible, and males from both strains were incompatible with uninfected *si*Wa females. *si*Ma behaved in crosses (Table 3) as an uninfected strain, as did *ma*79 (Table 4) infected by indistinguishable (on the basis of rDNA) *w*Ma symbiont: males are compatible with all females irrespective of infection status, and females are incompatible with males infected by a different symbiont type. From the *ma*79 and *si*Ma isofemale strains studied here, 10 isofemale sublines have been established and tested by PCR for the presence of symbiont 14 and 19 generations later, respectively. Only three *ma*79 and

Table 2. Origin and molecular types of strains used for crosses

Drosophila species	Origin	Strain	mtDNA type	*Wolbachia* type
D. simulans	Watsonville, CA	*si*Wa	*si*II	Uninfected
	Riverside, CA	*si*Ri	*si*II	*w*Ri
	Hawaii	*si*Ha	*si*I	*w*Ha
	Madagascar	*si*Ma	*si*III	*w*Ma
	New Caledonia	*si*No	*si*I	*w*No *w*Ha
D. sechellia	Seychelles	*se*81	*se*	*w*Sh
	Seychelles	*se*85	*se*	*w*Sh *w*Sn1
	Seychelles	*se*89	*se*	*w*Sh *w*Sn2
	Seychelles	*se*85TC	*se*	Antibiotic treatment
D. mauritiana	Mauritius	*ma*79	*ma*I	*w*Ma
	Mauritius	*ma*85	*ma*I	Uninfected

Table 3. Incompatibility relationships in *D. simulans*

Female strain	Male strain, symbiont(s)				
	*si*Wa, None	*si*Ri, *w*Ri	*si*Ha, *w*Ha	*si*No, *w*Ha and *w*No	*si*Ma, *w*Ma
*si*Wa	0.01 ± 0.01	1.00	0.99 ± 0.02	0.97 ± 0.06	0.04 ± 0.03
n	6	15	15	15	6
*si*Ri	0.15 ± 0.16	0.11 ± 0.04	0.92 ± 0.12	0.96 ± 0.06	0.09 ± 0.04
n	12	8	13	6	4
*si*Ha	0.17 ± 0.17	0.98 ± 0.03	0.14 ± 0.08	0.65 ± 0.12	0.09 ± 0.08
n	29	14	5	13	4
*si*No	0.05 ± 0.11	0.98 ± 0.02	0.12 ± 0.10	0.08 ± 0.12	0.07 ± 0.05
n	11	6	6	5	5
*si*Ma	0.05 ± 0.04	0.96 ± 0.06	0.94 ± 0.10	0.94 ± 0.04	0.05 ± 0.04
n	5	5	7	5	6

Percentage of unhatched eggs ± SE between replicates among eggs laid by the indicated number (*n*) of females. The strains *si*Wa, *si*Ri, and *si*Ha are representative of the formerly recognized incompatibility types W, R (6) and S_H (19). The name S_{HN} is proposed for the incompatibility type of strain *si*No.

five *si*Ma sublines were still infected. Four males of each subline were examined, and in all cases, between 50% and 100% of the sperm cysts were infected, showing that the crossing relationships of these males are not related to a particularly low infection level.

Incompatibility relationships of the *si*No strain carrying *w*Ha and *w*No symbionts were similar to those of the *si*Ha strain harboring only *w*Ha symbionts (Table 3), except that *si*No males were partially incompatible with *si*Ha females (65% unhatched eggs, compared with 14% for the *si*Ha intrastrain cross; one-tailed Mann–Whitney *U* test; $P = 0.0008$). This incompatibility seems to be due to the nature of symbionts themselves rather than to density effects, since the infection level of *si*No males (33% ± 25%; $n = 9$ males) is similar to that of *si*No males (29% ± 15%; ref. 19). Infected *D. sechellia* males are incompatible with uninfected females (Table 5, first line), and most crosses between infected flies were compatible. However, crosses between singly infected females and double infected males were less compatible than other crosses between infected *D. sechellia* ($P = 0.015$). In both species, double infection of males was correlated to partial incompatibility with females harboring only one of the two symbionts.

In interspecific crosses between *D. simulans* females and *D. sechellia* males (Table 6), sperm transfer may be low and sperm can be rapidly used up, so that viable eggs and eggs showing no development are often found together in the absence of cytoplasmic incompatibility, as shown in crosses with uninfected *se*85TC males. This obscures the comparisons, and only strong incompatibility can be detected, as in crosses with either *si*Wa or *si*Ri females (Table 6, first two lines; $P < 0.01$ in all comparisons between infected males and uninfected males crossed to the same females). This is expected if the incompatibility relationships of *w*Ha(*si*Wa) are similar to those of *w*Sh(*se*81) and those of *w*No(*si*No) are similar to those of *w*Sn1(*se*85) and *w*Sn2(*se*89), or if the presence of *w*Sn1/2 does not affect compatibility. The reciprocal crosses cannot be studied because *D. sechellia* females hardly mate with *D. simulans* (35).

Table 4. Incompatibility relationships between one infected and one uninfected *D. mauritiana* strain

Female strain	Male strain, symbiont	
	*ma*85, None	*ma*79, *w*Ma
*ma*85	0.13 ± 0.13	0.14 ± 0.12
n	25	15
*ma*79	0.08 ± 0.05	0.07 ± 0.06
n	10	14

See Table 3 legend.

Strains harboring *w*Ha symbionts isolated by experimental transfer from *si*No strains showed frequencies of infected sperm cysts (27% and 4%, each from nine males) comparable with those of strains HaWa3 and HaWa4 harboring *w*Ha symbionts isolated from *si*Ha (27% and 10%; ref. 19). The most infected of these two strains showed partial incompatibility with *si*Ri females (frequency of unhatched eggs: 0.52 ± 0.18) but not with *si*Ha or *si*No females (0.18 ± 0.04 and 0.11 ± 0.07, respectively).

DISCUSSION

The different sequences of *Wolbachia* found in the *D. simulans* complex can be grouped into two clearly defined phylogenetic clusters (Table 1), in agreement with a global phylogeny of *Wolbachia* (2). Divergence between *Drosophila* symbionts within each cluster is very limited, such that parallel changes and reversions are highly unlikely. The double infections described here are the only examples that could be detected by direct sequencing of *Wolbachia* symbionts in 12 host species among the Drosophilinae (F.R., unpublished data). However, a double infection can be suspected in three *Nasonia* species (7). In such a situation, sequences obtained after amplifying together closely related sequences and cloning the PCR products are subject to artifacts similar to recombination or conversion events between the original sequences (36, 37). Such artifacts yield sequences with an apparently very high level of homoplasy, as was indeed observed for *Nasonia* symbionts. By contrast, there is little homoplasy in sequences of symbionts from singly infected hosts (2, 3, 8, 38).

Incompatibility Types. In a previous study (24), *si*Ma was found to cause incompatibility. In contrast to this observation, males of the present *si*Ma strain are compatible even with uninfected females. The same result is obtained with *ma*79 males infected by *w*Ma. The absence of incompatibility in *D. mauritiana* has also been observed by R. Giordano (personal communication). Such absence of incompatibility may have favored the appearance of uninfected individuals in the original *si*Ma strain (see *Materials and Methods*) and its sublines.

Crosses of *si*Ha females with *si*Wa males infected by the *w*Ha symbionts of *si*No (obtained after experimental transfer) are compatible. Thus *w*Ha from *si*Ha and *w*Ha from *si*No may belong to the same incompatibility type, and the additional symbiont *w*No of doubly infected males is responsible for a partial incompatibility with females harboring only *w*Ha. This situation is analogous to the typical incompatibility observed between infected males and uninfected females. Incompatibility between singly infected females and doubly infected males is a likely explanation for the pattern of partial unidirectional incompatibility previously observed among *si*I strains

Table 5. Incompatibility relationships between four *D. sechellia* strains

Female strain	Male strain, symbiont(s)			
	*se*85TC, None	*se*81, *w*Sh	*se*85, *w*Sh and *w*Sn1	*se*89, *w*Sh and *w*Sn2
*se*85TC	0.23 ± 0.12	0.71 ± 0.15	0.81 ± 0.07	0.90 ± 0.06
n	12	12	12	12
*se*81	0.00	0.03 ± 0.01	0.35 ± 0.12	0.39 ± 0.02
n	6	12	12	12
*se*85	0.11 ± 0.07	0.12 ± 0.11	0.15 ± 0.05	0.12 ± 0.06
n	12	12	26	18
*se*89	0.07 ± 0.01	0.14 ± 0.07	0.22 ± 0.05	0.15 ± 0.05
n	12	12	12	12

See Table 3 legend.

(Table 6 of ref. 23, last column, from which it may be inferred that the *D. simulans* Seychelles 81 strain is doubly infected, as well as another strain from the Seychelles and at least three from New Caledonia).

***si*III/*ma*I mtDNA Lineages.** The *ma*I mitochondrial type is very closely related to *si*III (only one nt difference was found in 2527 bp; ref. 30), and its presence in *D. mauritiana* most probably results from cytoplasmic introgression by *D. simulans* females carrying the *si*III mtDNA (31, 39). The presence of indistinguishable (on the basis of rDNA sequences) *w*Ma bacteria in *ma*I and *si*III individuals strongly suggests that some of the *D. simulans* introgressing cytoplasms were infected. How the initial infection has been maintained up to now is not clear, since the symbionts do not (or no longer) cause incompatibility.

The mtDNA type closest to *si*III is *si*II, but the *w*Ri symbionts found associated with the latter are distantly related to *w*Ma. This can be explained only by independent infections of the *si*II and *si*III lineages. There is evidence that the *w*Ri infection is recent, as it is found associated with a particular subtype of *si*II mtDNA (40, 41), whose differentiation is itself recent (42).

Double Infections in *si*I/*se* mtDNA Lineages. *D. sechellia* flies harbor *w*Sh either alone or associated with *w*Sn1 or *w*Sn2. This situation closely parallels that found in *D. simulans si*I flies where *w*Ha is "equivalent" to the closely related type *w*Sh and *w*No to *w*Sn1/2, since all flies possess the *w*Ha sequence and sometimes *w*No. As *D. sechellia* mtDNA is related to *D. simulans si*I (Fig. 1), the results are best explained by considering that a cytoplasm ancestral to *si*I and *D. sechellia* flies possessed two types of bacteria—i.e., the ancestors of the two groups of equivalent symbionts—one of which was lost in some descendants in both species. For some reason—e.g., reduced bacterial density within males from natural populations (41)—incompatibility would not have been strong enough to prevent *w*No-free cytoplasms from becoming common in *D. simulans*

Table 6. Interspecific crossing relationships between *D. simulans* females and *D. sechellia* males

Female strain	Male strain, symbiont(s)			
	*se*85TC, None	*se*81, *w*Sh	*se*85, *w*Sh and *w*Sn1	*se*89, *w*Sh and *w*Sn2
*se*Wa	0.19 ± 0.23	0.93 ± 0.10	0.88 ± 0.11	ND
n	13	3	5	
*si*Ri(*w*Ri)	0.26 ± 0.24	0.90 ± 0.10	0.96 ± 0.05	0.93 ± 0.06
n	20	8	3	3
*si*Ha(*w*Ha)	0.70 ± 0.32	0.16 ± 0.13	ND	0.70 ± 0.14
n	21	9		4
*si*No(*w*Ha and *w*No)	0.54 ± 0.41	0.31 ± 0.26	0.47 ± 0.22	0.52 ± 0.24
n	22	7	4	15

See Table 3 legend. ND, not done (no insemination).

*si*I populations (7 of the 10 strains studied; data are lacking for *D. sechellia*).

An alternative explanation would be that one of the infections (*w*Ha/*w*Sh) would be ancestral to divergence of cytoplasmic lineages and the other infection had arisen independently in each host species, resulting in three distinct bacterial types (*w*No, *w*Sn1, and *w*Sn2). Their sequences share a C residue in position 561 which is not found in any other published *Wolbachia* sequence and additional sequences from symbionts of eight other *Drosophila* species and a mite (F.R., unpublished data). This explanation would imply that two (or even three) infections by symbionts harboring this autapomorphy occurred precisely in two closely related maternal lineages and only there.

A Synthesis. Congruencies between mitochondrial and bacterial relationships have been found at the level of the *si*III/*ma*I and *si*I/*se* lineages. At lest two infections occurred in *si*I/*se*. A third probably occurred in the *si*II lineage (by *w*Ri) (40, 41). Doubts remain as to whether *w*Ma, *w*No, *w*Sn1, and *w*Sn2 derive from a single infection of a common maternal ancestor (which would imply at least one more loss in the *si*II lineage) or by separate infections (implying a fourth infection by the ancestor of *w*Ma). Each of these infections can have occurred earlier than shown in Fig. 1 without any consequence other than implying additional losses. It should be noted that an infection event in a maternal lineage can be due to an occasional paternal inheritance within the ancestral host populations if it was polymorphic for mtDNA lineages, a rare situation at present (42) or to horizontal transfer from an extraspecific source.

Horizontal transfers are important for the subsistence of symbionts without favorable effects on their hosts (43), but their mechanisms are unknown and their frequency may be difficult to estimate; for any infection detected here many others have been rapidly eliminated because the probability of spread after infection of one female is small (44).

Differentiation of Incompatibility Types. Bidirectional incompatibility is a cause of postmating isolation between two *Nasonia* species (13, 17). Such incompatibility can be the consequence of two independent infections or the result of independent divergence of incompatibility types in populations separated after one infection event. *D. sechellia* and *D. simulans si*I populations are of particular interest in suggesting that such divergence is not straightforward; their separation occurred more than 0.5 million years ago (45), and in this time, independent evolution of *w*Ha from *w*Sh, and probably of *w*Sn1/2 from *w*No, has not caused divergence of incompatibility types leading to postmating isolation (Table 6). Within *D. simulans*, the different incompatibility types (Table 3) can be related to the different infection events, and there is little evidence for a variation of incompatibility properties after such events, beyond that related to fluctuations of bacterial density in individual hosts (19, 46). The most significant alternative would be that all symbionts of cluster I have

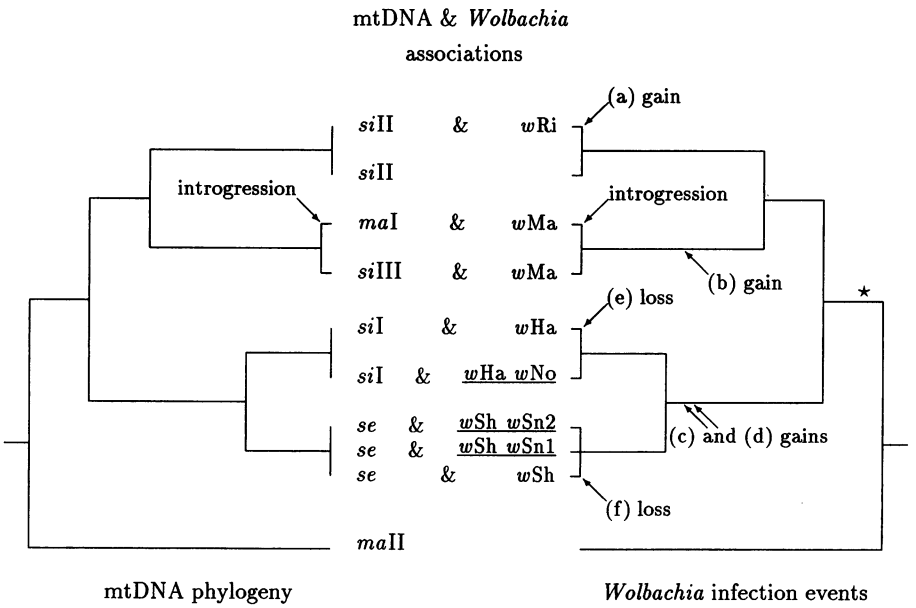

FIG. 1. History of cytoplasmic lineages and *Wolbachia* infections in the *D. simulans* complex. In the center are indicated the 10 different cytoplasmic genomes—associations of mitochondrial and bacterial types. Double infections are underlined. A firmly established mitochondrial phylogeny (*Left*, ref. 30) traces the evolutionary history of cytoplasmic lineages which occurred within 1–2 million years and *Wolbachia* infections (*Right*) can be placed in this context, as discussed in text. (a) and (b) are infections by ancestors of *w*Ri and *w*Ma respectively. The *si*III–*w*Ma association has invaded *D. mauritiana* by cytoplasmic introgression. Infection occurred in the ancestor of *si*I and *se* lineage by (c), leading to *w*Ha and *w*Sh, and probably by (d) leading to *w*No and *w*Sn1/2. A possible alternative to (b) and (d) gains is a single earlier infection—e.g., on the branch marked by a star, more than 1 million years ago—implying at least one loss in the *si*II lineage. An alternative to (d) gain and (e and f) losses is discussed in the text.

diversified from a single infection of the ancestral maternal lineage at least 1 million years ago (Fig. 1), in which case differentiation of incompatibility types within *Drosophila* populations would still be slow. It will be interesting to compare this situation with that found in other species, particularly *Culex pipiens*, where rapid divergence has been proposed to explain the much greater polymorphism of incompatibility types (21).

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