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Sylvain Charlat

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Présentée par

Sylvain Charlat

Pour obtenir le grade de

DOCTEUR de l'UNIVERSITÉ PARIS 6

***Evolution de l'incompatibilité cytoplasmique
associée à la bactérie Wolbachia***

Soutenue le 14 décembre 2002

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*L'histoire naturelle est un vrai roman d'aventures.
Quand je pense à l'interaction des forces et de la
matière, au terrible combat qu'elles se livrent, il me
semble que je pourrais écrire une épopée sur l'herbe.*

Jack London, *Martin Eden*, 1909.

Résumé

L'incompatibilité cytoplasmique (IC) est un phénomène lié à la bactérie endocellulaire *Wolbachia*, très répandue chez les Arthropodes. L'IC s'exprime par la mort précoce des embryons issus des croisements entre mâles infectés et femelles non infectées, alors que la viabilité est normale si la femelle porte la même bactérie, ou si le mâle lui-même n'est pas infecté. Les mécanismes moléculaires sont actuellement inconnus, mais ce phénomène peut être décrit par un modèle à deux fonctions, de type poison/antidote : le "poison" (appelé *mod*, pour "modification") serait produit par *Wolbachia* et déposé dans les spermatozoïdes en cours de maturation, induisant la mort des embryons, à moins que la bactérie ne soit également présente dans l'œuf fécondé, produisant un "antidote" (appelé *resc*, pour "rescue"). Du fait de la fonction *mod*, les femelles non infectées sont stérilisées par les mâles infectés présents dans la population. Au contraire, du fait de la fonction *resc*, les femelles infectées ne sont pas stérilisées. *Wolbachia* étant, transmise uniquement par voie femelle, avec le cytoplasme de l'œuf, l'IC lui permet d'envahir les populations hôtes et de s'y maintenir.

La diversité phylogénétique des *Wolbachia* se manifeste par une diversité phénotypique des fonctions *mod* et *resc*. D'une part, différents variants peuvent présenter différentes "intensités de *mod*", c'est-à-dire tuer une plus ou moins grande proportion de la descendance dans les croisements entre mâles infectés et femelles non infectées. D'autre part, différents variants peuvent être réciproquement incompatibles, démontrant que les fonctions *mod* et *resc* interagissent de manière spécifique. Le présent mémoire porte sur les processus évolutifs sous-jacents à cette diversité, étudiés par une combinaison d'approches théoriques et expérimentales.

De l'analyse théorique, il ressort que les types de compatibilité (les paires *mod* / *resc*) peuvent évoluer selon un processus en deux étapes. La première implique des variations neutres de la fonction *mod*, non soumises à sélection car *mod* n'est exprimée que chez les mâles. La deuxième étape implique la sélection de nouvelles fonctions *resc*.

L'approche expérimentale est fondée sur la confrontation, après injection cytoplasmique au sein de l'espèce modèle *Drosophila simulans*, de variants bactériens étroitement apparentés mais évoluant à l'état naturel dans des hôtes différents. Nos résultats suggèrent que des variants proches mais distinguables par les marqueurs moléculaires en usage peuvent être partiellement ou totalement incompatibles.

Nous nous intéressons également aux conséquences d'une perte secondaire de l'IC (phénotype [*mod*-]) sur la stabilité des associations *Wolbachia* / hôtes. Nos résultats suggèrent que chez les espèces *Drosophila simulans* et *Drosophila yakuba*, l'absence d'IC n'induit pas nécessairement la perte de l'infection.

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Introduction

Introduction

1. Des conséquences de l'anisogamie

La plupart des espèces à reproduction sexuée sont anisogames : la fécondation résulte de la rencontre d'un gamète mâle, de petite taille, et d'un gamète femelle, beaucoup plus gros et riche en réserves. Les gamètes mâles et femelles contribuent à parts égales au génome nucléaire de la descendance, mais à quelques exceptions près (Zouros 2000), la transmission du cytoplasme est assurée exclusivement par les gamètes femelles (Birky 1995). L'explication évolutive des effets de la bactérie *Wolbachia* réside dans cette asymétrie.

Tout comme les mitochondries (Yang *et al.* 1985), *Wolbachia* appartient au groupe des α -protéobactéries (O'Neill *et al.* 1992) et vit au sein du cytoplasme de ses hôtes. Elle infecte de nombreuses espèces d'Arthropodes, ainsi que des Nématodes du groupe des filaires (Werren *et al.* 1995a ; Bouchon *et al.* 1998; Wenseleers *et al.* 1998; West *et al.* 1998; Cook & Butcher 1999; Plantard *et al.* 1999; Taylor *et al.* 1999; Jeyaprakash & Hoy 2000; Werren & Windsor 2000). Ce succès repose très probablement sur son étonnante capacité à manipuler la reproduction des organismes hôtes à l'avantage des femelles, seul sexe transmettant la bactérie à la descendance. A l'heure actuelle, quatre phénomènes ont été décrits chez les Arthropodes qui s'inscrivent dans ce cadre théorique : la féminisation, l'induction de parthénogenèse thélytoque, le "male killing" et l'incompatibilité cytoplasmique (IC) (Stouthamer *et al.* 1999; Hurst & Jiggins 2000).

Les deux premiers phénomènes procèdent de la même logique : en interférant avec le déterminisme du sexe, *Wolbachia* pousse les femelles infectées à produire d'avantage de filles, au détriment des fils. On parle de féminisation lorsque des individus génétiquement mâles deviennent physiologiquement des femelles fertiles. Chez les crustacés isopodes, où ce phénomène est le mieux caractérisé, *Wolbachia* féminise les mâles en inhibant la formation de la glande androgène (Rigaud 1997). L'induction de parthénogenèse thélytoque s'observe chez des organismes haplo-diploïdes (Stouthamer 1997), tels que les hyménoptères, où le déterminisme du sexe repose sur le niveau de ploïdie. Dans ces

groupes, le mode de reproduction naturel est la parthénogenèse arrhénotoque où les mâles se développent à partir d'œufs non fécondés (haploïdes), tandis que les femelles sont issues des œufs fécondés (diploïdes). *Wolbachia* impose ici le développement femelle aux œufs non fécondés en rétablissant la diploïdie par une mitose incomplète après la méiose (Stouthamer & Kazmer 1994), ou plus en amont en empêchant la réduction méiotique (Weeks & Breeuwer 2001). Ce faisant, la bactérie induit le glissement de la parthénogenèse arrhénotoque vers un autre type de parthénogenèse, dit thélytoque, où les embryons issus d'œufs non fécondés sont femelles. Si la pénétrance du phénomène est totale, les femelles infectées auront finalement une descendance intégralement femelle.

Le "male-killing" conduit également les femelles infectées à produire d'avantage de femelles que de mâles, mais la cause est ici la mort des embryons mâles (Hurst & Jiggins 2000). Le bénéfice de *Wolbachia* est plus indirect : le nombre de filles à l'éclosion reste inchangé, mais la mort de leurs frères peut être avantageuse à bien des égards : elle offre une ressource alimentaire de proximité dans les espèces cannibales telles que les coccinelles, évite la consanguinité, diminue la compétition pour des ressources limitées ainsi que les interactions négatives au sein de la portée.

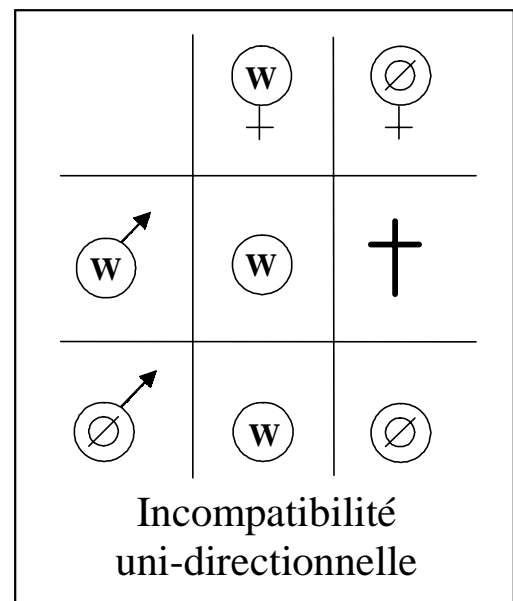
L'IC (Hoffmann & Turelli 1997), sujet de ce mémoire, diffère nettement des trois autres phénomènes. Au lieu de favoriser la descendance femelle des femelles infectées, *Wolbachia* adopte ici une stratégie indirecte : elle diminue le succès reproducteur des femelles non infectées, augmentant ainsi la valeur sélective relative des femelles infectées. Ce phénomène est présenté dans la suite de cette introduction, comprenant les éléments nécessaires à la compréhension de mes travaux. Pour une description plus détaillée, le lecteur pourra consulter l'article N°7 situé en annexe 1.

2. L'incompatibilité cytoplasmique

2.1. Incompatibilités uni- et bi-directionnelles

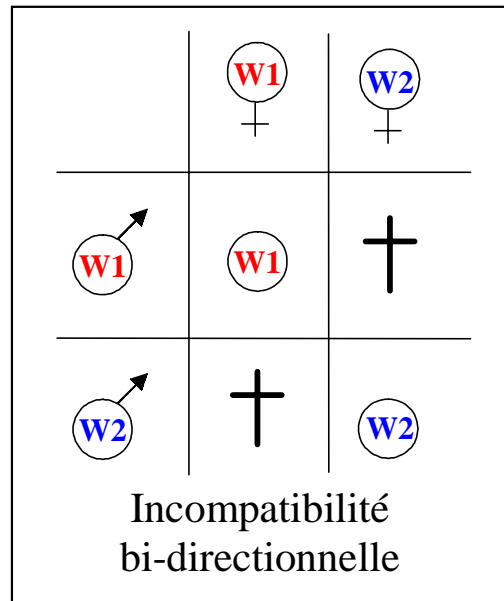
Sous sa forme la plus simple, l'IC se manifeste par une mortalité embryonnaire anormalement élevée dans la descendance des croisements entre mâles infectés par *Wolbachia* et femelles non infectées. Au contraire, les croisements entre mâles infectés et femelles infectées, ou entre mâles non infectés et femelles infectées, donnent une descendance viable. Ce type d'incompatibilité est dit uni-directionnel, car un

seul sens de croisement (mâle infecté × femelle non infectée) est incompatible. En conférant un gain de valeur sélective relative aux femelles infectées, l'incompatibilité uni-directionnelle permet à *Wolbachia* d'envahir les populations hôtes non infectées.



L'IC peut également s'exprimer dans des croisements entre mâles et femelles porteurs de variants bactériens différents (O'Neill & Karr 1990). La mortalité des embryons s'observe alors dans les deux sens de croisements, et on parle d'incompatibilité bi-directionnelle.

Cette distinction nous permet d'ores et déjà d'introduire deux notions importantes : l'intensité de l'IC et les types de compatibilité. L'intensité de l'IC se mesure dans le cadre de l'incompatibilité uni-directionnelle : elle correspond à la proportion des embryons tués par *Wolbachia* dans les croisements entre



mâles infectés et femelles non infectées. Au contraire, la notion de type de compatibilité est fondée sur les relations de compatibilité entre deux variants bactériens : on dira de deux variants incompatibles qu'ils portent des types de compatibilités différents.

2.2. Dynamique d'invasion

Avant même que le phénomène d'IC soit imputé, dans les années 70, à la bactérie *Wolbachia* (Yen & Barr 1971), son caractère invasif avait été démontré (Caspari & Watson 1959). Ce travail pionnier a par la suite été complété par d'autres modèles théoriques (Fine 1978; Hoffmann *et al.* 1990) et validé par l'étude de populations naturelles (Turelli & Hoffmann 1995). Nous en résumons ici les principaux enseignements.

Notons tout d'abord que l'avantage sélectif conféré par l'IC aux femelles infectées est fréquence-dépendant : plus la fréquence d'infection est élevée, plus le succès reproducteur des femelles non infectées est réduit, et plus le gain relatif des femelles infectées est important. En d'autres termes, aux stades initiaux de l'invasion d'une nouvelle population, quand la fréquence d'infection est par définition réduite, l'IC ne confère qu'un faible avantage sélectif aux femelles infectées. Cette observation permet de comprendre une conclusion majeure des modèles de dynamique d'invasion : si des phénomènes indépendants de l'IC réduisent la valeur sélective des cytoplasmes infectés, *Wolbachia* ne peut envahir les populations qu'à partir d'une fréquence d'infection initiale suffisamment élevée. Concrètement, deux phénomènes réduisant la valeur sélective des cytoplasmes infectés ont été mis en évidence : le coût de l'infection (représenté dans les modèles par une réduction de fécondité), et la transmission imparfaite de l'infection à la descendance.

Au delà de cette fréquence seuil, l'IC confère un avantage sélectif suffisamment important aux femelles infectées pour permettre une augmentation de la fréquence d'infection. Cependant, la fixation des cytoplasmes infectés ne sera finalement atteinte que si l'intensité de l'IC est de 100% (si aucun embryon ne survit aux croisements

incompatibles) et / ou si la transmission maternelle est parfaite (si les femelles infectées transmettent l'infection à tous leurs descendants). Si aucune de ces deux conditions n'est remplie, des embryons non infectés sont produits à chaque génération, dont une certaine proportion pourra survivre et se reproduire.

Pour résumer, trois paramètres doivent être pris en considération : l'intensité de l'incompatibilité uni-directionnelle, l'efficacité de la transmission maternelle et le coût de l'infection. Les valeurs respectives de ces trois paramètres déterminent un équilibre instable, une fréquence d'infection seuil, en deçà de laquelle *Wolbachia* est éliminée. Si ce seuil est dépassé, la fréquence d'infection atteint un équilibre stable, qui n'est la fixation que si l'intensité de l'IC et / ou la transmission maternelle sont de 100%.

2.3. Cytologie

Bien que les mécanismes moléculaires de l'IC soient encore inconnus, le phénomène est bien caractérisé au niveau cytologique. Des études menées chez le diptère *Drosophila simulans* et l'hyménoptère parasitoïde *Nasonia vitripennis* ont démontré que dans les croisements incompatibles, les chromosomes d'origine paternelle sont perdus lors de la première division embryonnaire (Reed & Werren 1995; Callaini *et al.* 1996; Lassy & Karr 1996; Callaini *et al.* 1997; Tram & Sullivan 2002). Des observations d'embryons fixés (Callaini *et al.* 1997) ainsi que des suivis en temps réel d'embryons vivants (Tram & Sullivan 2002) montrent que les chromosomes paternels entrent en anaphase quand les chromosomes maternels ont déjà ségrégués. De telles observations suggèrent, sans toutefois le démontrer, que les chromosomes paternels pourraient participer normalement à la mitose si le temps nécessaire leur était offert.

Il est intéressant de noter que chez les organismes haplo-diploïdes tels que les hyménoptères, l'IC n'entraîne pas nécessairement la mort des embryons : chez le parasitoïde *Nasonia vitripennis*, l'haploïdie imposée par la perte des chromosomes paternels dans les croisements incompatibles conduit à une "masculinisation" des embryons fécondés non infectés (Breeuwer & Werren 1990), ce qui, du point de vue d'un symbiote à transmission maternelle, équivaut à la mort. Compte tenu du système de déterminisme sexuel de ces organismes, fondé sur le niveau de ploïdie, ce phénomène n'a rien d'inattendu. Pour autant, il ne représente sans doute pas la règle : dans d'autres espèces haplo-diploïdes, la mort des embryons issus d'œufs fécondés est bel et bien observée dans les croisements incompatibles (Vavre *et al.* 2000; Bordenstein *et al.* 2003). La cause en est probablement une perte incomplète des chromosomes paternels, conduisant à un caryotype aneuploïde non viable. Cette interprétation attend néanmoins d'être confirmée par des études cytologiques.

2.4. Le modèle *mod* / *resc*

La présence de *Wolbachia* chez un mâle provoque la mort des embryons (tout au moins la perte des chromosomes paternels) à moins que la femelle ne soit infectée par la même bactérie. Ce constat est à l'origine du modèle *mod* / *resc*, soulignant le fait que les versants mâle et femelle de l'IC doivent être distingués (Werren 1997). La fonction *mod* (pour modification) serait exprimée dans la lignée germinale mâle pendant la spermatogenèse, avant l'élimination de *Wolbachia* et provoquerait la perte des chromosomes paternels après la fécondation. La fonction *resc* (pour rescue) serait exprimée dans la lignée germinale femelle, et rétablirait la viabilité des embryons.

Cette terminologie est purement conceptuelle et très générale : elle ne contient aucun présupposé quant à la nature des fonctions *mod* et *resc*. Elle sera abondamment utilisée dans le présent mémoire. Le terme "intensité de *mod*" sera utilisé pour décrire l'intensité de l'incompatibilité uni-directionnelle, afin de souligner le fait que ce paramètre ne dépend pas de la fonction *resc*. Notons que l'intensité de *mod* n'intervient pas dans la définition du type de compatibilité, qui dépendra lui de la reconnaissance entre les fonctions *mod* et *resc*.

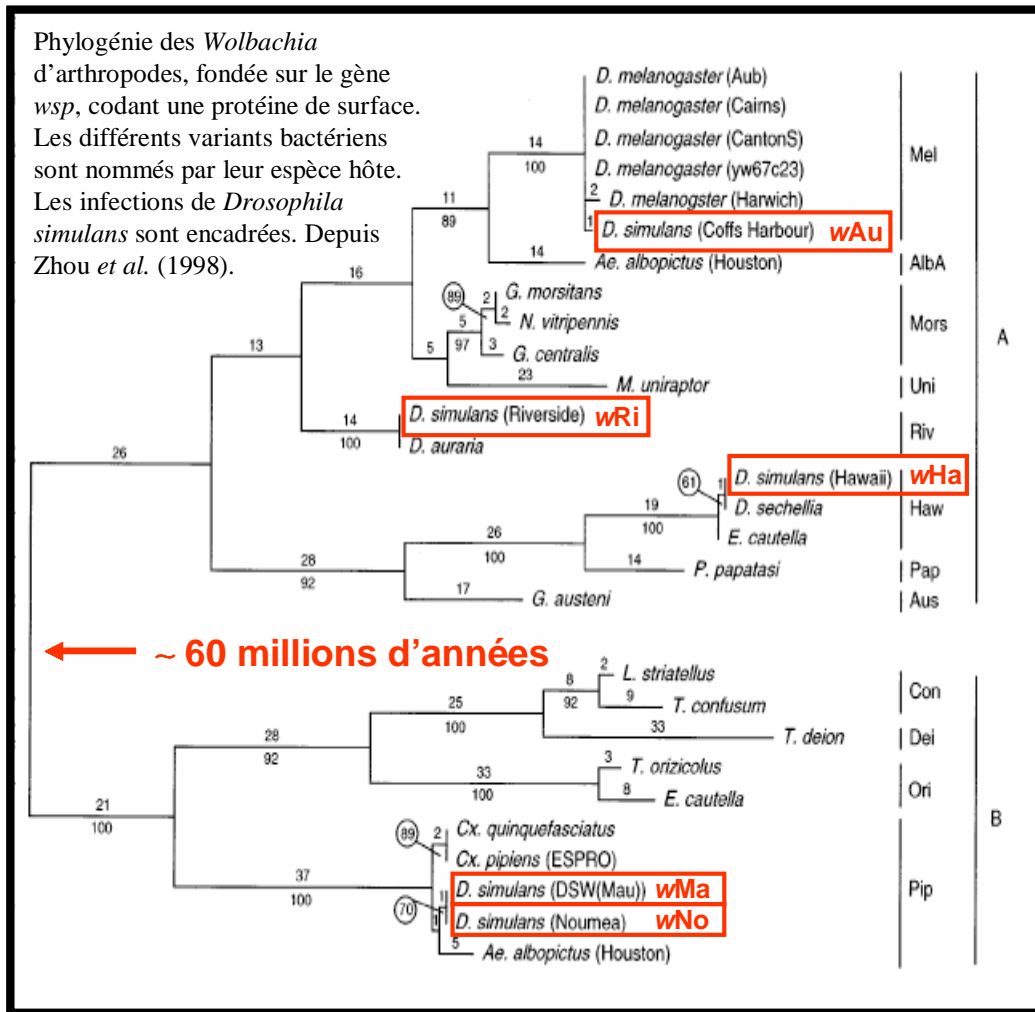
2.5. Diversité et évolution de l'incompatibilité cytoplasmique

Malgré un unique nom d'espèce, *Wolbachia pipientis* (Hertig 1936) représente un clade extrêmement diversifié. Les premières phylogénies, fondées sur l'ARN ribosomal 16S et le gène *ftsZ*, ont révélé un temps de coalescence d'approximativement 60 millions d'années pour les différents variants infectant les arthropodes (O'Neill *et al.* 1992; Werren *et al.* 1995b). Comme l'illustre l'exemple de *Drosophila simulans*, présenté dans la section suivante, cette diversité se manifeste au niveau phénotypique par une importante variation des fonctions *mod* et *resc*. En effet, il apparaît que des *Wolbachia* éloignées phylogénétiquement peuvent différer aussi bien par l'intensité de *mod* que par les types de compatibilité.

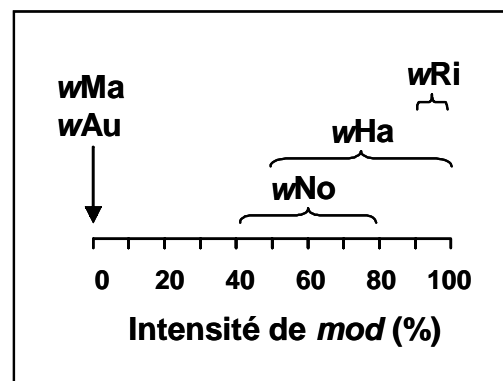
2.5.1. Un exemple parlant : les infections de *Drosophila simulans*

A l'heure actuelle, cinq différents variants ont été décrits chez l'hôte *Drosophila simulans*, espèce modèle étudiée dans notre laboratoire (Merçot & Charlat 2003) : *w*Ri (découvert à Riverside, Californie), *w*Ha (découvert à Hawaï), *w*No (découvert à Nouméa, Nouvelle Calédonie), *w*Au (découvert en Australie) et enfin *w*Ma (découvert à Madagascar). Ces différentes bactéries sont dispersées au sein de la phylogénie des *Wolbachia*, comme l'indique la figure ci-après (Zhou *et al.* 1998). Ce patron est compatible avec l'idée maintenant consensuelle que des transferts horizontaux entre différentes espèces hôtes peuvent se produire (Werren *et al.* 1995b; Heath *et al.* 1999; Vavre *et al.* 1999; Huigens *et al.* 2000).

Phylogénie des *Wolbachia* d'arthropodes, fondée sur le gène *wsp*, codant une protéine de surface. Les différents variants bactériens sont nommés par leur espèce hôte. Les infections de *Drosophila simulans* sont encadrées. Depuis Zhou *et al.* (1998).



Les intensités de leurs fonctions *mod* respectives diffèrent nettement : wMa et wAu n'induisent aucune IC détectable, et les trois autres induisent de 40 à 100% de mortalité embryonnaire (voir figure ci-contre). D'autre part, elles présentent des types de compatibilité différents, les trois variants inducteurs d'IC étant bi-directionnellement incompatibles. Cet exemple nous conduit au thème central de ce mémoire : l'étude des processus sous-jacents à la diversification des fonctions *mod* et *resc*.



2.5.2. Evolution de l'intensité de *mod*

L'intensité de *mod* est un paramètre mesurable expérimentalement par des croisements entre mâles infectés et femelles non infectées. Il s'agit donc d'un phénotype, dépendant en tant que tel des déterminants génétiques de la bactérie mais également des conditions environnementales. Des expériences de transferts artificiels de *Wolbachia* entre différentes espèces ont permis de mettre en évidence que le génome de l'hôte pouvait être une composante majeure de la variance environnementale de ce phénotype. Ainsi, *w*Ri induit plus de 90% de mortalité embryonnaire dans son hôte naturel *Drosophila simulans*, mais seulement 30% après injection dans *Drosophila melanogaster* (Boyle *et al.* 1993). De même, le variant *w*Mel induit environ 30% de mortalité embryonnaire dans son hôte naturel *Drosophila melanogaster*, contre 95% après injection dans *Drosophila simulans* (Poinsot *et al.* 1998). Pour comprendre l'évolution de l'intensité de *mod*, il convient donc de s'intéresser à fois aux facteurs hôtes et bactériens.

2.5.2.1. Evolution des facteurs bactériens

Une forte intensité de *mod* facilite l'invasion des populations et le maintien de l'infection : elle réduit la valeur de l'équilibre instable (la fréquence seuil d'invasion) et augmente la valeur de l'équilibre stable. Beaucoup moins intuitive est la conclusion selon laquelle, au sein des populations infectées, les facteurs bactériens déterminant l'intensité de *mod* ne sont pas soumis à sélection (Prout 1994; Turelli 1994). Pour la comprendre, il faut garder à l'esprit que la valeur sélective d'une *Wolbachia* ne dépend que de son effet sur les femelles : l'induction d'une mortalité embryonnaire élevée par un mâle infecté augmente la fréquence de toutes les bactéries portant la fonction *resc* adéquate, mais ne bénéficie pas particulièrement au clone bactérien responsable. A l'extrême, une bactérie perdant totalement sa capacité à induire de l'IC, mais conservant une fonction *resc* intacte (phénotype [*mod-resc*+]) n'est pas contre sélectionnée. En accord avec cette analyse, une évolution cyclique de l'intensité de *mod* a été suggérée (Hurst & McVean 1996) : (i) invasion des populations par un phénotype [*mod+resc*+] , (ii) fixation d'un phénotype [*mod-resc*+] , (iii) perte de l'infection due à la réduction de l'intensité de *mod* (précédée ou non de la perte de la fonction *resc*). A l'appui de ce scénario, le phénotype [*mod-resc*+] a par la suite été découvert chez *Drosophila simulans* : la *Wolbachia w*Ma, bien que n'induisant pas d'IC, protège de la mortalité induite par sa proche parente *w*No (Merçot & Poinsot 1998; Poinsot & Mercot 1999). Dans le cadre de cette hypothèse, le maintien de l'IC chez les arthropodes serait uniquement du à la composante "invasion de nouvelle population" : seuls les clones bactériens induisant une forte mortalité embryonnaire peuvent envahir de nouvelles populations (par transferts horizontaux ou par migrations) avant la perte intra-populationnelle de l'infection.

D'autres travaux révèlent cependant qu'un facteur important est négligé dans ces analyses : la structuration des populations (Frank 1998). En substance, si la structuration des populations n'est pas nulle, des intensités de *mod* élevées peuvent être maintenues par sélection de parentèle. Trois points sont nécessaires à la compréhension de cette conclusion : (i) dans une population structurée, l'apparementement entre clones bactériens est plus important au sein des sous-populations (les patches) qu'entre les sous-populations, (ii) un variant induisant une forte mortalité embryonnaire augmente la fréquence d'infection dans sa propre sous-population, et finalement (iii) les cytoplasmes infectés "débordent" des sous-populations infectées à haute fréquence vers les sous-populations infectées à basse fréquence.

Pour résumer, les déterminants bactériens de l'intensité de *mod* ne sont pas soumis à sélection dans des populations non structurées. En revanche, une forte intensité de *mod* est sélectionnée dans les populations structurées. Mentionnons finalement la suggestion selon laquelle les variants présentant une forte intensité de *mod* peuvent être également délétères pour les femelles qui les portent (Turelli 1994) ; suggestion fondée sur l'hypothèse (non vérifiée) qu'un fort coût métabolique et une forte intensité de *mod* résultent tous deux d'une densité bactérienne élevée. Sous cette dernière hypothèse, les variants présentant une forte intensité de *mod* sont contre-sélectionnés. En d'autres termes, la structuration des populations et la relation coût / intensité de *mod* imposent des pressions de sélections opposées.

2.5.2.1. Evolution des facteurs hôtes

Au sein d'une population infectée, si la fréquence stable d'infection est inférieure à 1, des croisements incompatibles (entre mâles infectés et femelles non infectées) se produisent à chaque génération. Les facteurs hôtes réduisant la mortalité embryonnaire dans la descendance de tels croisements sont sélectionnés (Turelli 1994). En théorie, cette réduction peut procéder de deux phénomènes bien distincts : (i) une diminution directe de l'intensité de *mod* par les mâles infectés et (ii) l'apparition d'une fonction hôte de type *resc* chez les femelles non infectées. L'analyse de la distribution de *Wolbachia* au cours de la spermatogenèse chez *Drosophila simulans* et *Drosophila melanogaster* suggère plutôt un contrôle direct de l'intensité de *mod* par les mâles infectés (Poinot *et al.* 1998). En effet, chez *Drosophila simulans*, où l'intensité de *mod* est forte, *Wolbachia* est présente dans une grande proportion des spermatocystes. Au contraire, chez *Drosophila melanogaster*, où l'intensité de *mod* est faible, *Wolbachia* n'est présente que dans une faible proportion des spermatocystes.

Pour résumer, si la fréquence d'infection à l'équilibre est inférieure à 1, la sélection sur les facteurs hôtes favorise une réduction de l'intensité de *mod*. Les effets combinés de l'évolution des facteurs hôtes et bactériens sur l'évolution de l'intensité de *mod* sont difficiles à prévoir. On suspecte cependant qu'en l'absence d'une forte structuration des populations,

la tendance sera globalement à la baisse, et ce d'autant plus si le maintien d'une forte intensité de *mod* se fait au prix d'un coût métabolique sur les femelles infectées.

2.5.3. Evolution des types de compatibilité

L'incompatibilité bi-directionnelle démontre la spécificité de l'interaction *mod* / *resc*. Pour expliquer l'existence de plusieurs types de compatibilité, deux hypothèses peuvent être proposées : (i) des origines indépendantes ou (ii) la divergence de plusieurs types de compatibilité à partir d'un type ancestral. La première hypothèse n'est pas exclue, mais apparaît comme la moins parcimonieuse. Elle implique en effet des apparitions multiples du phénomène d'IC au cours de l'évolution des *Wolbachia*, menant chaque fois à un type de compatibilité particulier. Nous l'écartons donc pour nous attacher à la seconde.

Dans le cadre d'une étude plus générale, Turelli (1994) s'est penché sur l'évolution des types de compatibilité, sans toutefois y apporter une explication suffisante. En accord avec des travaux plus anciens démontrant que la valeur sélective d'un type de compatibilité dépend positivement de sa fréquence (Rousset *et al.* 1991), Turelli note en effet qu'un type de compatibilité mutant apparaissant au sein d'une population infectée est fortement contre-sélectionné. En d'autres termes, son analyse suggère implicitement que les types de compatibilité sont évolutivement stables. L'explication de l'évolution des types de compatibilité constitue un des éléments principaux de ce mémoire.

3. Objectifs et méthodes

J'ai développé au cours de ma thèse des approches théoriques et expérimentales pour l'étude de l'évolution de l'IC, et de ses conséquences sur la stabilité des associations *Wolbachia* / hôtes. Le travail de modélisation, est présenté au chapitre 1. Il est principalement fondé sur la mise à profit du modèle *mod* / *resc* pour l'exploration des conditions permettant l'évolution des types de compatibilité. L'étude expérimentale de l'évolution de l'IC est présentée dans le second chapitre. Elle est basée sur un principe général simple : la confrontation, par injections artificielles dans l'espèce modèle *Drosophila simulans*, de bactéries apparentées mais évoluant séparément à l'état naturel. Dans le troisième chapitre, nous nous intéressons plus particulièrement à des variants n'exprimant pas (n'exprimant plus) d'IC (le phénotype [*mod*-]), afin d'étudier les conséquences d'une réduction drastique de l'intensité de *mod* sur la stabilité des associations *Wolbachia* / hôtes.

Chapitre 1.

Approche théorique

Chapitre 1.

Approche théorique

Dans ce chapitre sont présentés les résultats de notre analyse théorique de l'évolution de l'IC, fondée sur une innovation principale : l'intégration d'un modèle à deux fonctions, de type *clef-serrure*. Ce modèle est issu d'un examen critique des différents mécanismes proposés dans la littérature comme explications possibles de l'IC. Cette analyse, initiée par Denis Poinot et complétée au cours de ma thèse a donné lieu à la rédaction de l'article N°8, présenté en annexe 2. Pour résumer, trois modèles moléculaires ont été proposés : le modèle "*ralentisseur*" (Callaini *et al.* 1997; Tram & Sullivan 2002), le modèle "*titration-restitution*" (Kose & Karr 1995) et le modèle "*clef-serrure*" (Hurst 1991). Selon le modèle *ralentisseur*, l'induction de mortalité embryonnaire (la fonction *mod*) serait liée à un ralentissement de la cinétique des chromosomes d'origine paternelle lors de la première division cellulaire. Le sauvetage des embryons (la fonction *resc*) résulterait d'un effet identique sur les chromosomes d'origine maternelle. La compatibilité entre mâles et femelles infectés par la même bactérie résulterait donc simplement d'une mise en phase des chromosomes paternels et maternels. Selon le modèle *titration-restitution*, *Wolbachia* perturberait les chromosomes paternels en séquestrant des molécules nécessaires au déroulement normal de la première mitose (la *titration*). Dans l'œuf infecté, *Wolbachia* aurait une action identique *avant la fécondation*, ce qui lui permettrait de libérer la molécule manquante *après la fécondation* (la *restitution*), restaurant ainsi la fonctionnalité des deux lots chromosomiques. Finalement, le modèle *clef-serrure* postule que l'IC procède de la fixation d'une molécule bactérienne (la *serrure*) sur les chromosomes paternels pendant la spermatogenèse, et de la production dans l'œuf d'une autre molécule bactérienne (la *clef*), rétablissant la fonctionnalité des chromosomes paternels par une inhibition directe de l'action de la molécule serrure.

Confrontés aux différentes propriétés de l'IC (incompatibilité bi-directionnelle, effets des multi-infections, phénotype [*mod-resc+*], etc.), les modèles *ralentisseur* et *titration-restitution* rencontrent des difficultés, rendant nécessaire un certain nombre d'hypothèses supplémentaires, telles que la fixation des produits bactériens sur une grande variété de

cibles, ou l'extinction sexe-spécifique de leur expression. Au contraire, le modèle *clef-serrure* nous est apparu satisfaisant et parcimonieux. Ce modèle a d'importantes implications concernant l'évolution de l'IC. En particulier, en postulant que les fonctions *mod* et *resc* sont contrôlées par des gènes différents, il ouvre la possibilité de variations indépendantes de ces deux fonctions. C'est en analysant les conséquences de telles variations que nous avons élaboré le modèle présenté dans ce chapitre.

Article N°1.

On the *mod resc* model, and the evolution of *Wolbachia* compatibility types

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Genetics, **159**:1415-1422.

En bref...

L'incompatibilité bi-directionnelle démontre la spécificité de l'interaction mod / resc et la diversité des types de compatibilité. Nous nous intéressons ici aux processus évolutifs sous-jacents à cette diversité. Nous proposons un modèle fondé sur l'hypothèse que les fonctions mod et resc sont contrôlées par des gènes différents, et peuvent par conséquent varier indépendamment. Selon ce modèle, le remplacement d'un type de compatibilité mod_{ArescA} par un type mod_{BrescB} se fait en deux étapes. La première implique l'apparition d'un mutant de type mod_{BrescA}, et son augmentation de fréquence sous l'effet de la dérive. Cette dérive est possible car les variations affectant la fonction mod ne sont pas soumises à sélection. La seconde étape est la fixation par sélection d'un type mod_{BrescB}, rendue possible par la présence au sein de la population du variant mod_{BrescA}. Nous montrons qu'un processus similaire peut mener au maintien stable de Wolbachia "suicides" de types mod_{BrescA} et mod_{ArescB}.

On the *mod resc* Model and the Evolution of Wolbachia Compatibility Types

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ABSTRACT

Cytoplasmic incompatibility (CI) is induced by the endocellular bacterium Wolbachia. It results in an embryonic mortality occurring when infected males mate with uninfected females. The mechanism involved is currently unknown, but the *mod resc* model allows interpretation of all observations made so far. It postulates the existence of two bacterial functions: modification (*mod*) and rescue (*resc*). The *mod* function acts in the males' germline, before Wolbachia are shed from maturing sperm. If sperm is affected by *mod*, zygote development will fail unless *resc* is expressed in the egg. Interestingly, CI is also observed in crosses between infected males and infected females when the two partners bear different Wolbachia strains, demonstrating that *mod* and *resc* interact in a specific manner: Two Wolbachia strains are compatible with each other only if they harbor the same compatibility type. Here we focus on the evolutionary process involved in the emergence of new compatibility types from ancestral ones. We argue that new compatibility types are likely to evolve under a wider range of conditions than previously thought, through a two-step process. First, new *mod* variants can arise by mutation and spread by drift. This is possible because *mod* is expressed in males and Wolbachia is transmitted by females. Second, once such a *mod* variant achieves a certain frequency, it can create the conditions for the deterministic invasion of a new *resc* variant, allowing the invasion of a new *mod resc* pair. Furthermore, we show that a stable polymorphism might be maintained in natural populations, allowing the long-term existence of "suicidal" Wolbachia strains.

CYTOPLASMIC incompatibility (CI; reviewed in HOFFMANN and TURELLI 1997; CHARLAT *et al.* 2001) is induced by the maternally inherited endocellular bacterium Wolbachia, widespread in Arthropods (WERREN *et al.* 1995; JEYAPRAKASH and HOY 2000). This phenomenon results in a more or less intense host embryonic mortality, occurring when infected males mate with uninfected females, while the three other types of crosses are fully fertile (unidirectional incompatibility, Figure 1A). As a consequence of unidirectional incompatibility, infected females are normally fertile when mating with both infected and uninfected males, while uninfected females suffer a fertility deficit when mating with infected males. The more frequent the infected males, the more frequent are the crosses detrimental to uninfected females. Because Wolbachia is transmitted by females only, infected cytoplasm is selected for in a positively frequency-dependent manner, allowing the bacterium to spread through the population and then maintain itself. Considering the invasion dynamics in more detail, theoretical analysis (CASPARI and WATSON 1959; FINE 1978; HOFFMANN *et al.* 1990), together with empirical data (TURELLI and HOFFMANN 1995), highlighted the importance of three main parameters: (i)

CI level (the percentage of embryos killed by CI in incompatible crosses); (ii) the fitness effect of infection on hosts (apart from CI); and (iii) the bacterial transmission efficiency from mothers to offspring. The above studies showed that the frequency of infected individuals presents a stable equilibrium depending on these three parameters. This stable equilibrium frequency is 1 if maternal transmission is perfect and CI level exceeds 0% or if CI level is 100%. Furthermore, the infection frequency can only increase toward this equilibrium value if it first reaches a threshold frequency, the level of which also depends upon these three parameters.

The mechanism of CI induction is currently unknown. However, the *mod resc* model allows interpretation of the various patterns observed so far (WERREN 1997). It postulates the existence of two bacterial functions: *mod* (for modification) and *resc* (for rescue). The *mod* function acts on the nucleus in the males' germline, before Wolbachia are shed from maturing sperm (PRESGRAVES 2000). If sperm is affected by *mod*, zygote development will fail unless *resc* is expressed in the egg.

Interestingly, CI is also observed in crosses between infected males and infected females, when the two partners bear different Wolbachia strains (O'NEILL and KARR 1990). In such cases, CI occurs in both directions of cross and is thus termed bidirectional (Figure 1B). Bidirectional CI demonstrates that *mod* and *resc* interact in a specific manner. Two Wolbachia strains are compatible with each other only if they harbor the same compat-

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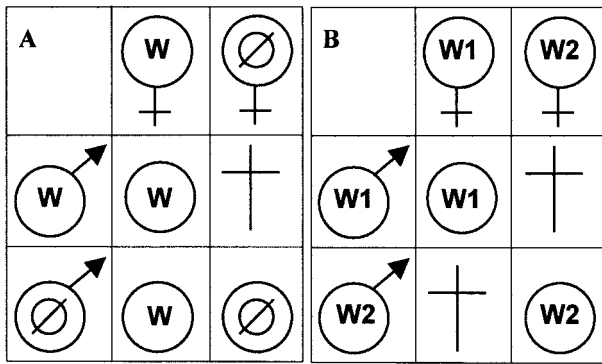


FIGURE 1.—Cytoplasmic incompatibility. Infection statuses of parents and offspring are indicated in circles. Crosses (†) symbolize embryonic mortality. (A) Unidirectional incompatibility. Infected females are fully fertile when mating with infected (*w*) as well as uninfected (\emptyset) males, while embryonic mortality occurs when uninfected females mate with infected males. (B) Bidirectional incompatibility. When males and females are infected, crosses are compatible only if the two partners bear the same *Wolbachia* variant.

ibility type, defined by a given *mod resc* pair. Two hypotheses can be proposed to account for the existence of different compatibility types. First, CI might have emerged many times independently, giving rise to different independent *mod resc* pairs. Alternatively, the different CI systems existing today might derive from one or a few ancestral ones, in which case bidirectionally incompatible strains must have evolved from compatible ancestors. This second hypothesis should be preferred, because it is far more parsimonious. This leaves a problem to solve: How can new compatibility types evolve? This article provides insights into this question.

ARE *MOD* AND *RESC* CONTROLLED BY THE SAME GENES?

A biochemical model has been proposed, according to which *mod* and *resc* are controlled by the same genetic determinant(s) (CALLAINI *et al.* 1997). It is out of the scope of this article to discuss in depth the validity of this model, but let us consider its theoretical consequences on the evolution of compatibility types. If *mod* and *resc* are controlled by the same determinant(s), no asymmetrical changes can occur between the two functions. As a consequence, any *mod resc* mutant is necessarily self-compatible and bidirectionally incompatible with the original strain (fully or only partially). Previous models on the dynamics of bidirectionally incompatible strains showed that a variant cannot invade when rare (ROUSSET *et al.* 1991; FRANK 1998). Thus, if *mod* and *resc* are controlled by the same determinant(s), new compatibility types cannot invade, unless selection is counteracted by stochastic events. One might suggest that the spread of such *mod resc* mutants is facilitated if the mutant clones are at the same time advantaged in

terms of transmission efficiency and/or fitness effects to the host (a similar, but not strictly identical, proposition is given in TURELLI 1994). However, there is no *a priori* reason to think that mutations affecting compatibility types should also affect transmission efficiency and/or fitness effects.

Actually, some empirical evidence suggests that different genes control the *mod* and *resc* functions. Indeed, some *Wolbachia* strains that are unable to induce CI but are capable of rescuing it were discovered (BOURTZIS *et al.* 1998; MERÇOT and POINSOT 1998; POINSOT and MERÇOT 1999). This finding strongly suggests that *mod* and *resc* are genetically separate: if not different genes, at least different gene domains. WERREN (1998), discussing the process involved in the evolution of compatibility types, assumed that asymmetrical changes could occur between *mod* and *resc*. Thus, although not explicitly stated, *mod* and *resc* are considered as genetically separate. Werren argued that the emergence of a new compatibility type can occur through an intermediate stage, involving a mutant able to rescue its own CI as well as the one induced by the resident bacterium. If *mod* and *resc* are considered independently, two mutations are necessary for such a bacterium to emerge: (i) one change in the *mod* function (making the original strain unable to rescue the CI induced by the mutant bacterium) and (ii) one change in the *resc* function, allowing the mutant bacterium to rescue both its own CI and the original strain's one. Such double mutations are highly unlikely. As a consequence, Werren's explanation (in its present form and following our interpretation) is not fully satisfactory. We describe below a process that allows the emergence of new compatibility types under a wider range of conditions, which is based on the hypothesis that *mod* and *resc* are genetically separate.

NOTATIONS AND ASSUMPTIONS

For the purpose of this article, *Wolbachia* strains are defined by four parameters: *mod compatibility* (mod_c), *mod intensity* (mod_i), *resc compatibility* ($resc_c$), and *resc intensity* ($resc_i$). mod_c and $resc_c$ are qualitative traits that define the compatibility type. mod_i is a quantitative trait referring to the frequency of embryo death in incompatible crosses. mod_i can vary from 0 (CI level = 0%) to 1 (CI level = 100%). Finally, $resc_i$ is a quantitative trait referring to the frequency of rescued embryos when the compatibility between mod_c and $resc_c$ is complete. $resc_i$ can vary from 0 (nonfunctional *resc*) to 1 (fully functional *resc*).

To illustrate our notation, let us describe the following strain, referred to as "strain 0" (S0) in the sections below. Its properties are noted as follows: For $M_{A,y}R_{A,z}$, *M* refers to *mod*; the two subscripts refer to mod_c (capital letter) and mod_i (small letter), respectively. *R* refers to *resc*; the two subscripts give $resc_c$ (capital letter) and $resc_i$ (small letter), respectively. A given mod_c is compatible

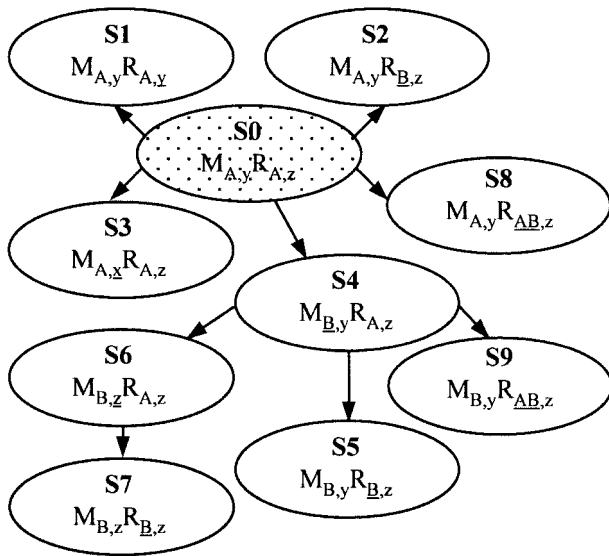


FIGURE 2.—Identity of the different Wolbachia variants and mutational relationships between them. New mutations are underlined.

with a given *resc_c* if *M* and *R* bear the same capital subscript (*i.e.*, *M_{A,y}* is compatible with *R_{A,x}*, *R_{A,y}*, or *R_{A,z}*). Thus, in subscripts, capital letters refer to qualitative traits (A or B in the sections below, with *M_A* ≠ *M_B* and *R_A* ≠ *R_B*), and small letters refer to quantitative traits (*x* or *y* or *z* in the sections below, with $0 \leq M_x < M_y < M_z \leq 1$ and $0 \leq R_x < R_y < R_z \leq 1$).

We analyze the emergence of new compatibility types under the following list of assumptions:

1. Any mutation affecting *mod_c* or *resc_c* renders these two totally incompatible (no partial compatibility).
2. As previously mentioned, we suppose that *mod* (*i.e.*, *mod_c* + *mod_i*) is independent from *resc* (*i.e.*, *resc_c* + *resc_i*). Furthermore,
3. *mod_i* is independent from *mod_c*, as well as *resc_i* from *resc_c*.
4. Mutations affecting *mod* and *resc* do not interfere with the efficiency of maternal transmission or the effect of Wolbachia on host fitness (although maternal transmission might not be perfect and Wolbachia might have an effect on host fitness).
5. Recombination between Wolbachia strains cannot occur.
6. A given individual host is homogeneous with regard to Wolbachia infections (when a mutation gives rise to a new clone, its host is infected by this clone only). Finally,
7. host populations are considered as panmictic,
8. with unbiased sex ratio, and
9. nonoverlapping generations.

The results discussed below are qualitatively robust to relaxing assumptions 1 and 3 (data not shown).

A	$\begin{matrix} \text{S0} \\ M_A R_A \end{matrix}$	$\begin{matrix} \text{S2} \\ M_A R_B \end{matrix}$
	+	+
$\begin{matrix} \text{S0} \\ M_A R_A \end{matrix}$	$\begin{matrix} \text{S0} \\ M_A R_A \end{matrix}$	+
$\begin{matrix} \text{S2} \\ M_A R_B \end{matrix}$	$\begin{matrix} \text{S0} \\ M_A R_A \end{matrix}$	+
B	$\begin{matrix} \text{S0} \\ M_A R_A \end{matrix}$	$\begin{matrix} \text{S4} \\ M_B R_A \end{matrix}$
	+	+
$\begin{matrix} \text{S0} \\ M_A R_A \end{matrix}$	$\begin{matrix} \text{S0} \\ M_A R_A \end{matrix}$	$\begin{matrix} \text{S4} \\ M_B R_A \end{matrix}$
$\begin{matrix} \text{S4} \\ M_B R_A \end{matrix}$	+	+

FIGURE 3.—Patterns of compatibility when *resc_c* or *mod_c* are affected. Infection statuses of parents and offspring are indicated in circles. Crosses (+) symbolize embryonic mortality. For simplicity, *mod_i* and *resc_i* are not shown. (A) Patterns of compatibility between S0 and S2. Note that females bearing S2 suffer a fertility deficit when mating with both types of males, so that S2 is counterselected. (B) Patterns of compatibility between S0 and S4. Note that females bearing S0 and females bearing S4 show the same compatibility patterns, so that S0 and S4 have the same fitness.

EVOLUTIONARY FORCES ACTING ON MOD AND RESC VARIATIONS

Variations of *resc*: Let us first discuss the probable fate of variations affecting the *resc* function. Consider a host population (population 1, harboring a unique Wolbachia strain S0, *M_{A,y}R_{A,z}*; Figure 2) and a strain S1 (*M_{A,y}R_{A,y}*, with *R_y* < *R_z*) arising by a mutation affecting the *resc_i* function of an S0 bacterium (Figure 2). S1 is selected against since females bearing S1 suffer a fertility deficit when mating with males infected by S0 or S1. Similarly, a strain S2 (*M_{A,y}R_{B,z}*, with *R_B* ≠ *R_A*) arising by a mutation affecting the *resc_c* function of an S0 bacterium (Figure 2), would be eliminated. Indeed, as illustrated in Figure 3A, females bearing S2 are not fully fertile when mating with males infected by S0 or S2. Thus, the efficiency of *resc* is expected to be optimized: Any reduction of *resc_i* or change in *resc_c* is limited by selection. As a consequence, the evolution of new compatibility types cannot start from changes in the *resc* function. As discussed below, variations affecting the *mod* function are far less constrained.

Variations of *mod*: Consider population 1 (infected by S0, *M_{A,y}R_{A,z}*) and a strain S3 (*M_{A,x}R_{A,z}*, with *M_x* < *M_y*), arising by a mutation affecting the *mod_i* function of an S0 bacterium (Figure 2). In crosses involving infected males and uninfected females, S3 will induce a lower CI than S0. As a consequence, the overall infection frequency will decrease (indeed, if maternal transmission is not perfect, the infection frequency at equilibrium depends on CI level). However, given that *resc* is not affected, females bearing S3 and females bearing S0 are equally compatible with all types of males. Thus, S3 and S0 have the same fitness: As previously stated (PROUT 1994; TURELLI 1994), variations of *mod_i* are neutral. Note, however, that such a conclusion has to be

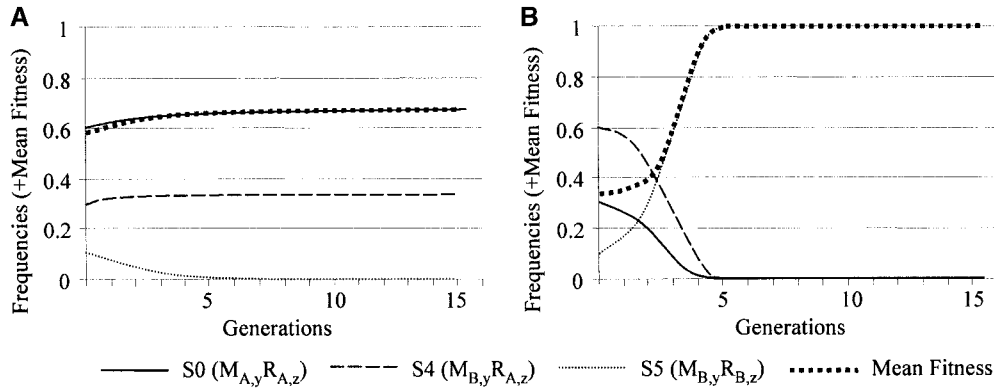


FIGURE 4.—Fate of S5 when occurring in population 2 (harboring S0 and S4). These numerical examples were obtained with the following conditions: $M_y = 1$; $R_z = 1$; overall infection frequency = 1; perfect maternal transmission; no cost to the host. Algebraic details are in APPENDIX A. (A) Initial situation 1: $f(M_B) < f(M_A)$. $f(S0) = 0.6$; $f(S4) = 0.3$; $f(S5) = 0.1$. (B) Initial situation 2: $f(M_B) > f(M_A)$. $f(S0) = 0.3$; $f(S4) = 0.6$; $f(S5) = 0.1$.

tempered if the host population is structured. Indeed, in structured populations, high CI levels are selected for through a kin selection process (FRANK 1997).

What about variations affecting mod_c ? Consider population 1 (infected by S0, $M_{A,y}R_{A,z}$) and a strain S4 ($M_{B,y}R_{A,z}$, with $M_B \neq M_A$) arising by a mutation affecting the mod_c function of an S0 bacterium (Figure 2). As illustrated in Figure 3B, fertility is reduced in crosses between males bearing S4 and females bearing S4 or S0. However, given that the $resc$ function did not change, females bearing S4 or S0 are equally compatible with all types of males. Thus, S4 and S0 have the same fitness: Variations of mod_c are neutral. This provides conditions for the emergence of new compatibility types, which we now consider.

THE EMERGENCE OF NEW COMPATIBILITY TYPES

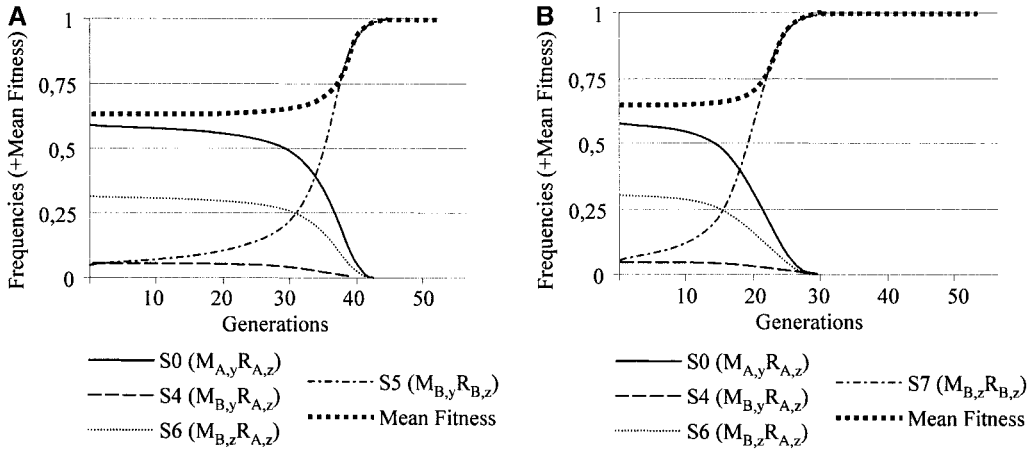
Consider a host population (population 2, harboring S0, $M_{A,y}R_{A,z}$, and S4, $M_{B,y}R_{A,z}$). The relative proportion of these two bacterial variants changes through genetic drift only. Consider a strain S5 ($M_{B,y}R_{B,z}$, with $R_B \neq R_A$), self-compatible, arising by a mutation affecting the $resc_c$ function of an S4 bacterium (Figure 2). As illustrated in Figure 4A, S5 is counterselected if the frequency of M_A variants exceeds that of M_B variants, that is, if $f(S0) > f(S4 + S5)$. In contrast, S5 will invade the population deterministically if $f(M_B) > f(M_A)$ (Figure 4B). Simply speaking, the bacteria selected for are the ones bearing the $resc_c$ function compatible with the most frequent mod_c function. Thus, provided that drift resulted in $f(S4)$ exceeding $f(S0)$, any S5 strain will deterministically invade the population, leading to a shift of compatibility type from $M_A R_A$ to $M_B R_B$. Let us emphasize that this process does not imply several simultaneous mutational events. Note also that, at any time, natural populations are likely to be polymorphic with regard to mod_c , given that variations are neutral. If several mod_c functions co-exist, a new compatibility type will invade as soon as the appropriate mutation affecting $resc_c$ occurs in a Wolbachia bearing the most frequent mod_c function.

Invasion by a new compatibility type may be facilitated by mutations affecting mod_i . Indeed, consider population 2 (harboring S0, $M_{A,y}R_{A,z}$, and S4, $M_{B,y}R_{A,z}$) and a strain S6 ($M_{B,z}R_{A,z}$, with $M_z > M_y$) arising by a mutation affecting the mod_i function of an S4 bacterium (Figure 2). In such a population (population 3, harboring S0, S4, and S6), the relative proportion of the three variants changes through genetic drift only. If S5 ($M_{B,y}R_{B,z}$) occurs in population 3, it may invade the population even if $f(M_B) < f(M_A)$, as illustrated in Figure 5A. The bigger the difference between M_z and M_y , the lower the frequency of M_B that must be reached for S5 to invade deterministically.

Interestingly, the process involved in the shift to a new compatibility type might also lead to an overall increase of CI levels. Indeed, consider population 3, harboring S0 ($M_{A,y}R_{A,z}$), S4 ($M_{B,y}R_{A,z}$), and S6 ($M_{B,z}R_{A,z}$). Consider now that instead of S5 ($M_{B,y}R_{B,z}$, bearing M_y), a strain S7 ($M_{B,z}R_{B,z}$, with $R_B \neq R_A$) arises by a mutation affecting the $resc_c$ function of an S6 bacterium (Figure 2). This strain invades population 3 in the same general conditions as S5, as described in the above paragraph, although more rapidly (Figure 5B). However, in the present case, the CI level is finally higher than in the previous situation, given that $M_z > M_y$. Thus, the process involved in the evolution of compatibility types might not simply be facilitated by mutations increasing mod_i ; it might also induce by itself an increase of CI level. Higher transmission efficiency or lower cost to the host might also favor the spread of new compatibility types. However, these two parameters are expected to be optimized by selection in natural populations (TURELLI 1994) so that mutants with increased transmission efficiency or decreased cost to the host are less likely to appear than mutants with increased CI level.

EVOLUTION AND STABLE MAINTENANCE OF SUICIDAL WOLBACHIA

Consider population 2 (harboring S0, $M_{A,y}R_{A,z}$, and S4, $M_{B,y}R_{A,z}$) and the strain S2 ($M_{A,y}R_{B,z}$, with $R_B \neq R_A$)



Note that S5 invades although $f(M_B) < f(M_A)$, because $M_z > M_y$. (B) S7 occurs in a population harboring S0, S4, and S6. In the initial situation, $f(S0) = 0.59$; $f(S4) = 0.05$; $f(S6) = 0.31$; $f(S7) = 0.05$. Note that S7 invades although $f(M_B) < f(M_A)$, because $M_z > M_y$.

FIGURE 5.—Consequences of mod intensity variations on the emergence of new compatibility types. These numerical examples were obtained with the following conditions: $M_y = 0.6$; $M_z = 1$; $R_z = 1$; overall infection frequency = 1; perfect maternal transmission; no cost to the host. Algebraic details are in APPENDIX B. (A) S2 occurs in a population harboring S0, S4, and S6. In the initial situation, $f(S0) = 0.59$; $f(S4) = 0.05$; $f(S6) = 0.31$; $f(S5) = 0.05$.

arising by a mutation affecting the $resc_C$ function of an S0 bacterium (Figure 2). Remember that S2, when arising in population 1 (infected by S0 only) is selected against. Different outcomes may occur in population 2. As illustrated in Figure 6A, S2 is lost if $f(M_A) > f(M_B)$, that is, if $f(S0 + S2) > f(S4)$. In contrast, the S2 frequency will increase if $f(M_A) < f(M_B)$. Indeed, if $f(M_A) < f(M_B)$, S2 bears the $resc_C$ function compatible with the most frequent mod_C . As $f(S2)$ increases, $f(M_B)$ decreases and $f(M_A)$ increases, until $f(M_A) = f(M_B)$; that is, $f(S0 + S2) = f(S4)$, which is a stable equilibrium (Figure 6B). The population (population 4, harboring S0, S2, and S4) thus presents a stable polymorphism of Wolbachia strains: one self-compatible strain (S0) and two “suicidal” strains (S4 and S2), unable to rescue their own CI phenotype, but able to rescue the one induced by another strain. This polymorphism is stable in that any deviations of frequencies are limited by selection. However, note that the equilibrium might be broken if an S5 ($M_{B,y}R_{B,z}$) strain occurs in population 4, as S5 could invade the population.

GENERALIZATION OF THE *RESC* FUNCTION

Consider population 2 (harboring S0, $M_{A,y}R_{A,z}$, and S4, $M_{B,y}R_{A,z}$) and a strain S8 ($M_{A,y}R_{AB,z}$, with $R_{AB} \neq R_A$) arising by a mutation affecting the $resc_C$ function of an S0 bacterium, or a strain S9 ($M_{B,y}R_{AB,z}$, with $R_{AB} \neq R_A$) arising by a mutation affecting the $resc_C$ function of an S4 bacterium (Figure 2). S8, as well as S9, bears a $resc_C$ function compatible both with M_A and M_B . Such strains are selected for, regardless of $f(M_A)$ and $f(M_B)$. In other words, generalization of $resc_C$ is always selected for. Interestingly, POINSOT *et al.* (1998) reported the case of a Wolbachia strain able to rescue two different mod functions, suggesting the existence of such super- $resc$ functions. If S8 gets fixed, selection on R_B is relaxed, which might eventually lead to its loss. Similarly, if S9 gets fixed, R_A might eventually be lost, leading to a shift of compatibility type from $M_A R_A$ to $M_B R_B$.

This process, involving an intermediate Wolbachia strain harboring a specific mod_C and a “double” $resc_C$, can be compared to WERREN’S (1998) hypothesis. It is important to stress two original facets of the present

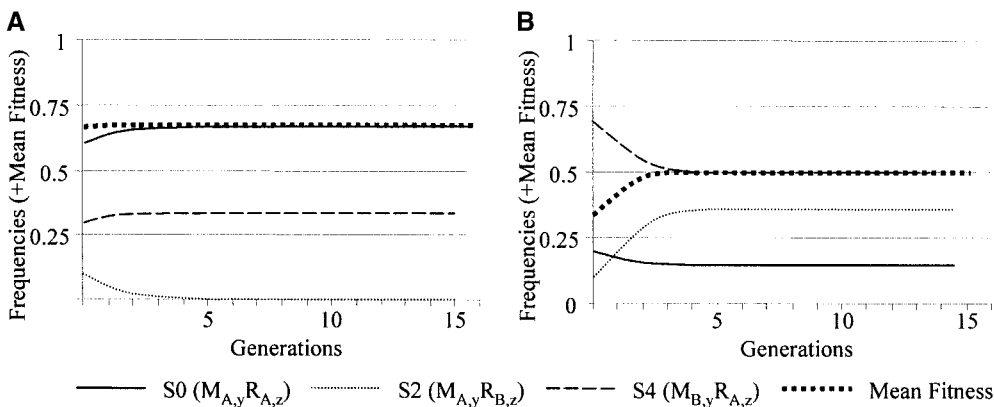


FIGURE 6.—Fate of S2 when occurring in population 2 (harboring S0 and S4). These numerical examples were obtained with the following conditions: $M_y = 1$; $R_z = 1$; overall infection frequency = 1; perfect maternal transmission; no cost to the host. Algebraic details are in APPENDIX C. (A) Initial situation 1: $f(M_B) < f(M_A)$. $f(S0) = 0.6$; $f(S4) = 0.3$; $f(S2) = 0.1$. (B) Initial situation 2: $f(M_B) > f(M_A)$. $f(S0) = 0.2$; $f(S4) = 0.7$; $f(S2) = 0.1$.

proposition, making it more satisfactory: (i) The two mutational events do not have to be simultaneous, since variations of *mod_C* are neutral, and (ii) for a shift of compatibility type to occur, there is no need that two mutations leading to a double *resc_C* function occur in a *different* manner and in *different* populations (isolated in space or in time).

CONSEQUENCES ON HOST MEAN FITNESS

CI can affect host population mean fitness in various ways. First, CI-inducing Wolbachia may be costly to their host (negative effect on host fitness) and yet be maintained at high frequencies through the effect of CI (CASPARI and WATSON 1959). Second, when infection is not fixed, a proportion of crosses within the population are incompatible. Finally, the occurrence of suicidal Wolbachia strains can greatly affect population mean fitness. Any population harboring non-self-compatible strains suffers a mean fitness reduction because of these latter. As an example, population 2, harboring S0 ($M_{A,y}R_{A,z}$) and S4 ($M_{B,y}R_{A,z}$) suffers a mean fitness reduction owing to the presence of the S4 strain (see also Figures 4A and 6A, where the mean fitness is much lower than 1, because of S4). In population 2, S4 frequency varying under drift, the population can eventually go extinct if S4 gets fixed (at fixation, mean fitness = 0 if $M_y = 1$). The stable equilibrium described above (population 4, harboring S0, S2, and S4) is also interesting in this respect. Indeed, as illustrated in Figure 6B, population mean fitness is fixed to 0.5 at equilibrium: On average, half of the eggs do not hatch because of CI (if $M_y = 1$).

Mean fitness reductions of this magnitude are very likely to affect population demography and might render suicidal strains rare, through the extinction of populations bearing them. If suicidal mutants occur frequently, Wolbachia-infected populations might indeed go extinct frequently because of reduced mean fitness. The actual consequences of embryonic mortality caused by CI on population viability will depend on the type of ecological factors limiting population size. If population size is limited mainly by density-dependent factors, such as competition for food, the population demography is likely to be less affected than if population size is limited mainly by non-density-dependent factors. Consequently, Wolbachia infections might be rarer in species where population size is limited mainly by non-density-dependent factors.

CONCLUSION AND PROSPECTS

Our analysis suggests that if *mod* and *resc* are genetically separate, new compatibility types are likely to evolve under a wide range of conditions, through a process involving drift and selection. This being so, compatibility types cannot be considered as evolutionarily stable

in finite populations. Generalization of the *resc* function might represent an intermediate stage in the evolution of new compatibility types, although it is not an indispensable step. Finally, we have shown that stable polymorphism can be maintained, allowing the long-term existence of suicidal Wolbachia strains, with heavy consequences on population mean fitness.

For this analysis, we assumed that when a mutation occurs, the individual host is infected by the mutant clone only [assumption (6)]. The underlying hypothesis is that the effective bacterial population size is very small within an individual host. This assumption might be justified if Wolbachia clones get through tight bottlenecks at every generation, during the germ cells' colonization within the developing embryo. Yet, multiple infections are stably maintained in natural populations (MERÇOT *et al.* 1995; ROUSSET and SOLIGNAC 1995; WERREN *et al.* 1995), suggesting that population size is not that small. Double infections can even be maintained for many generations in experiments where selection for the presence of both strains is relaxed (POINSOT *et al.* 2000). Taking this fact into consideration might reveal interesting features with regard to the evolution of compatibility types.

Future models concerned with the evolution of *mod* and *resc* will undoubtedly have to include nondeterministic processes, as these seem to play a fundamental role. Simulation programs, combining the effects of mutation, selection, and drift, should tell us how plausible are the different outcomes described here. Empirical tests are also required. In particular, the rate at which bidirectional incompatibility evolves must be estimated. For now, complete bidirectional incompatibility has been reported only from evolutionarily distant strains. It should not be hastily concluded from this (lack of) observation that the evolution of compatibility types is a slow process, given that only very few closely related strains have been confronted. This issue could be more deeply investigated through artificial injections of several Wolbachia strains, more or less closely related, within a single host. Finally, if the suicidal Wolbachia is to be found, it will come from the field.

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APPENDIX A: ALGEBRAIC DETAILS FOR FIGURE 4

The fitness of the different variants is the probability that females bearing them mate with compatible males. If $f(S_0) = P$, $f(S_4) = Q$ and $f(S_5) = R$, then

$$W_{S_0} = P, \quad W_{S_4} = P, \quad W_{S_5} = 1 - P,$$

and the population mean fitness is

$$\bar{W} = P(P + Q) + R(1 - P).$$

The frequencies of the different variants at generation $N + 1$ are functions of the frequencies at generation N :

$$P_{N+1} = P_N^2 / \bar{W}$$

$$Q_{N+1} = P_N Q_N / \bar{W}$$

$$R_{N+1} = R_N(1 - P_N) / \bar{W}.$$

APPENDIX B: ALGEBRAIC DETAILS FOR FIGURE 5

Figure 5A: If $f(S_0) = P$, $f(S_4) = Q$, $f(S_6) = T$, and $f(S_5) = R$, then

$$W_{S_0} = W_{S_4} = W_{S_6} = P + (Q + R)(1 - M_y)$$

$$W_{S_5} = Q + T + R + P(1 - M_y),$$

and the population mean fitness is

$$\bar{W} = (P + Q + T)(P + (Q + R)(1 - M_y)) + R(Q + T + R + P(1 - M_y)).$$

The frequencies of the different variants at generation $N + 1$ are functions of the frequencies at generation N :

$$P_{N+1} = P_N(P + (Q + R)(1 - M_y)) / \bar{W}$$

$$Q_{N+1} = Q_N(P + (Q + R)(1 - M_y)) / \bar{W}$$

$$T_{N+1} = T_N(P + (Q + R)(1 - M_y)) / \bar{W}$$

$$R_{N+1} = R_N(Q + T + R + P(1 - M_y)) / \bar{W}$$

Figure 5B: If $f(S_0) = P$, $f(S_4) = Q$, $f(S_6) = T$, and $f(S_7) = R$, then

$$W_{S_0} = W_{S_4} = W_{S_6} = P + Q(1 - M_y)$$

$$W_{S_7} = Q + T + R + P(1 - M_y),$$

and the population mean fitness is

$$\bar{W} = (P + Q + T)(P + Q(1 - M_y)) + R(Q + T + R + P(1 - M_y)).$$

The frequencies of the different variants at generation $N + 1$ are functions of the frequencies at generation N :

$$P_{N+1} = P_N(P + Q(1 - M_y)) / \bar{W}$$

$$Q_{N+1} = Q_N(P + Q(1 - M_y)) / \bar{W}$$

$$T_{N+1} = T_N(P + Q(1 - M_y)) / \bar{W}$$

$$R_{N+1} = R_N(Q + T + R + P(1 - M_y)) / \bar{W}.$$

APPENDIX C: ALGEBRAIC DETAILS FOR FIGURE 6

If $f(S_0) = P$, $f(S_4) = Q$, and $f(S_2) = R$, then

$$\begin{aligned}W_{S_0} &= 1 - Q \\W_{S_4} &= 1 - Q \\W_{S_2} &= Q\end{aligned}$$

and the population mean fitness is

$$\bar{W} = (1 - Q)(P + Q) + QR.$$

The frequencies of the different variants at generation $N + 1$ are functions of the frequencies at generation N :

$$P_{N+1} = P_N(1 - Q_N)/\bar{W}$$

$$Q_{N+1} = Q_N(1 - Q_N)/\bar{W}$$

$$R_{N+1} = Q_N R_N/\bar{W}.$$

Article N°2 (manuscrit en préparation).
Exploring the evolution of *Wolbachia*-induced cytoplasmic incompatibility : a simulation approach

Charlat, S., Calmet, C. & Merçot, H.

En bref...

Nous présentons ici les premiers résultats d'un programme simulant l'évolution de l'IC sous l'effet de la mutation, de la dérive et de la sélection. Cet outil permet d'estimer sous quelles conditions le scénario d'évolution précédemment proposé demeure réaliste. Le modèle est explicitement fondé sur l'hypothèse d'un mécanisme de type clef serrure : la clef et la serrure sont codées par des séquences de caractère, comparables à des séquences nucléiques ou protéiques. Les analyses déjà effectuées suggèrent que la longueur des séquences clef et serrure, ainsi que le nombre d'états possibles pour les caractères qui la composent, sont des paramètres déterminant. Il apparaît également que le taux de mutation affecte non seulement la vitesse, mais également les profils d'évolution des types de compatibilité. Précisons que d'autres analyses sont actuellement en cours, qui viendront compléter le présent manuscrit.

**EXPLORING THE EVOLUTION OF *WOLBACHIA* COMPATIBILITY TYPES:
A SIMULATION APPROACH**

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ABSTRACT

Wolbachia-induced cytoplasmic incompatibility (CI) shows itself when males bearing the bacterium mate with uninfected females, or with females bearing a different *Wolbachia* variant: in such crosses, paternal chromosomes are lost at the first embryonic mitosis, most often resulting in developmental arrest. The molecular bases of CI are currently unknown, but it is useful to distinguish conceptually the male and female sides of this phenomenon: in males, *Wolbachia* must do something before it is shed from maturing sperm, that will disrupt paternal chromosome functionality (this is usually termed “the *mod* function”, for modification); in females, *Wolbachia* must somehow restore embryonic viability, through what is usually called “the *resc* function”, for rescue. The occurrence of embryonic mortality between males and females bearing different *Wolbachia* variants demonstrates that the *mod* and *resc* functions interact in a specific manner; different *mod resc* pairs make different compatibility types. We are interested in the evolutionary process allowing the diversification of compatibility types. In an earlier model, based on the main assumption that the *mod* and *resc* functions are determined by different bacterial genes (and can thus mutate independently), we have showed that compatibility types can evolve through a two step process, the first involving drift on *mod* variations, and the second involving selection on *resc* variations. This previous study has highlighted the need for simulation based models that would include the effects of non deterministic evolutionary forces. The present study is based on a simulation program fulfilling this condition.

Maternally inherited elements are subject to sex-dependent selective pressures: their fitness is increased if females produce more females or better surviving females, regardless of possible detrimental effects to males (Cosmides & Tooby 1981; Frank & Hurst 1996). The endocellular bacterium *Wolbachia* nicely illustrates the possible outcomes of such selection, having evolved a variety of “sex manipulation strategies” that can be interpreted within this theoretical frame (reviewed in O'Neill *et al.* 1997; Stouthamer *et al.* 1999). Cytoplasmic Incompatibility (CI) is one of them, probably the most common (reviewed in Hoffmann & Turelli 1997; Charlat *et al.* 2002). In embryos resulting from crosses between males that bear a CI *Wolbachia*, and females that don't, paternal chromosomes are lost at the first mitosis (Callaini *et al.* 1996; Lassy & Karr 1996; Callaini *et al.* 1997; Tram & Sullivan 2002), resulting in death in most cases (that is, in diploid species), more rarely in male development (in some haplo-diploids) . On the contrary, if the female bears the bacterium, paternal chromosomes are not lost. Infected females thus produce in average more females than uninfected ones, allowing infected cytoplasmic lines to invade uninfected populations. Infected males suffer a fertility deficit if uninfected females remain in the populations, since some proportion of their mating will be partially or fully sterile. But *Wolbachia* does not bother: it is transmitted by females only.

The underlying mechanism remains to be elucidated. In males, *Wolbachia* must somehow affect the paternal nucleus before it is shed from maturing sperm, resulting in paternal chromosomes loss after fertilization. In females, the bacterium must somehow prevent this loss, and thereby rescue the embryo. This conceptual distinction between the male and female sides of CI was formalized by Werren (1997) through the modification / rescue (*mod* / *resc*) terminology.

Interestingly, crosses between infected males and infected females can also be incompatible, if the two partners bear different bacteria. The *mod* and *resc* functions thus seem to interact specifically: different *Wolbachia* can harbor different *mod resc* pairs, that is, different compatibility types. We are interested in the process allowing compatibility types to evolve. In an earlier study (Charlat *et al.* 2001), we showed that compatibility types are not constrained by stabilizing selection, if *mod* and *resc* are determined by different genes, which, we think, is a reasonable assumption (Poinsot *et al.* 2003). We suggested that compatibility types could change through a two steps process: the first involving drift on *mod* variations, and the second involving selection on *resc* variations. This work highlighted the need for simulation-based models that would incorporate the effects of non-deterministic evolutionary

forces. The present paper is based on a simulation program developed in an attempt to fulfill this condition.

THE MODEL

Basically, the model allows to follow the frequency of different *Wolbachia* variants, in their host population, under drift, selection and mutation. The algorithm is described in more details in this section.

What defines a *Wolbachia* variant

Attempts have been made in earlier literature to translate the *mod resc* general formalization into more concrete models (Kose & Karr 1995; Callaini *et al.* 1997; Tram & Sullivan 2002). To our opinion, the most attractive model assumes that *mod* and *resc* can be respectively seen as a lock and a key (Poinsot *et al.* 1998). In fertilized embryos, the lock, fixed on paternal material, would interact with the key, produced by *Wolbachia* in the egg. Depending on the conformation of the lock and the key, compatibility would range from 0 (total incompatibility) to 1 (total compatibility). This view was implicitly the basis of our earlier theoretical work (Charlat *et al.* 2001). The symbolism used here refers to the lock-and-key model more explicitly.

A *Wolbachia* variant is defined here by 5 parameters. Two parameters define the compatibility type: the *Lock* and the *Key*. In practice, *Lock* and *Key* are sequences of n_c characters, with n_s possible states for each site (states 1, 2, ..., n_s). In a cross between a male bearing *Wolbachia* i and a female bearing *Wolbachia* j , a compatibility score (C_{ij}) is calculated as the number of matching sites in the *Lock* / *Key* alignment. For example, with $n_c = 10$, we can have $Lock_i = 2222211111$ and $Key_j = 2222222222$, which gives the compatibility score $C_{ij} = 0.5$. *Lock* and *Key* are comparable to the mod_C and $resc_C$ parameters in our earlier study (Charlat *et al.* 2001), but with the notable difference that the current model allows partial compatibility if $n_c > 1$. *MI* (*mod* intensity) is the efficiency of the *mod* function; it corresponds to what is often referred to as “CI level”, which can be measured in crosses between infected males and uninfected females. Physically, *MI* can be seen as the proportion of infected males’ sperm that is actually affected by CI. *TE* (transmission efficiency) is the proportion of infected eggs among those laid by infected females. Finally, *FE* is the fecundity of infected females relative to that of uninfected females.

It must be noted that the evolution of the TE and FE parameters is not in the focus of the present study, as their evolutionary trajectories have been perfectly described by the analytical approach (Turelli 1994): selection on *Wolbachia* always acts to increase infected females fecundity and maternal transmission efficiency. In other words, starting from any initial condition, these parameters will rapidly reach their maximum values under mutation and selection. However, FE and TE are still relevant to the model, as we can investigate the effect of fixing some maximum values lower than 1 for these two parameters. For example, the model allows to investigate the evolution of the *Lock* and *Key* parameters in populations where TE cannot exceed 0.9, that is, in populations where uninfected individuals can persist.

From one generation to the next

N_e is the number of males and females that actually reproduce at every generation. N_e females and N_e males are randomly chosen as parents on the basis of their frequencies at generation n . Sampling errors allow frequencies to drift. For every parents pair, progeny is determined as illustrated in Figure 1, allowing natural selection to act. The *Wolbachia* variants present at generation $n + 1$ are then submitted to mutation. Thus, generations n and $n + 1$ are separated by a round of drift, selection and mutation. The 5 above listed parameters are allowed to mutate independently from each other. Depending on the questions addressed, different mutation rates (Mu_{rate} : how frequent are mutations), and mutation steps (Mu_{step} : how wide is the effect of mutations) will be used. Throughout the paper, these values will be made explicit when relevant.

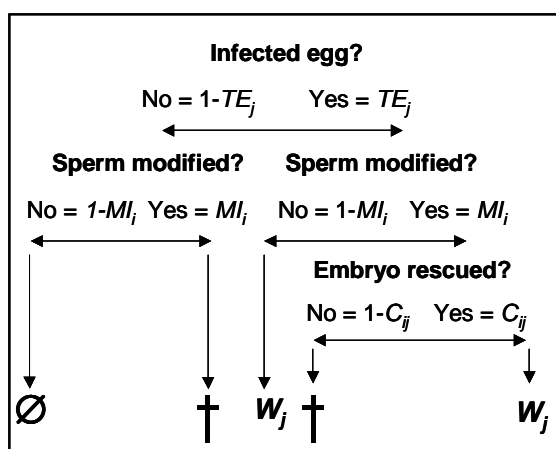


Figure 1.

The Figure illustrates how progeny is determined for any given cross. The male is infected by *Wolbachia* i , with parameters $Lock_i$, Key_i , Mli , TE_i and FE_i (but only Mli and $Lock_i$ are relevant, since the other parameters are not expressed in males). The female is infected by *Wolbachia* j , with parameters $Lock_j$, Key_j , Mlj , TE_j and FE_j (but only Key_j , TE_j and FE_j are relevant, since the other parameters are not expressed in females). If $negg$ is the normal number of eggs laid per female, then E_{dead} (the number of dead embryos; Figure symbol: †), E_{uninf} (the number of living uninfected embryos; Figure symbol: ∅), and E_{inf} (the number of living infected embryos; Figure symbol: W_j) are the following:
 $E_{dead} = negg \times FE [Mli (1-TE_j) + TE_j \times Mli (1-C_{ij})]$
 $E_{uninf} = negg \times FE \times (1-TE_j) \times (1-Mli)$
 $E_{inf} = negg \times FE \times TE_j [(1-Mli) + (Mli \times C_{ij})]$

Assumptions

Our model implies the following list of assumptions: (1) unbiased sex ratio, (2) non overlapping generations, (3) no population structure, (4) random mating, (5) no multiple infections (a given individual host is homogeneous with regard to *Wolbachia* infections; when a mutation gives rise to a new variant, its host is infected by this clone only), (6) no recombination between *Wolbachia* variants, (7) no variations of host effects.

VALIDATING THE MODEL

As a first step, we were interested in testing whether the model was able to retrieve earlier results, obtained through the analytical approach. We first verified the basic prediction that CI allows *Wolbachia* to invade uninfected host populations, and more efficiently so if *MI* is high (Figure 2). We then investigated the combined effects of *MI*, *TE* and *FE* on invasion dynamics. Caspari and Watson (1959), Fine (1978) and Hoffmann *et al.* (1990) showed that if *TE* and / or *FE* differ from 1, *Wolbachia* does not invade unless it first reaches a threshold frequency depending on *MI*, *TE* and *FE*. Above that point, infection frequency increases toward a stable infection frequency, which is not fixation if *MI* and *TE* are lower than 1. As illustrated in Figure 3, the simulation and analytical approaches provide perfectly congruent predictions.

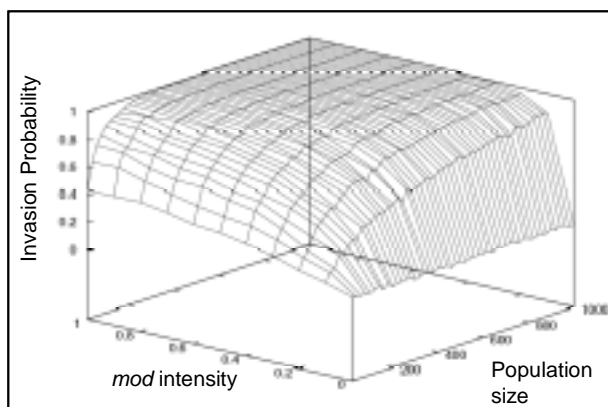


Figure 2.

The Figure is a plot of invasion probability (estimated as the number of times, over 1000 runs where *Wolbachia* finally got fixed) as a function of CI level (*MI*) and population size, for an initial frequency of 0.1. As expected, we observe that stronger CI will allow *Wolbachia* to invade populations more efficiently. This is best seen for big populations, where drift has negligible effect as compared to selection.

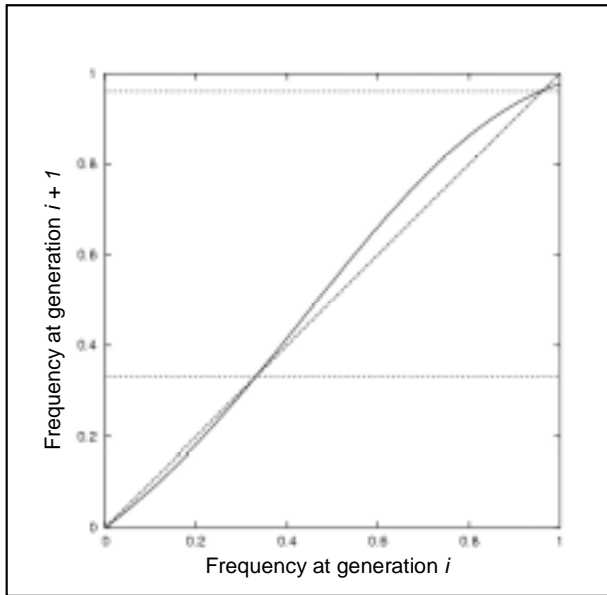


Figure 3. The curve is a plot of infection frequency at generation $i + 1$ as a function of frequency at generation i , for a *Wolbachia* with the following properties: $MI = 0.9$, $TE = 0.8$, $FE = 0.96$. Any point below the $x = y$ line (dashed) indicates that infection frequency is decreasing; any point above indicates that it is increasing. Horizontal lines indicate the values predicted by the analytical approach for the lowest (unstable) and highest (stable) equilibriums (Hoffmann *et al.* 1990). As expected, the curve crosses the $x = y$ line precisely for these values. These results were obtained with a big population ($N_e = 106$), so that drift has negligible effects.

ILLUSTRATING RELEVANT PROCESSES WITHOUT RANDOM MUTATION

Before considering “realistic evolution”, where an initial population can freely change under mutation, selection and drift, we present here the results of simulations conducted under controlled conditions. These examples will allow the reader to understand which sequence of events can lead to which population state. With these processes in mind, the evolution of populations will be analyzed more globally in the next section.

Infection loss

Analytical models have revealed that elevated values of the MI , TE and FE parameters facilitate the stable maintenance of *Wolbachia* in host populations (Hoffmann *et al.* 1990). They further showed that mutations decreasing TE or FE are always selected against (Turelli 1994), so that the long term evolution of these two parameters should stabilize the presence of *Wolbachia*. On the contrary, mutations reducing MI are not selected against in panmictic populations (Prout 1994; Turelli 1994), so that MI is supposed to evolve under drift only (unless it is linked to other traits through pleiotropic effects). Increasing MI will stabilize the infection, but decreasing MI will have the opposite effect. Figure 4a illustrates how a random decrease of the average MI in the population can lead to infection loss: when *Wolbachia* variants with low MI get too frequent, the overall infection frequency (the stable equilibrium predicted by analytical models) decreases, while the threshold infection frequency (below

which *Wolbachia* is lost deterministically) increases. Eventually, the population can get out of the conditions allowing infection to be maintained.

Less explicit in earlier analyses is the fact that variations affecting the *Lock* parameter can also lead to infection loss. As detailed in our earlier work (Charlat *et al.* 2001), mutations affecting the *Lock* are not subject to selection, although they give rise to self-incompatible, or “suicidal” *Wolbachia*. Figure 4b illustrates how this can lead to infection loss: as new *Lock* variants (totally or partially self-incompatible) get too frequent by drift, the fitness gain provided by CI to infected females is lowered.

In summary, neutral variations of *MI* make CI less deleterious to uninfected females, while variations of the *Lock* make CI more deleterious to infected females. The final effect is the same in the two cases: bearing *Wolbachia* becomes less advantageous for females.

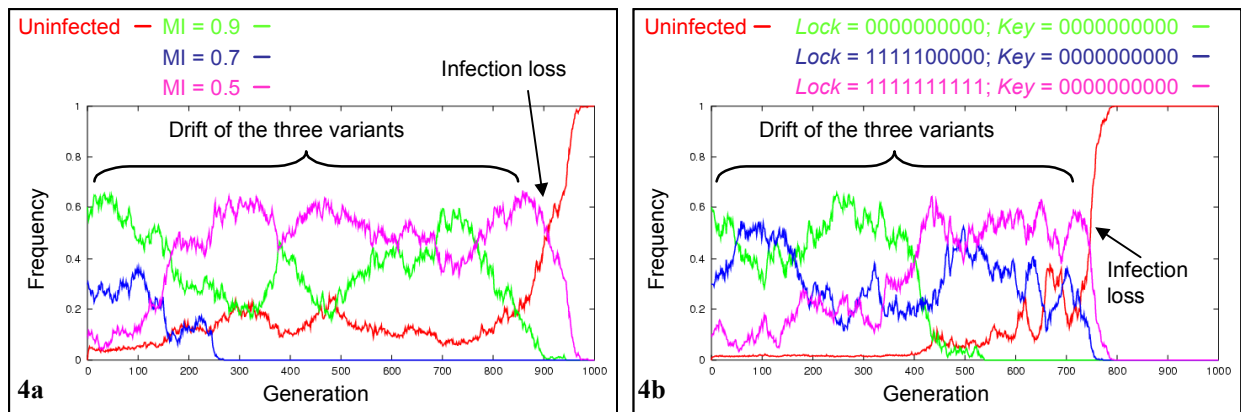


Figure 4.

The Figure illustrates the neutrality of *MI* and *Lock* variations, and their possible consequences on infection loss. Population size: $N_e = 1000$.

4a. The consequences of *MI* polymorphism. The three variants ($MI = 0.9; 0.6; 0.3$) are initially introduced with respective frequencies 0.6, 0.3 and 0.1. The other parameters are not polymorphic: $TE = 0.9, FE = 1, Lock = 1111111111, Key = 1111111111$. Uninfected individuals rapidly reach the frequency expected based on these parameters values. As expected from earlier analysis (Prout 1994; Turelli 1994), the frequencies of the three variants change through drift. These random changes affect the frequency of uninfected individuals, which increases as variants with low *MI* get more frequent through drift. After generation 900, the respective frequencies of the three variants are such that the unstable equilibrium value gets above the overall infection frequency, leading to the loss of *Wolbachia*.

4b. The consequences of *Lock* polymorphism. The three variants ($Lock = 1111111111; 2222211111; 2222222222$) are initially introduced with respective frequencies 0.6, 0.3 and 0.1. The other parameters are not polymorphic: $MI = 0.9, TE = 0.9, FE = 1, Key = 1111111111$. Uninfected individuals rapidly reach the frequency expected based on these parameters values. The frequencies of the three variants changes through drift. These random changes affect the frequency of uninfected individuals, which increases as the two variants with *Lock* different from 1111111111 get more frequent through drift. After generation 700, the respective frequencies of the three variants are such that the unstable equilibrium value gets above the overall infection frequency, leading to the loss of *Wolbachia*.

Fixation of new compatibility types

As previously described (Charlat *et al.* 2001), random variations of *Lock* can create the conditions for new compatibility types to invade populations. For the purpose of this section, let us define two different *Lock* sequences: $Lock_A$ (111111111) and $Lock_B$ (222222222) and two different *Key* sequences: Key_A (111111111) and Key_B (222222222). Consider a

population harboring two *Wolbachia* variants:

$Lock_A / Key_A$ (AA) and $Lock_B / Key_A$ (BA) This

is a neutral polymorphism: the relative proportion of the two variants changes through drift only. A third variant

($Lock_B / Key_B$ or BB) is introduced in the population. This new variant gets more frequent if the overall frequency of $Lock_B$

exceeds that of $Lock_A$, that is, if

$f(BA) + f(BB) > f(AA)$. As $f(BB)$ increases, $Lock_B$ becomes more frequent, so that the fitness of BB increases. Eventually, BB will get fixed, so that the compatibility type will have evolved from $Lock_A / Key_A$ to $Lock_B / Key_B$. Figure 5 shows how such a process can be visualized with our model.

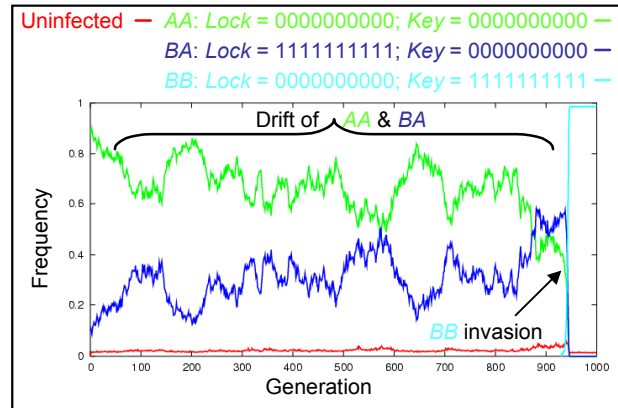


Figure 5.

The Figure illustrates the sequence of events leading to the invasion of new compatibility types. Population size: $N_e = 1000$. Two variants (AA: $Lock_A / Key_A$; BA: $Lock_B / Key_A$; with $Lock_A = 111111111$, $Lock_B = 222222222$, $Key_A = 111111111$, $Key_B = 222222222$) are initially introduced with respective frequencies 0.9 and 0.1. The other parameters are not polymorphic: $MI = 0.9$, $TE = 0.9$, $FE = 1$. As illustrated in Figure 4b, this is a neutral polymorphism. A new variant (BB: $Lock_B / Key_B$) is introduced after generation 900, at a time where $f(BA) > f(AA)$. BA rapidly invades the population because $Lock_B$ is more frequent than $Lock_A$.

Balanced suicidal polymorphism

Consider a population including two variants: $Lock_A / Key_A$ (AA) and $Lock_B / Key_A$ (BA). This is a neutral polymorphism: the relative proportion of AA and BA changes through drift only.

Consider a new variant ($Lock_A / Key_B$ or AB) arising by mutation of the *Key* in the AA variant. AB gets more frequent if the overall frequency of $Lock_B$ exceeds that of $Lock_A$, that is, if $f(BA) > f(AA) + f(AB)$. However, as AB becomes more frequent, $Lock_B$ becomes less frequent, so that selection for AB is reduced. This leads to a situation where the respective fitness of the three variants are equal, when the frequency of $Lock_A$ equals that of $Lock_B$, which we previously described as a stable equilibrium (Charlat *et al.* 2001).

Simulation runs such as that presented in Figure 6 show that not all variants are actually maintained at stable frequencies when such equilibrium is reached. The frequency of BA appears to be stable, but $f(AA)$ and $f(AB)$ vary randomly, and symmetrically, leading either to the loss of AB (as in Figure 6a) or AA (as in Figure 6b). If AB is lost, the population goes back to the initial neutral polymorphism with AA and BA . On the contrary, if AA is lost, the population reaches a stable polymorphism, with BA and AB at equal frequencies. The rationale behind these variations is illustrated in Figure 7.

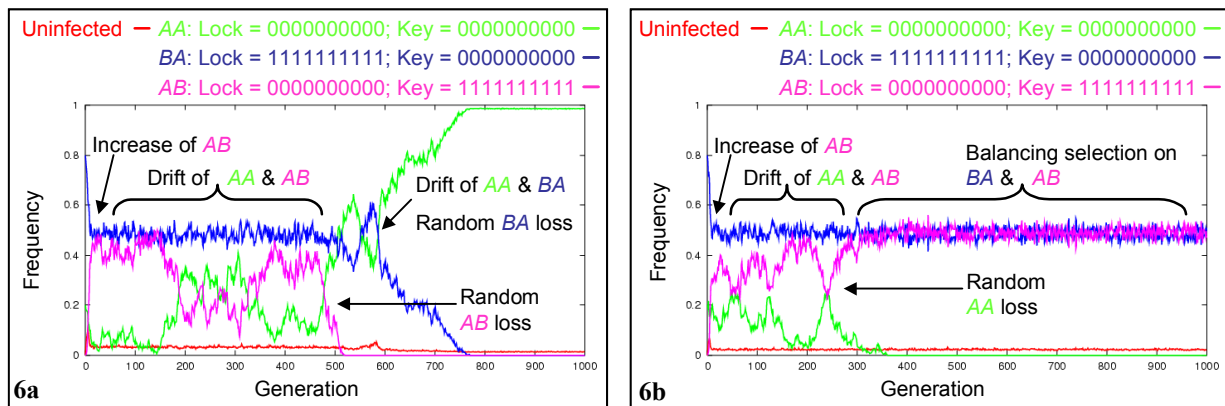


Figure 6.

The Figure illustrates how a “suicidal” polymorphism can be maintained by balancing selection. Population size: $N_e = 1000$. Two variants (AA : LockA / KeyA; BA : LockB / KeyA; with LockA = 1111111111, LockB = 2222222222, KeyA = 1111111111, KeyB = 2222222222) are initially introduced with respective frequencies 0.2 and 0.8. The other parameters are not polymorphic: $MI = 0.9$, $TE = 0.9$, $FE = 1$. As illustrated in Figure 4b, this is a neutral polymorphism. A third variant (AB : LockA / KeyB) is introduced at the first generation, with frequency 0.001. In the very first generations, its frequency increases rapidly, because it bears KeyB in a population where LockB is more frequent than LockA. This increase stops when $f(\text{LockA}) = f(\text{LockB})$, that is, when $f(BA) = f(AA) + f(AB)$. At that point $f(BA)$ is stably maintained, while $f(AA)$ and $f(AB)$ vary randomly and symmetrically, leading to the loss of AB (in 6a) or AA (in 6b). In 6a, the population goes back to the initial neutral polymorphism (with AA and BA), whereas in 6b, a stable polymorphism is reached, with $f(BA)$ and $f(AB)$ being maintained equal by balancing selection.

Neutral suicidal polymorphism and population extinction

Neutral variations of the *Lock* function can greatly reduce host population mean fitness: as a $Lock_B / Key_A$ type gets frequent by drift, many crosses in the population are incompatible. Eventually, nothing opposes the fixation by drift of such a suicidal *Wolbachia*. If $MI < 1$, some proportion of the eggs can still survive. However, if $MI = 1$, and if $Lock_B$ and Key_A are totally incompatible, no viable progeny is produced, so that the host population simply goes extinct.

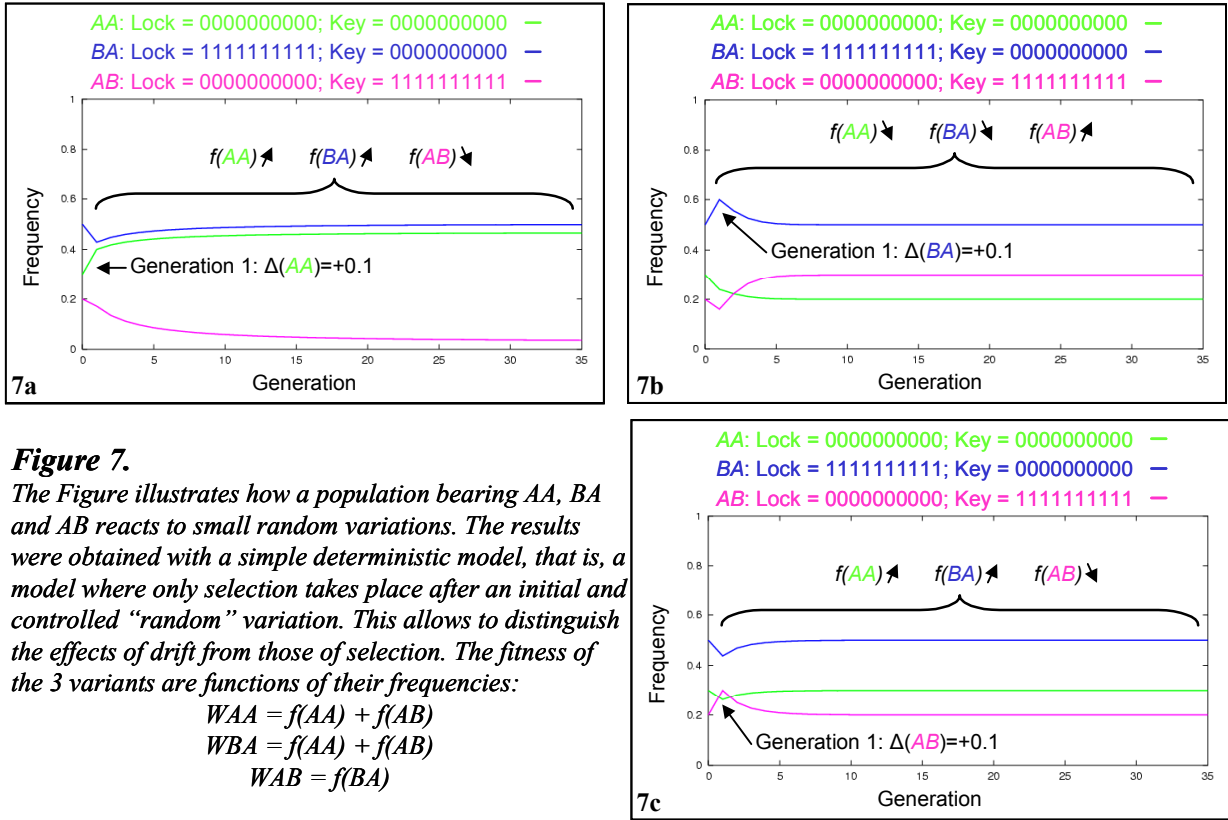


Figure 7.

The Figure illustrates how a population bearing AA, BA and AB reacts to small random variations. The results were obtained with a simple deterministic model, that is, a model where only selection takes place after an initial and controlled “random” variation. This allows to distinguish the effects of drift from those of selection. The fitness of the 3 variants are functions of their frequencies:

$$\begin{aligned} WAA &= f(AA) + f(AB) \\ WBA &= f(AA) + f(AB) \\ WAB &= f(BA) \end{aligned}$$

The variants are initially introduced with respective frequencies $f(AA) = 0.3$, $f(BA) = 0.5$, $f(AB) = 0.2$. At that point, $WAA = WBA = WAB$ but small random variations can occur. In 7a, 7b and 7c, we consider the effect of an initial “random” increase ($\Delta = 0.1$) of $f(AA)$, $f(BA)$ and $f(AB)$, respectively. We assume that this “random” increase is accompanied by an evenly distributed decrease of the two other variants. In other words, if $f(AA)$ increases by $\Delta f(AA)$ then:

$$\Delta f(BA) = -\Delta f(AA) \frac{f(BA)}{f(BA) + f(AB)} \quad \text{and} \quad \Delta f(AB) = -\Delta f(AA) \frac{f(AB)}{f(BA) + f(AB)}$$

7a. A random increase of $f(AA)$ induces variations in the fitness of the three variants: WAA and WBA increase while WAB decreases. Thus, following $\Delta f(AA)$, $f(AB)$ will decrease and $f(AA)$ and $f(BA)$ will increase deterministically, until a point where $f(BA) = f(AA) + f(AB)$, where selection ceases as all fitness are equal. The longer the time the population takes to get back equal to this point, the stronger the increase of $f(AA)$ relative to $f(AB)$ due to selection. A decrease of $f(AA)$ would have the exact symmetrical effect.

7b. A random increase of $f(BA)$ induces variations in the fitness of the three variants: WAA and WBA decrease, while WAB increases. Thus, following $\Delta f(BA)$, $f(AB)$ will increase, and $f(AA)$ and $f(BA)$ will decrease deterministically until a point where $f(BA) = f(AA) + f(AB)$, where selection ceases. The longer the time the population takes to get back equal to this point, the stronger the increase of $f(AB)$ relative to $f(AA)$ due to selection. A decrease of $f(BA)$ would have the exact symmetrical effect.

7c. A random increase of $f(AB)$ induces variations in the fitness of the three variants: WAA increases, WBA increase and WAB decreases. Thus, following $\Delta f(AB)$, $f(AB)$ will decrease and $f(AA)$ and $f(BA)$ will increase deterministically, until a point where $f(BA) = f(AA) + f(AB)$, where selection ceases as all fitness are equal. This will always lead back to the initial situation, before the “random” increase of $f(AB)$.

In summary, AA and AB are globally neutral relative to each other (although locally selection takes place) while the frequency of BA is locally and globally stabilized by selection.

EVOLUTION UNDER MUTATION, DRIFT AND SELECTION

Six discrete population states

Having illustrated several possible sequences of events using controlled conditions, we will now analyze how these different processes will globally affect the evolution of populations, by allowing all parameters to mutate randomly. For the purpose of this analysis, let us define six discrete “population states”:

- *State 1*: infection loss, when all individuals are uninfected
- *State 2*: population extinction, when no offspring is produced, due to the fixation by drift of a suicidal *Wolbachia*
- *State 3*: stability of compatibility types, when the initial *Lock / Key* pair is still “fixed”. Arbitrarily, we define “fixation” as a frequency exceeding 0.9, so that “fixation” of a type does not mean that there is no cryptic polymorphism, due to recurrent mutation
- *State 4*: “fixation” of a new compatibility type, when a new *Lock / Key* type, more compatible with it-self than with the initial type, is at a frequency exceeding 0.9
- *State 5*: balanced suicidal polymorphism, when two types with higher cross-compatibility than self compatibility (like $Lock_B / Key_A$ and $Lock_A / Key_B$), and only these two, are at frequencies exceeding 0.1.
- *State 6*: this population state is peculiar in that it corresponds to all possible situations that are not described by the 5 other states. In practice, *State 6* will mainly describe (i) populations harboring a neutral polymorphisms (like $Lock_A / Key_A$ and $Lock_B / Key_A$) and (ii) populations where a suicidal type (like $Lock_B / Key_A$) is at a frequency exceeding 0.9. However, we do not rule out that *State 6* might include others types of situations, potentially interesting but unidentifiable based on our current understanding of the system.

This being defined, the evolution of populations can be depicted as transitions between population states over generations. Starting from an initial condition, different simulations can lead to very different patterns, because of random mutation and drift. To describe general tendencies, we can compile the results of a sufficient number of simulation runs, which will allow to estimate the probability of the different population states over generations. In the following sections, we will use this approach to explore the evolution of compatibility types under various conditions. Preliminary analyses (not shown) have been performed using high mutation rates over a short number of generations, in order to moderate calculation time.

These have allowed to identify three important factors: (1) the structure of the *Lock* and *Key* sequences (the length of the sequence and the number of possible states at each site), (2) the mutation rate and (3) the maximum possible values for *TE*, *FE* and *MI*, that will condition the possible maintenance of uninfected individuals in the populations. At the time of writing, only the two first factors have been investigated using a sufficient number of simulations. The effect of the third is currently under analysis, and will complement the current version of this manuscript.

The effect of the Lock / Key structure

We defined *Lock* and *Key* as sequences of n_c characters, with n_s possible states for each site (states 1, 2, ..., n_s). In a cross between a male bearing *Wolbachia i* and a female bearing *Wolbachia j*, the compatibility score (C_{ij}) is calculated as the number of matching sites in the *Lock / Key* alignment. To investigate the effect of varying n_c and n_s on the evolution of compatibility types, we followed 500 populations over 100,000 generations in four different conditions: (1) $n_c = 1$ and $n_s = 2$ (Figure 8a), (2) $n_c = 1$ and $n_s = 10$ (Figure 8b), (3) $n_c = 10$ and $n_s = 1$ (Figure 8c), and finally (4) $n_c = 10$ and $n_s = 10$ (Figure 8d).

As visible on Figure 8, the values of n_c and n_s deeply affect the evolution of populations. Let us consider first the effect of the n_c parameter. When $n_c = 1$ (Figures 8a and 8b), we observe that the probability of the initial state (*State 3*: fixation of the initial type) decreases very slowly over generations. Indeed, after 100,000 generations, fixation of the initial type is still observed in 90% of the simulations. Furthermore, we observe that among the remaining 10%, most populations have gone extinct (*State 2*: population extinction). Thus, it appears that in these conditions, compatibility types cannot evolve. As illustrated in Figures 8c and 8d, things are clearly different when $n_c = 10$. Indeed, after 100,000 generation, less than 40% of the populations are still in the initial state. Depending on the value of n_s ($n_s = 2$ in Figure 8c, $n_s = 10$ in Figure 8d), the respective proportions of populations in *State 4* (fixation of a new compatibility types) and *State 6* (the imprecisely defined state) vary. To apprehend the rationale behind these effects of n_c variations, one must distinguish two aspects of the differences observed between Figures 8a & b *versus* Figures 8c & d: (1) the slow *versus* rapid decrease of *State 3* (the initial state) and (2) the replacement of *State 3* by *State 2* (population extinction) *versus* *States 4* and *6*. The explanation of the first difference is the following: the mutation rate of the *Lock* and *Key* sequences is defined here as 10^{-6} per site; in other words, the overall mutation rate of the *Lock* and *Key* sequences is lower when the sequence is short

(Figures 8a & b) than when it is long (Figures 8c & d); the initial state is lost faster in Figures 8c & d because the overall mutation rates of the *Lock* and *Key* sequences is higher. The explanation of the second difference (population extinction *versus* other states) is very different. In Figures 8a & b, where $n_c = 1$, compatibility between a given *Lock* and a given *Key* can only be 0 or 1. In other words, if the intensity of *mod* is maximum (if $MI = 1$), which is initially the case in these simulations, not a single viable egg is produced in incompatible crosses. Thus, if a suicidal type (with $Lock = 2$ and $Key = 1$) is fixed by drift, the host population goes extinct. On the contrary, in Figures 8c & d, where $n_c = 10$, mutations of the *Lock* and *Key* sequences only lead to partial incompatibility. In other words, a suicidal type (partially suicidal) can reach fixation without leading the host population to extinction. In summary, the fact that populations go extinct in Figures 8a & b suggests that mutations of the *Lock* sequence often reach fixation before a compatible *Key* occurs by mutation. This readily leads the host population to extinction if $n_c = 1$, that is, if mutations of the *Lock* sequence result in complete incompatibility.

Let us consider now the effect of n_s . We do not observe any marked difference between Figures 8a and 8b, suggesting that the value of n_s has virtually no effect when $n_c = 1$. In contrast, we observe a clear difference between Figures 8c and 8d. In Figure 8c, *State 3* (fixation of the initial type) is replaced by *State 4* (fixation of a new compatibility type) in more than 50% of populations after 100,000 generations, while 10% constantly remain in *State 6*. On the contrary, in Figure 8d, *State 3* has been replaced by *State 4* in only 30% of the populations, while *State 6* is observed in near 40% of the cases, after a gradual increase tending to slow down over generations. The explanation of this difference is the following. When $n_s = 2$, as in Figure 8c, mutations affecting the different sites of the *Lock* and *Key* sequences can only change the character from 1 to 2 or from 2 to 1. Thus, when a (partially) suicidal type (e.g. with $Lock = 2111111111$ and $Key = 1111111111$) is frequent in the population (or when it is fixed), the mutations of the *Key* sequence will give rise to the appropriate *Key* (2111111111) with probability 1/10. On the contrary, when $n_s = 10$, such a mutation occurs only with probability 1/90. By noting that such mutations lead to the fixation of new compatibility types (*State 4*), one understands that the bigger the value of n_s , the less probable the transition between *State 6* and *State 4*.

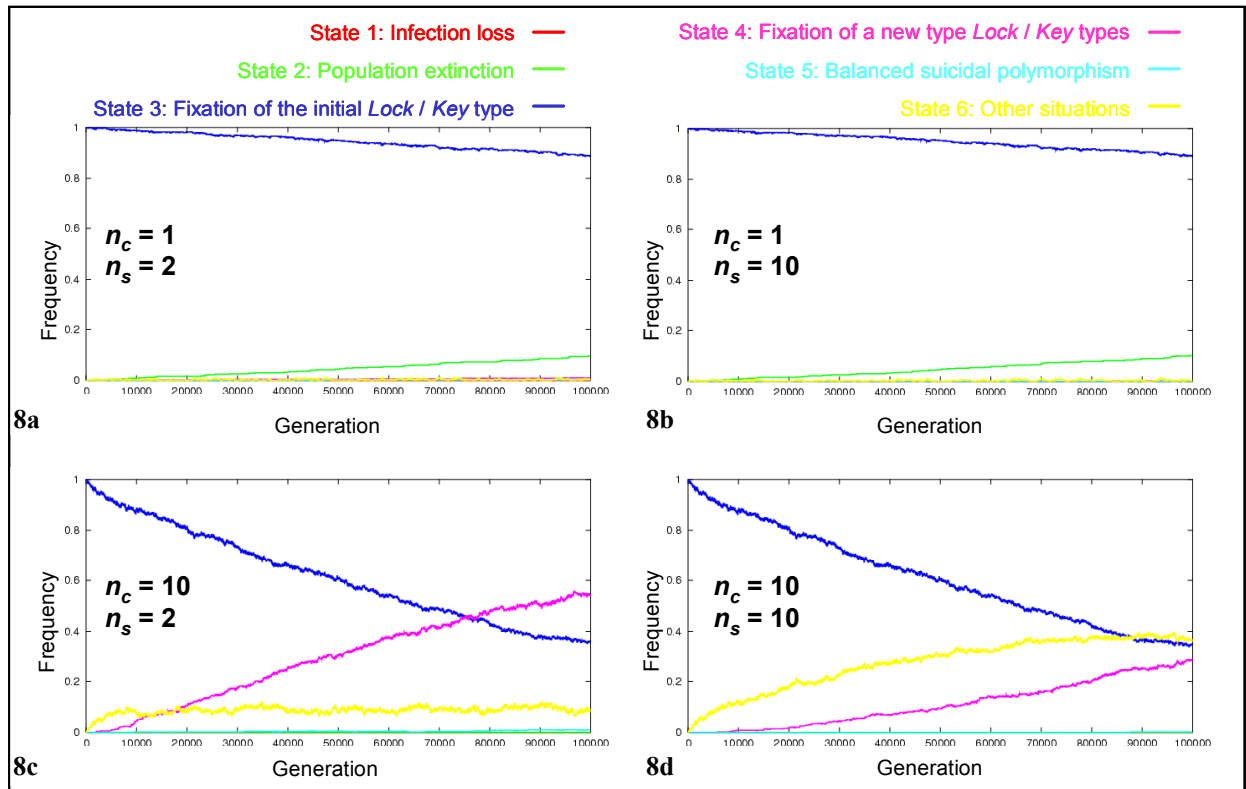


Figure 8.

The Figure shows the evolution of population states over 100,000 generations, for four different combinations of n_c (the length of the Lock and Key sequences) and n_s (the number of possible character states at each site). The curve plots the frequency of population states, calculated over 500 simulation runs that were performed under the following conditions:

Initial population: one single variant, totally self compatible, with $MI = 1$, $TE = 1$, $FE = 1$.

Population size: $N_e = 1000$

Mutation rate: $Murate = 10^{-6}$. For the Lock and the Key parameters, $Murate$ is multiplied by n_s so that $Murate$ gives the mutation rate per site.

Mutation steps: $Mustep = \pm 0.05$ for MI , TE and FE . For the Lock and Key parameters, mutation of a given site in the sequence leads to a new character state at this site, randomly chosen among the $n_s - 1$ remaining possible character states.

8a. $n_c = 1$, $n_s = 2$. Initial type: Lock = 1, Key = 1.

8b. $n_c = 1$, $n_s = 10$. Initial type: Lock = 1, Key = 1.

8c. $n_c = 10$, $n_s = 2$. Initial type: Lock = 1111111111, Key = 1111111111.

8d. $n_c = 10$, $n_s = 10$. Initial type: Lock = 1111111111, Key = 1111111111.

The effect of mutation rates

The results discussed so far were obtained with a mutation rate of 10^{-6} . To investigate the effects of mutation rates on the evolution of compatibility types, we repeated similar simulations as those presented in Figure 8 (that is, with four different n_c / n_s combinations), with a ten times higher mutation rate (10^{-5}). The results are presented in Figure 9. By comparing Figures 8 and 9, we can determine the effect of varying mutations rates for each n_c / n_s combination.

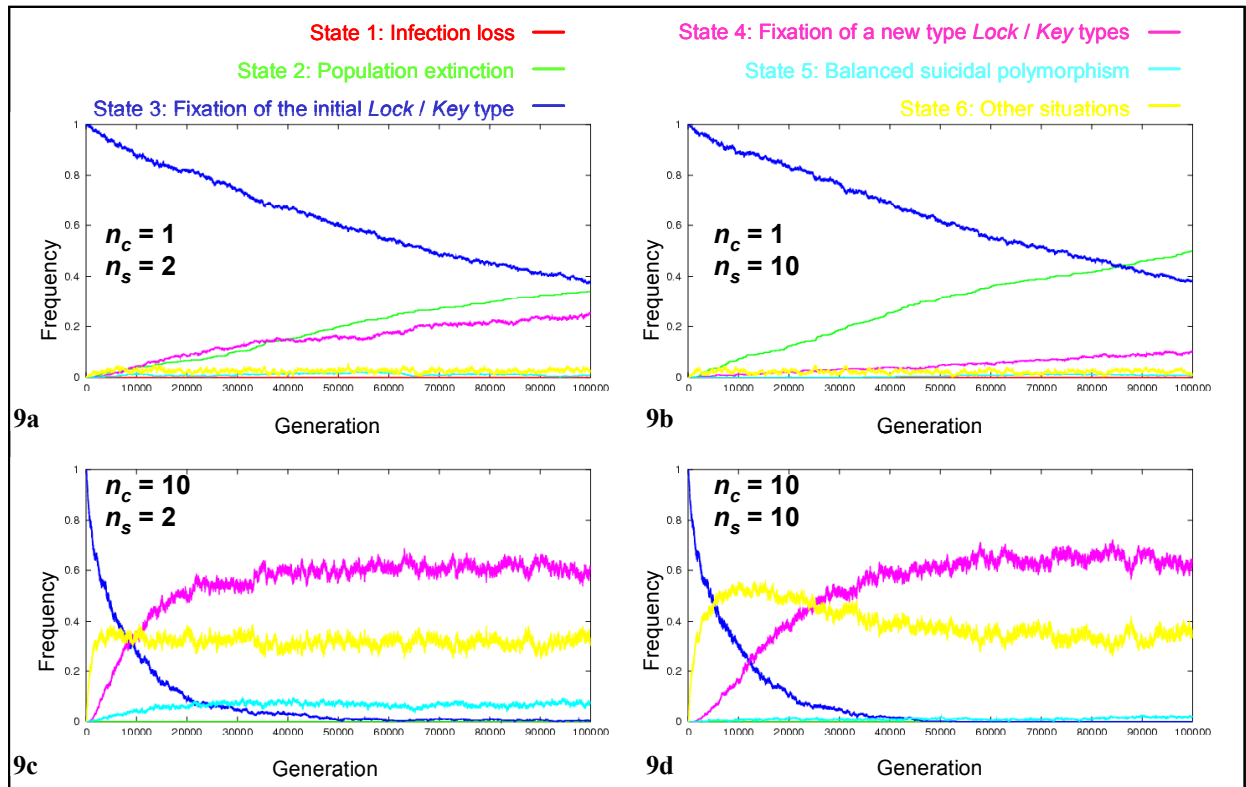


Figure 9.

Same legend as Figure 8, apart from the fact that the mutation rate is ten times higher ($Mutate = 10-5$).

Three notable differences can be seen between Figures 8a and 9a: (1) in Figure 9a, less than 40% of populations are still in *State 3* after 100,000 generations, as compared to 90% in Figure 8a; (2) in Figure 9a, more than 30% of the populations have gone extinct (*State 2*) after 100,000 generations, as compared to 10% in Figure 8a; finally (3) in Figure 9a, more than 20% of populations harbor a new *Lock / Key* pair after 100,000 generations, as compared to 1% in Figure 8a. Difference 1 (that is, *State 3* less frequent in Figure 9a than 8a) suggests that the initial *Lock / Key* type is lost faster when mutation rates are higher, which is not a very surprising result. It is notable that the rate of decrease of *State 3* in Figure 9a is exactly the same as that observed in Figure 8c. This is consistent with the interpretation that we gave when comparing Figure 8a with Figure 8c: *State 3* is lost faster when n_c is bigger simply because the overall mutation rate of the *Lock* and *Key* sequences is increased. Difference 2 (that is, more frequent *State 2* in Figure 9a than 8a) suggests that extinction risk increases with mutation rate. This can be understood by remembering that extinction results from the fixation of a suicidal type (e.g. with $Lock = 2$ and $Key = 1$). Mutations giving rise to such suicidal types being neutral, their rate of fixation only depends on the mutation rate (Kimura 1983). Difference 3 (that is, more frequent *State 4* in Figure 9a than 8a) suggests that the evolution of compatibility types is facilitated by increased mutation rates, which might seem

straightforward. A subtle aspect deserves however to be discussed: in Figure 9a, the ratio *State 4 / State 2* (new type / extinction) is much higher than in Figure 8a. To understand why, one must remember that fixation of new type (e.g. with *Lock* = 2 and *Key* = 2) can only occur in populations where a suicidal type (e.g. with *Lock* = 2 and *Key* = 1) is sufficiently frequent. When the mutation rate is increased (as in Figure 9a), mutations giving rise to this new type have more chance to occur before the suicidal type has reached fixation (that is, before populations have gone extinct).

Comparison between Figures 8b and 9b does not provide new information. Note nevertheless that the ratio *State 4 / State 2* (new type / extinction) is lower in Figure 9b than 9a. This is because the probability that a suicidal *Lock / Key* types gives a new self compatible type by mutation is decreased when n_s is increased, as already mentioned.

Four notable differences can be seen between Figures 8c and 9c: (1) in Figure 9c, only a tiny remnant of populations are still in *State 3* after 50,000 generations, as compared to 60% in Figure 8c, (2) in Figure 9c, the frequency of *State 4* reaches a plateau of 60% after 50,000 generations, while no plateau was reached in Figure 8c after 100,000 thousands generations; (3) in Figure 9c, the frequency of *State 6* is fixed at 35% after less than 5,000 generations, while this plateau was lower (less than 10%) in Figure 8c; finally (4) in Figure 9c, a significant and stable proportion of populations is in *State 5* (balanced suicidal polymorphism), while this state was negligible in Figure 8c. Difference 1 (that is, the complete loss of *State 3* in Figure 9c) suggests that *State 3* (fixation of the initial compatibility type) is unstable in the long term. Difference 2 (that is, *State 4* reaches a plateau in Figure 9c) suggests that even with high mutation rates, the proportion of populations harboring a new compatibility type can never reach 1. Difference 3 (that is, frequency of *State 6* stable and higher in Figure 9c) suggests that increasing mutation rates will increase the probability of neutral and transitory polymorphism, which is straightforward. Difference 4 (that is, *State 5* is more frequent in Figure 9c) suggests that high mutation rates facilitate the occurrence of a balanced suicidal polymorphism.

We will not develop the comparison between Figures 8d and 9d, as it leads to similar conclusions as that between 8c and 9c. Overall, it is interesting to note that the conditions used in Figure 9c and d ($\mu_{rate} = 10^{-5}$ and $n_c = 10$) are such that 100,000 generations seem to be sufficient for the equilibrium distribution of population states to be observed. At equilibrium, populations are either in *State 4* (fixation of new type), *State 5* (balanced suicidal polymorphism) or *State 6* (neutral polymorphism or fixation of partially suicidal *Wolbachia*). This equilibrium is clearly dynamic: populations themselves are not stable, but the probability

of transitions between states 4, 5 and 6 are stable. It is likely that a similar equilibrium would be observed with a mutation rate of 10^{-6} , or even less, if populations were followed over a sufficient number of generations. This remains to be investigated.

A PRELIMINARY CONCLUSION

The results presented in the current version of this manuscript allow to illustrate how the model can be used to study the evolution of compatibility types under various conditions. For now, it appears that the values of n_c and n_s , as well as mutation rates, are important parameters. It is difficult to predict which value for these parameters are the most realistic. Assuming a maximum mutation rate of 10^{-6} is probably reasonable. Concerning, the n_c and n_s parameters, it is difficult to fix the most sensible values, as the actual genetic determinants of the *mod* and *resc* functions are unknown. However, current knowledge on bi-directional incompatibility brings insights into this question. First, it is well established from several host species that more than two compatibility types do exist. For example, *Drosophila simulans* harbors three variants showing a total bi-directional incompatibility (reviewed in Merçot & Charlat 2003). This would suggest that n_s cannot be smaller than 3. Furthermore, it is known that compatibility between variants (what we call here the “compatibility score”) can be different from 0 or 1. Indeed, the *w*Ri variant (naturally infecting *D. simulans*), is partially compatible with the *w*Mel variant (injected from its natural host *Drosophila melanogaster* into *D. simulans*) (Poinsot *et al.* 1998). Similarly, the *w*Cer2 variant (injected from its natural host *Rhagoletis cerasi* into *D. simulans*; (Riegler *et al.* in prep), is partially compatible with *w*Mel and *w*Ri (Charlat *et al.* in prep). These results would suggest that n_c must be bigger than 1.

Aside from the effect of n_c , n_s , and mutation rates, fixing some maximum values for the *TE*, *FE* and *MI* parameters will be of particular interest, as this will allow the persistence of uninfected individuals in the populations, and possibly the loss of infection in the long term. It will also be interesting to determine if, as we previously suggested (Charlat *et al.* 2001), the processes underlying the evolution of compatibility types (the *Lock* and *Key* parameters) can affect the evolution of CI levels (the *MI* parameter). With this tool in hands, one can investigate the evolution of compatibility in virtually any conditions.

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Chapitre 2.

Approche expérimentale

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Approche expérimentale

D'une manière générale, le problème de l'échelle temporelle rend l'étude expérimentale des processus évolutifs difficile. Il nous reste l'approche comparative : utiliser la phylogénie pour inférer l'évolution des caractères.

L'exemple des infections de *Drosophila simulans*, présenté en introduction, démontre la diversité des types de compatibilité. Cependant, les *Wolbachia* présentes chez cette espèce sont trop éloignées phylogénétiquement pour nous renseigner sur les étapes initiales de la divergence des types de compatibilité, et sur les processus sous-jacents. Comme l'illustrent les expériences présentées dans ce chapitre, nous avons cherché à contourner cette difficulté en injectant dans *Drosophila simulans* des variants bactériens étroitement apparentés, bien qu'évoluant à l'état naturel dans des espèces hôtes différentes.

Article N°3.

Evolution of *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila simulans* and *D. sechellia*

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Evolution, **56**:1735-1742.

En bref...

Drosophila simulans et *Drosophila sechellia* sont deux espèces étroitement apparentées. L'analyse de leur génome mitochondrial suggère que les lignages cytoplasmiques des deux espèces divergent depuis moins d'un million d'années (Ballard 2000b). La présence de *Wolbachia* étroitement apparentées dans les deux espèces est en accord avec l'hypothèse d'une co-spéciation, c'est-à-dire d'une divergence parallèle des hôtes et de leurs infections (Rousset & Solignac 1995). Afin de tester si la séparation "récente" de ces *Wolbachia* s'est accompagnée d'une divergence significative des déterminants de l'IC (intensité de mod et types de compatibilité) nous avons injecté les bactéries de *Drosophila sechellia* dans *Drosophila simulans*. Nos résultats indiquent que les bactéries des deux espèces induisent des niveaux de mortalité embryonnaire similaires, et demeurent parfaitement compatibles.

EVOLUTION OF *WOLBACHIA*-INDUCED CYTOPLASMIC INCOMPATIBILITY IN *DROSOPHILA SIMULANS* AND *D. SEHELLIA*

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Abstract.—The intracellular bacterium *Wolbachia* invades arthropod host populations through various mechanisms, the most common of which being cytoplasmic incompatibility (CI). CI involves elevated embryo mortality when infected males mate with uninfected females or females infected with different, incompatible *Wolbachia* strains. The present study focuses on this phenomenon in two *Drosophila* species: *D. simulans* and *D. sechellia*. *Drosophila simulans* populations are infected by several *Wolbachia* strains, including *wHa* and *wNo*. *Drosophila sechellia* is infected by only two *Wolbachia*: *wSh* and *wSn*. In both *Drosophila* species, double infections with *Wolbachia* are found. As indicated by several molecular markers, *wHa* is closely related to *wSh*, and *wNo* to *wSn*. Furthermore, the double infections in the two host species are associated with closely related mitochondrial haplotypes, namely *sII* (associated with *wHa* and *wNo* in *D. simulans*) and *se* (associated with *wSh* and *wSn* in *D. sechellia*). To test the theoretical prediction that *Wolbachia* compatibility types can diverge rapidly, we injected *wSh* and *wSn* into *D. simulans*, to compare their CI properties to those of their sister strains *wHa* and *wNo*, respectively, in the same host genetic background. We found that within each pair of sister strains CI levels were similar and that sister strains were fully compatible. We conclude that the short period for which the *Wolbachia* sister strains have been evolving separated from each other was not sufficient for their CI properties to diverge significantly.

Key words.—Cytoplasmic incompatibility, *Drosophila*, endocellular bacteria, evolution, symbiosis, *Wolbachia*.

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Wolbachia is a maternally transmitted endocellular symbiont of arthropods and nematodes, belonging to the α -proteobacteria group (reviewed in Stouthamer et al. 1999; Stevens et al. 2001). An intriguing feature of this bacterium is that, in arthropods, it can induce various alterations of its hosts' reproduction (namely, thelytokous parthenogenesis, feminization, male killing, and cytoplasmic incompatibility), all of which favor its invasion of and maintenance in host populations.

Cytoplasmic incompatibility (CI) is the most commonly observed *Wolbachia*-induced phenotype (reviewed in Hoffmann and Turelli 1997; Charlat et al. 2001a). Its distribution within *Wolbachia* phylogeny suggests that it might be ancestral relative to the other phenotypes (Werren et al. 1995). Basically, CI occurs in crosses between infected males and uninfected females, resulting in a more or less intense embryonic mortality. The three other possible crosses (male infected \times female infected, male uninfected \times female infected, and male uninfected \times female uninfected) show normal fertility. Because crosses show CI in only one direction, it is termed "unidirectional." As a consequence of unidirectional CI, infected females produce, on average, more offspring than uninfected ones. Because *Wolbachia* are transmitted only maternally, this phenomenon allows *Wolbachia* to spread through uninfected populations and then maintain itself (Caspari and Watson 1959; Turelli and Hoffmann 1995). Interestingly, CI can also occur in crosses between males and females that are both infected, if the two partners bear different, incompatible *Wolbachia* strains (O'Neill and Karr

1990). In this latter case, CI occurs in both directions and is thus termed "bidirectional."

The mechanism of CI is still unknown. A formal model proposes that CI involves at least two distinct bacterial functions: *mod* (for modification) and *resc* (for rescue; Werren 1997). The *mod* function would somehow modify the sperm nucleus (Presgraves 2000), before *Wolbachia* are shed from the maturing sperm, and the *resc* function, expressed in the egg, would rescue the embryo through an interaction with the modified sperm.

Here we focus on two sibling *Drosophila* species infected by CI-inducing *Wolbachia*: *D. simulans* and *D. sechellia*. *Drosophila simulans* is an extensively studied *Wolbachia* host. This species harbors at least five *Wolbachia* strains, exhibiting diverse CI phenotypes (reviewed in Merçot and Charlat 2002). We are here interested in two of these variants: *wHa* (O'Neill and Karr 1990) and *wNo* (Merçot et al. 1995; Rousset and Solignac 1995). These two strains express a [*mod*+*resc*+] phenotype: They induce CI when present in males and rescue their own modification when present in females, in all host genetic backgrounds tested so far. In populations from the Seychelles archipelago and New Caledonia, the *wHa* strain can be found as the only *Wolbachia* infection, but *wNo* is almost always found in association with *wHa* in doubly infected individuals (Rousset and Solignac 1995). Initially, *wNo* was separated from *wHa* under laboratory conditions (Merçot and Poinot 1998a; Poinot et al. 2000), but very rare lines singly infected by *wNo* were recently found in the wild (James et al. 2002).

D. sechellia is endemic in the Seychelles archipelago. It

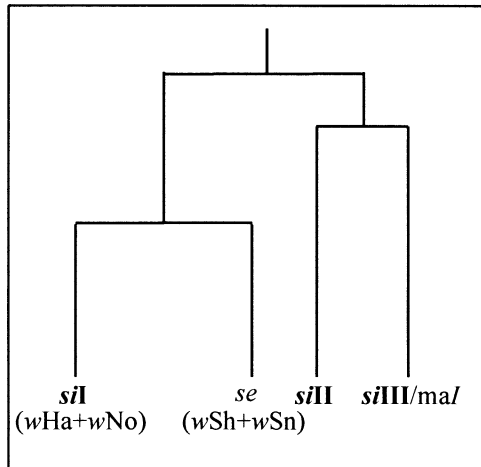


FIG. 1. Phylogenetic relationships between mitochondrial haplotypes harbored by *Drosophila simulans* (*siI*, *siII*, *siIII*), *D. sechellia* (*se*), and the *malI* haplotype of *D. mauritiana* (Solignac and Monnerot 1986; Satta and Takahata 1990; Ballard 2000a,b). *Drosophila simulans* haplotypes are in bold. *malI* and *siIII* are virtually identical, a pattern due to a recent introgression (Solignac and Monnerot 1986; Ballard 2000c). *Drosophila simulans* is paraphyletic relative to *D. sechellia* (the *siI* + *siII* + *siIII* group is paraphyletic, and the *siI* + *se* group is monophyletic). It is not clear whether this is due to *D. simulans* having retained some ancestral polymorphism or to some introgression having occurred after speciation. The *Wolbachia* double infections associated with *siI* and *se* are given in parentheses.

harbors only two *Wolbachia* strains, *wSh* and *wSn*, which both express a [*mod*⁺ *resc*⁺] phenotype (Rousset and Solignac 1995; Giordano et al. 1995; Bourtzis et al. 1996). The infection pattern in *D. sechellia* is similar to *D. simulans*: *wSh* can be found on its own, but *wSn* seems to occur only in association with *wSh* in doubly infected individuals (Rousset and Solignac 1995), although segregation can occur in laboratory strains (S. Charlat, P. Bonnavion, and H. Merçot, unpubl. data).

Rousset and Solignac (1995) showed that *wSh* and *wHa*, as well as *wNo* and *wSn*, are closely related based on their 16S rRNA sequences. They also found that the mitochondria associated with *wHa* and *wNo* (namely, the *siI* mitochondrial haplotype of *D. simulans*) are closely related to those associated with *wSh* and *wSn* (namely, the *se* mitochondrial haplotype of *D. sechellia*; Fig. 1). These observations led Rousset and Solignac (1995) to propose that a double infection was present prior to the split between the *siI* and *se* cytoplasmic lineages, so that the *Wolbachia* in the two species would have diverged together with their associated mitochondria. Although less straightforward, an alternative interpretation would be that horizontal transfer occurred between the *siI* and *se* cytoplasmic lineages, in which case the bacteria would have diverged for a correspondingly shorter period than the mitochondria with which they are at present associated. But whichever of these scenarios is correct, the important observation is that the *Wolbachia* of the two species are closely related, sufficiently so to be undistinguishable as judged by their 16S rRNA locus or the less conserved *wsp* gene (Zhou et al. 1998; this study).

In an attempt to test the theoretical prediction that *Wolbachia* compatibility types can diverge rapidly (Charlat et al.

2001b), we transferred *wSh* and *wSn* from their natural host *D. sechellia* into *D. simulans*, to compare their CI properties to those of their sister strains *wHa* and *wNo* in the same host background.

MATERIALS AND METHODS

Drosophila sechellia Strains

S9 is a strain naturally infected by *wSh* (Bourtzis et al. 1996). *Dsech* is a strain infected by *wSh* + *wSn*, founded in the 1980s, originating from the Seychelles archipelago (kindly provided by the Population Genetics and Evolution [PGE] group, from Gif sur Yvette, France).

Drosophila simulans Strains

STC is an inbred uninfected strain, *Wolbachia*-cured by antibiotic treatment (tetracycline), derived from the Seychelles strain, naturally infected by *wHa* + *wNo*, collected on Mahe island (Seychelles archipelago) in 1981. *AHa* and *BHa* are isofemale lines, infected by *wHa*, obtained in 1996 by segregation in the Seychelles strain (Poinsot et al. 2000). *ANo* and *BNo* are isofemale lines infected by *wNo*, obtained in 1996 by segregation in the Seychelles strain (Poinsot et al. 2000). *Ash* is an isofemale line, infected by *wSh*, obtained by cytoplasmic injection from the *Dsech* strain into the *D. simulans STC* strain. *CSh* is an isofemale line, infected by *wSh*, obtained by cytoplasmic injection from the *D. sechellia S9* strain into the *D. simulans STC* strain. *ASn* and *BSn* are isofemale lines, infected by *wSn*, obtained by cytoplasmic injections from the *Dsech* strain into the *D. simulans STC* strain. *KC9* is an isofemale line infected by *wKi*, founded using flies collected in 1996 in Tanzania by D. Lachaise (Merçot and Poinsot 1998b; Poinsot and Merçot 1999).

Rearing Conditions

To ensure optimal conditions for the maintenance of *Wolbachia* infections, host strains were maintained at 25°C, on axenic medium (David 1962), at low larval density, by crossing young adults (3–5 days old).

Wolbachia Detection and Identification

In all experiments, detection of *Wolbachia* and the distinction between different *Wolbachia* variants were done by polymerase chain reaction (PCR). DNA was obtained according to O'Neill et al. (1992) and the *wsp* gene was amplified according to Zhou et al. (1998). Primer specificity allowed us to distinguish *wHa* and *wSh* (primer 178F and 691R) from *wNo* and *wSn* (primers 183F and 691R).

Sequencing

wsp PCR fragments were obtained with primer pair 81F and 691R (Zhou et al. 1998). 16S PCR fragments were obtained using primers 76–99F and 1012–994R (O'Neill et al. 1992) for *wNo* and *wSn* and primers 8–27F (5'-AGAGTTTGA-TCCTGGCTCA-3') and 704–685R (5'-TTTACGAATTC-ACCTCTAC-3') for *wHa* and *wSh* (Rousset 1993). PCR products were cloned into pGEM-T (Promega, Madison, WI) following the manufacturer's instructions. Plasmid DNA was pu-

rified using the QIA-prep Spin plasmid kit (Qiagen GmbH, Hilden, Germany). Sequencing reactions were performed using the d-Rhodamine dye-terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Norwalk, CT) and run on an ABI377 sequencer (Perkin-Elmer Applied Biosystems), all according to the manufacturer's instructions. Sequences have been deposited in the EMBL database under accession numbers AF468031–AF468036.

Wolbachia Transfer from Drosophila sechellia into D. simulans

Wolbachia was injected from *D. sechellia* into the *D. simulans* *STC* strain. Injections were performed using dechorionated embryos aged less than 1 h, following Santamaria (1987), with Femtotips needles (Eppendorf, Hamburg, Germany). Cytoplasm was taken from the posterior pole of donor eggs and injected at the posterior pole into recipient eggs. Females deriving from injected eggs represent the generation G0 postinjection. G0 females were each crossed with two *STC* males.

Measurement of Embryonic Mortality

Embryonic mortality was measured using individual crosses, between males aged 3–4 days and females aged 4–7 days. Mating was controlled, and crosses where copulation lasted for less than 15 min were discarded to ensure insemination. Inseminated females were individually placed at 25°C on axenic medium colored with neutral red, making egg counting easier. Females were removed after 48 h of laying. Eggs were left for an additional 24 h at 25°C to allow hatching of all viable embryos and finally placed at 4°C until egg counting. Embryonic mortality was then determined as the percentage of unhatched eggs. Samples with less than 20 eggs were discarded (the average egg count was 102, ranging from 20 to 248). For crosses showing 0% hatching, a fertility test was performed by crossing each parent with individuals of compatible infection status to distinguish between crosses where CI is 100% and crosses involving intrinsically sterile individuals, which were excluded from analysis. Finally, the infection status of parents was checked by PCR.

Cytoplasmic Incompatibility Intensity and Compatibility Relationships

CI intensity is defined here as the percentage of embryos that fail to hatch in crosses between infected males and uninfected females (strain *STC*). In each experiment, control crosses involving uninfected males were also performed to determine the control cross mortality (CCM). This allows calculation of a corrected CI (CI_{corr}), taking into account the embryonic mortality not caused by CI. Where EM stands for the observed embryonic mortality (Poinsot et al. 1998), $CI_{\text{corr}} = (EM - CCM)/(1 - CCM)$.

The compatibility relationships between sister *Wolbachia* strains were estimated by crossing infected males with uninfected females in all directions of cross.

Statistical Analysis

All statistical tests were performed on SAS (ver. 6.12; SAS Institute, Cary, NC) after arcsine transformation. Statistical details are given in the following section.

TABLE 1. Injections in *Drosophila simulans*. The number of females having transmitted *Wolbachia* to G1 is given in parentheses.

Donor line (infection status)	<i>S9</i> (<i>wSh</i>)	<i>Dsech</i> (<i>sSh</i> + <i>wSn</i>)
Nb eggs	679	1220
Nb G0 females	21	32
Nb G0 females uninfected	4	2
Nb G0 females <i>wSh</i>	17 (3)	6 (0)
Nb G0 females <i>wSn</i>	—	0 (0)
Nb G0 females <i>wSh</i> + <i>wSn</i>	—	24 (10)
Total G0 females infected	17 (3)	30 (10)

RESULTS

Injections and Segregation of wSh and wSn

Injections of the *D. simulans* *STC* strain were performed using *D. sechellia* donor lines *S9* (infected by *wSh*) and *Dsech* (infected by *wSh* + *wSn*). A total of 1899 embryos were injected, resulting in 53 adult females (G0), which were allowed to lay before their infection status was determined. Forty-seven G0 females were found to be infected but only 13 transmitted *Wolbachia* to their offspring (to test the ability of infected G0 females to transmit the bacteria, pools of three G1 individuals were tested by PCR). These results are summarized in Table 1. Among the offspring of each of these 13 G0 females, 10 G1 females were left to lay before their infection status was determined by PCR. This analysis provided interesting results regarding the ability of *Wolbachia* to colonize the germ cells following the injection. After injection of *wSh* cytoplasm, of 30 G1 females (the offspring of the three G0 females infected by *wSh*), 25 were found to be infected and five to be uninfected. Following injection of doubly infected cytoplasm, of 100 G1 females (the offspring of the 10 G0 females infected by *wSh* + *wSn*), 35 were found doubly infected, 44 infected by *wSn* only, nine infected by *wSh* only, and 12 uninfected. Thus, a significant segregation occurred between G0 and G1, allowing separation of *wSh* and *wSn*.

Sequences

A 576-bp fragment of the *wsp* gene was cloned from three *wSh* lines (the *D. sechellia* *S9* line, as well as two *D. simulans* lines that were injected with material from *S9*, not used in the present study). A 558-bp fragment was cloned from three *wSn* lines (the *D. simulans* *ASn* line, as well as two other *D. simulans* lines that were injected with material from *ASn*, not used in the present study). At least three clones were sequenced for each line. The *wsp* sequences obtained from the three *wSh* lines strains were identical to each other and to the published *wHa* *wsp* gene sequence (Zhou et al. 1998, accession number AF020068). Equally, the *wsp* sequences obtained from the three *wSn* lines were identical to each other and to the published *wNo* *wsp* gene sequence (Zhou et al. 1998, accession number AF020074).

Based on this perfect identity on the *wsp* gene, previously reported differences in the 16S rRNA, a highly conserved locus, was surprising (Giordano et al. 1995; Rousset and Solignac 1995; see also Bourtzis et al. 1996). Furthermore, the different sequences deposited in the EMBL database ap-

TABLE 2. Is there any variability of the 16S rRNA? The X64265 sequence (*wHa*) differs by two nucleotides (positions 84 and 578) from the corresponding sequence presented in Rousset and Solignac (1995, table 1). Nucleotide positions are defined as in Rousset et al. (1992b). Nucleotides that we interpret as sequencing errors are in italics. Note that our *wHa* and *wSh* sequences are identical to the *wHa* sequence from O'Neill et al. (1992), and our *wNo* and *wSn* sequences are identical to the *wNo* sequence from James and Ballard (2000).

<i>wHa</i> and <i>wSh</i>			Potentially variable positions					
Variant	ID	Reference	84	310	578	616	620	654
<i>wHa</i>	X64265	Rousset et al. (1992a)	<i>T</i>	<i>G</i>	<i>C</i>	<i>G</i>	<i>C</i>	<i>G</i>
<i>wSh</i>	X80977	Rousset and Solignac (1995)	<i>T</i>	<i>G</i>	<i>T</i>	<i>G</i>	<i>C</i>	<i>G</i>
<i>wHa</i>	X61769	O'Neill et al. (1992)	<i>C</i>	<i>G</i>	<i>T</i>	<i>G</i>	<i>C</i>	<i>G</i>
<i>wSh</i>	U17059	Giordano et al. (1995)	<i>C</i>	<i>A</i>	<i>T</i>	<i>T</i>	<i>T</i>	<i>T</i>
<i>wHa</i>	AF468032	this study	<i>C</i>	<i>G</i>	<i>T</i>	<i>G</i>	<i>C</i>	<i>G</i>
<i>wSh</i>	AF468031	this study	<i>C</i>	<i>G</i>	<i>T</i>	<i>G</i>	<i>C</i>	<i>G</i>

<i>wNo</i> and <i>wSn</i>			Potentially variable positions			
Variant	ID	Reference	169	257	539	760
<i>wSn1</i>	X80978	Rousset and Solignac (1995)	<i>C</i>	<i>A</i>	<i>G</i>	<i>A</i>
<i>wSn2</i>	X80979	Rousset and Solignac (1995)	<i>T</i>	<i>A</i>	<i>G</i>	<i>A</i>
<i>wNo</i>	X64267	Rousset et al. (1992a)	<i>C</i>	<i>A</i>	<i>G</i>	<i>G</i>
<i>wNo</i>	AF312372	James and Ballard (2000)	<i>C</i>	<i>G</i>	<i>A</i>	<i>A</i>
<i>wNo</i>	AF468034	this study	<i>C</i>	<i>G</i>	<i>A</i>	<i>A</i>
<i>wSn</i>	AF468033	this study	<i>C</i>	<i>G</i>	<i>A</i>	<i>A</i>

peared to provide incongruent information. To clarify this issue, we sequenced 16S fragments for the different variants. Material from one line was sequenced for each variant, and at least four clones were sequenced for each line. Each nucleotide from the final consensus sequence was present in at least three of the four sequences for every site. Primer pairs were chosen to obtain reliable information for the sites that had been previously found variable. The results (Table 2)

suggest that *wHa* and *wSh* are in fact identical, as are *wNo* and *wSn*, and that previously reported differences are most likely due to sequencing errors. Thus, although other loci might reveal some variability, the *Wolbachia* from *D. simulans* and *D. sechellia* cannot be distinguished based on the 16S and *wsp* sequences.

Cytoplasmic Incompatibility Intensity: *wSh* and *wHa* in *Drosophila simulans*

The comparison between *wSh* and *wHa* in *D. simulans* was performed using two lines infected by *wHa* (*AHa* and *BHa*) and two lines infected by *wSh* (*ASh* and *CSh*). Two sets of experiments were carried out, the first with flies from G7 to G10 postinjection (Table 3a, dataset A) and the second with flies from after G30 postinjection (Table 3a, dataset B). *wSh* was found to induce CI in both lines for both experiments. The results were analyzed by ANOVA (Table 4a). The experiment-by-line interaction was found significant ($F_{1,111} = 35.31$, $P = 0.0001$). Thus, CI intensity differed between the two experiments, but this did not affect all the lines in the same way. Indeed, it appears that CI intensity is clearly lower in the second experiment for two lines of four (*AHa* and *ASh*). The other factors were not found to differ significantly. Thus, most importantly, our data do suggest that CI levels in *D. simulans* induced by *wSh* and *wHa* do not differ from each other.

Cytoplasmic Incompatibility Intensity: *wSn* and *wNo* in *Drosophila simulans*

The comparison between *wSn* and *wNo* in *D. simulans* was performed using two lines infected by *wNo* (*ANo* and *BNo*) and two lines infected by *wSn* (*ASn* and *BSn*). Two sets of experiments were carried out, the first with flies from G7 and G10 postinjection (Table 3b, dataset A) and the second with flies after G30 postinjection (Table 3b, dataset C). *wSn* was

TABLE 3. Cytoplasmic incompatibility (CI) intensity: descriptive statistics. All males were crossed with uninfected females from the *STC* strain. (a, b) Corrected CI was calculated using the corresponding control cross mortality, given in 3. (c) Datasets with the same letter were obtained in the same experiment; 0, uninfected; SE, standard error.

Dataset	Male (infection/line)	<i>n</i> crosses	<i>n</i> eggs	CI _{cor} (%)	SE (%)
(a) <i>wHa</i> and <i>wSh</i> (corrected CI)					
A	<i>wHa/AHa</i>	12	1595	93.9	2.4
A	<i>wHa/BHa</i>	12	1107	71.9	5.2
A	<i>wSh/ASh</i>	12	1525	90.1	2.8
A	<i>wSh/CSh</i>	9	849	49.7	5.8
B	<i>wHa/AHa</i>	16	1877	48.1	5.4
B	<i>wHa/BHa</i>	23	2952	75.9	2.8
B	<i>wSh/ASh</i>	18	2348	59.1	4.2
B	<i>wSh/CSh</i>	17	1205	59.1	3.5
(b) <i>wNo</i> and <i>wSn</i> (corrected CI)					
A	<i>wNo/ANo</i>	7	910	67.6	5.4
A	<i>wNo/BNo</i>	9	852	48.5	2.9
A	<i>wSn/ASn</i>	6	748	50.9	10.5
A	<i>wSn/BSn</i>	4	223	55.7	9.2
C	<i>wNo/ANo</i>	17	2048	58.8	2.6
C	<i>wNo/BNo</i>	20	2123	52.5	2.7
C	<i>wSn/ASn</i>	24	5658	49.4	3.7
C	<i>wSn/BSn</i>	21	2463	41.8	4.7
(c) Control cross mortality (raw embryonic mortality)					
A	0/ <i>STC</i>	16	1253	19.5	8.4
B	0/ <i>STC</i>	26	2934	11.8	2.2
C	0/ <i>STC</i>	24	2334	10.4	2.0

TABLE 4. Cytoplasmic incompatibility (CI) intensity: ANOVAs W, *Wolbachia*; L, line; Ex, experiment. L is nested within W. When two factors are noted in the *F* denominator, the latter was calculated as the mean of the mean squares weighted by their degrees of freedom.

Source	df	Mean square	<i>F</i>	<i>F</i> denominator	Pr > <i>F</i>
(a) <i>wHa</i> and <i>wSh</i>					
W	1	0.30997946	0.39	Ex × L	ns
L	2	0.41122834	0.35	Ex × W	ns
Ex	1	1.29459319	1.09	E × L	ns
Ex × W	1	0.1366767	0.17	Ex × L	ns
Ex × L	2	1.18595925	35.31	Error	0.0001
Error	111	0.03358365			
(b) <i>wNo</i> and <i>wSn</i>					
W	1	0.13329951	4.06	Error	0.0466
L	2	0.1008245	4.06	Error	ns
Ex	1	0.06188041	3.07	Error	ns
Ex × W	1	0.01554748	1.88	Error	ns
Ex × L	2	0.0487602	0.47	Error	ns
Error	100	0.03283871	1.48		

found to induce CI in both lines for both experiments. The results were analyzed by ANOVA (Table 4b). The *Wolbachia* factor was the only one found just significant ($F_{1,100} = 4.06$, $P = 0.0466$). However, note that the line factor is very close to being significant ($F_{2,100} = 3.07$, $P = 0.0508$). If a line effect had been detected, the *F*-value for the *Wolbachia* factor would have been calculated differently (with the line mean square as denominator). Consequently, the *Wolbachia* factor would not have been found significant. Thus, *wNo* induces a CI very similar to and possibly slightly higher than that of *wSn* in the *D. simulans* *STC* strain (mean $CI_{cor} = 55.8\%$ for *wNo* vs. 47.2% for *wSn*).

Compatibility Relationships: *wHa* and *wSh*

The compatibility relationships between *wHa* and *wSh* were investigated using the lines *AHa* and *BHa* (*wHa*), *ASh* and *CSh* (*wSh*). Sixteen different types of crosses were performed (all possible crosses between the four lines). For clarity, Table 5 does not present the results for these 16 crosses. Instead, the lines for a given *Wolbachia* strain were pooled. Note however that for the ANOVA (Table 6a) the lines were not pooled.

Results show that *wHa* and *wSn* are compatible. This can be seen from the fact that the interaction between *WM* (*Wolbachia* in male) and *WF* (*Wolbachia* in female) is not found significant, which shows that embryonic mortality in crosses involving a male or a female bearing a given *Wolbachia* strain does not depend on the infection status of its partner. Interestingly, fertility was reduced in crosses involving *wSh* females, regardless of the *Wolbachia* present in the male. Indeed, the *WF* factor was found significant ($F_{1,323} = 16.81$, $P < 0.025$). In crosses involving *wHa* females, the mean embryonic mortality is 11.1% with *wHa* males and 8.6% with *wSh* males, whereas in crosses involving *wSh* females, the mean embryonic mortality is 20.9% with *wHa* males and 20.9% with *wSh* males.

Compatibility Relationships: *wNo* and *wSn*

The compatibility relationships between *wNo* and *wSn* were investigated using the lines *ANo* and *BNo* (*wNo*), *ASn*

TABLE 5. Compatibility relationships, descriptive statistics. EM, mean embryonic mortality; SE, standard error.

Male infection	Female infection	<i>n</i> crosses	<i>n</i> eggs	EM (%)	SE (%)
<i>wHa</i>	<i>wHa</i>	81	8216	11.1	1.8
<i>wSh</i>	<i>wHa</i>	96	9148	8.6	1.2
<i>wHa</i>	<i>wSh</i>	84	8175	20.9	2.4
<i>wSh</i>	<i>wSh</i>	78	7168	20.9	2
<i>wNo</i>	<i>wNo</i>	41	3616	16.9	2.2
<i>wSn</i>	<i>wNo</i>	44	4458	18	2
<i>wNo</i>	<i>wSn</i>	39	3397	18.4	2.6
<i>wSn</i>	<i>wSn</i>	41	3959	16.4	2.6

and *BSn* (*wSn*). Sixteen different types of crosses were performed (all possible crosses between the four lines). The results are summarized in Table 5, the ANOVA is presented in Table 6b. No factor was found significant. Thus, no incompatibility was detected between *wNo* and *wSn*.

Compatibility Relationships: *wKi* and *wSn*

wKi is known from previous studies to rescue the CI induced by *wNo*, although it is unable to induce CI itself (*[mod- resc+]* phenotype; Merçot and Poinso 1998b; Poinso and Merçot 1999). We were therefore interested in determining whether *wKi* was also able to rescue the CI induced by *wSn*. To answer this question, males infected by *wNo* (lines *ANo* and *BNo*) and males infected by *wSn* (lines *ASn* and *BSn*) were crossed with females infected by *wKi* (line *KC9*) and with uninfected females (strain *STC*). The results, summarized in Table 7a, were analyzed by ANOVA (Table 8). Only the female infection status was found to have a significant effect ($F_{1,43} = 54.11$, $P = 0.0001$). Thus, *wKi* rescues both *wNo* and *wSn* with the same efficiency.

In the same experiment, uninfected females (*STC*) were crossed with uninfected males (*STC*) and with *wKi* males (*KC9*; Tab. 7b). Embryonic mortality was not found significantly different in the two types of cross (*t*-test, $P = 0.555$). Thus, as expected from previous studies (Merçot and Poinso 1998b; Poinso and Merçot 1999), we observe that *wKi* does not induce CI.

TABLE 6. Compatibility relationships, ANOVAs. WM, *Wolbachia* in male; WF, *Wolbachia* in female; LM, line male; LF, line female. LM and LF are nested within WM and WF, respectively.

Source	df	Means square	<i>F</i>	Pr > <i>F</i>
(a) <i>sHa/wSh</i>				
WM	1	0.01482943	0.1	ns
WF	1	2.50696252	16.81	<0.025
LM	2	0.06155595	0.41	ns
LF	2	0.23984259	1.61	ns
WM × WF	1	0.01127337	0.08	ns
Error	323	0.14916442		
(b) <i>wNo/wSn</i>				
WM	1	0.00012255	0	ns
WF	1	0.00574036	0	ns
LM	2	0.04856927	0.04	ns
LF	2	0.2382015	0.21	ns
WM × WF	1	0.00626302	0.01	ns
Error	149	1.15812401		

TABLE 7. *wKi mod* and *resc* functions. The different lines for a given *Wolbachia* strain were pooled in (a). EM, mean embryonic mortality; SE, standard error.

Male	Female	<i>n</i> crosses	<i>n</i> eggs	EM	SE
(a) Test of <i>wKi resc</i> function versus <i>wSn</i> and <i>wNo</i>					
<i>wNo</i>	KC9	13	1074	27.9%	6.8%
<i>wSn</i>	KC9	14	1315	21.9%	5.0%
<i>wNo</i>	<i>STC</i>	10	965	71.0%	10.1%
<i>wSn</i>	<i>STC</i>	14	1368	81.2%	2.9%
(b) Test of <i>wKi mod</i> function					
<i>STC</i>	<i>STC</i>	10	849	23.5%	4.1%
KC9	<i>STC</i>	8	629	20.0%	2.2%

DISCUSSION

Injection and Segregation

Singly infected or doubly infected cytoplasm from *D. sechellia* was injected into the *D. simulans STC* strain. Among the offspring of doubly infected G0 females, we observed doubly infected females (35%), uninfected females (12%), and singly infected females at a high percentage (44% infected by *wSn* only, 9% infected by *wSh* only). Thus, segregation occurred at a very high rate between the G0 and G1 following injection. This result is in contrast to classical segregation rates, previously estimated around 3% for *wHa* and *wNo* and around 1% for *wSh* and *wSn*, in experiments where doubly infected females were crossed with uninfected males for several generations (Poinset et al. 2000; S. Charlat, P. Bonnavion, and H. Merçot, unpubl. data). Of course, the experimental conditions are very different here, because segregation occurs after cytoplasmic injection. It is likely that our observation illustrates the fact that the bacteria go through a severe bottleneck during the process of injection. The number of bacteria that colonize a given polar cell during injection must be very limited, much more so than during the normal process of transmission from mothers to offspring. We therefore suggest that cytoplasmic injections might represent an efficient method for separating *Wolbachia* strains naturally present as multiple infections.

G1 females singly infected by *wSn* were observed more frequently than those singly infected by *wSh*. We see two possible explanations, which are not mutually exclusive. First, this proportion might reflect the respective concentration of the two strains within donor cytoplasm. Alternatively, the percentages of infection observed in G1 might be due to differences in the ability to colonize polar cells. Interestingly, S. Charlat, P. Bonnavion, and H. Merçot also observed that in the original host *D. sechellia*, *wSn* seemed more efficiently transmitted than *wSh*, although the difference was not statistically significant (unpubl. data).

The proportion of uninfected offspring produced by singly infected G0 mothers (*wSh*) and doubly infected G0 mothers (*wSh* + *wSn*) were not significantly different (16.7% vs. 12.0%; $\chi^2 = 0.449$, *df* = 1, *P* < 0.9). On the contrary, the proportion of G1 flies having lost *wSh* was significantly higher in the offspring produced by doubly infected G0 mothers than in the offspring produced by singly infected G0 mothers (16.7% vs. 56%; $\chi^2 = 14,371$; *df* = 1, *P* < 0.001). If one assumes that the rate of loss is proportional to the total

TABLE 8. Test of *wKi resc* function versus *wSn* and *wNo* by ANOVA. WM, *Wolbachia* in male; FS, female infection status (*wKi*/uninfected); LM, line male. LM is nested within WM.

Source	<i>df</i>	Mean square	<i>F</i>	Pr > <i>F</i>
FS	1	3.59445355	54.11	0.0001
WM	1	0.03461307	0.52	ns
LM	2	0.09105409	1.37	ns
WM × FS	1	0.16660223	2.51	ns
FS × LM	2	0.11363608	1.71	ns
Error	43	0.06642904		

amount of bacteria injected into the recipient egg, these two results suggest that the total amount of bacteria is similar in doubly infected and singly infected donor cytoplasm and, accordingly, that each *Wolbachia* strain is present at lower concentrations in doubly infected cytoplasm.

wSh Bearing Females Show Reduced Hatching Rates in Compatibility Experiments

We observed that embryonic mortality was higher in crosses involving females infected by *wSh*, regardless if the male was infected by *wSh* or *wHa*. We see two hypotheses to interpret this result. First, *wSh* might be less efficiently transmitted from mothers to embryo than *wHa*. Indeed, if a significant part of the eggs laid by *wSh* females do not bear the *Wolbachia*, embryonic mortality is likely to occur because of the CI induced by *wSh* or *wHa* in males. Secondly, *wSh* might reduce the intrinsic female fertility. We investigated the first hypothesis by measuring the transmission efficiency of *wHa* and *wSh* in the *STC* strain, using the *AHa*, *BHa*, *ASh*, and *CSH* lines. Infected females were crossed to uninfected males (*STC*) and the infection status of sons and daughters was determined (*n* = 32 for each line). *Wolbachia* was very efficiently transmitted to the offspring in the four lines (31 infected of 32 for *AHa*, 30/32 for *BHa*, 31/32 for *ASh*, and 30/32 for *CSH*), suggesting that *wSh* is not less efficiently transmitted to offspring than *wHa*. To test the second hypothesis, uninfected males will have to be crossed with uninfected and infected females, which remains to be done.

Cytoplasmic Incompatibility Phenotypes

wSh and *wSn* were injected from *D. sechellia* into *D. simulans* to compare their CI phenotypes to those expressed by *wHa* and *wNo*, respectively. When placed in the same genomic background, *wSh* and *wHa* were not found to induce significantly different CI levels. In contrast, *wNo* was found to induce a higher CI level than *wSn*. However, this difference was small (less than 10%) and the α probability was just below the 5% threshold. Most notably, *wHa* and *wSh* showed a more variable CI expression than *wNo* and *wSn*. Accordingly, quantitative differences appear between previous estimations of *wHa* intensity (Merçot and Poinset 1998a; Poinset and Merçot 2001). This variability weakens the statistical power of the comparison between *wHa* and *wSh* and highlights the importance of using more than one line for estimating CI levels.

Concerning the evolution of CI levels, two theoretical analysis showed that the bacterial determinants are not directly

subject to selection (Prout 1994; Turelli 1994). Turelli (1994) further showed that host factors decreasing CI levels are selected for. A decrease of CI levels due to host evolution is thus expected to occur faster than a decrease due to bacterial factors, which are presumably driven by drift only. Accordingly, *w*Sn induces a lower CI in its natural host than in *D. simulans* (S. Charlat, P. Bonnavion, and H. Merçot, unpubl. data), whereas *w*Sn and *w*No induce similar CI levels when in the same host (this study), suggesting that the CI level differences between *D. simulans* and *D. sechellia* are due to the evolution of hosts factors affecting CI levels rather than bacterial factors.

In the compatibility tests, we observed that embryonic mortality for a given type of male or female did not depend on its partner's infection status. In other words, *w*Ha and *w*Sh were fully compatible, as were *w*No and *w*Sn. Furthermore, *w*Ki, which is known to rescue the CI induced by *w*No (Merçot and Poinot 1998b; Poinot and Merçot 1999), also fully rescued the CI induced by *w*Sn. Interspecific crosses realized between *D. simulans* females and *D. sechellia* males have previously been done, suggesting that *w*Ha and *w*No were compatible, at least partially, with *w*Sh and *w*No, respectively (Rousset and Solignac 1995). However, from these results, a partial incompatibility could not be excluded, owing to elevated hybrid mortality. Furthermore, because *D. sechellia* females do not mate with *D. simulans* males, only the ability of *w*Ha and *w*No to rescue the CI induced by *w*Sh and *w*Sn could be tested for (Rousset and Solignac 1995). Our results show that compatibility is complete, in both crossing directions.

Theory suggests that *Wolbachia* compatibility types are not constrained by stabilizing selection, suggesting that they might evolve rapidly (Charlat et al. 2001b), but empirical studies testing this prediction are only beginning. As a first step, we focused here on two pairs of very closely related *Wolbachia*, having evolved separately for about 500,000 years (if double infection predates the split between the *siI* and *se* cytoplasmic lineages, as suggested by Rousset and Solignac 1995) or even less than this, if subsequent horizontal transfer took place. We observe that such a short isolation was not sufficient for compatibility types to diverge. As a second step, more divergent *Wolbachia* should be confronted in a single host. A case of potential interest involves *D. melanogaster* and the tephritid cherry fruit-fly *Rhagoletis cerasi*. These species are infected by CI inducing *Wolbachia* that differ by only five substitutions in the *wsp* locus (Zhou et al. 1998; Riegler and Strauffer 2002; M. Riegler, pers. comm.). Whether these two bacteria are still compatible with each other is an open question.

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Incipient evolution of *Wolbachia* compatibility types

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En bref...

Drosophila melanogaster, *Drosophila simulans* (diptères *Drosophilidae*), et *Rhagoletis cerasi* (diptère *Tephritidae*) sont infectés par des *Wolbachia* étroitement apparentées, respectivement nommées *wMel*, *wAu* et *wCer2*.
Nous décrivons ici les relations de compatibilité entre ces variants, déterminées après injection dans *Drosophila simulans*. Il apparaît que la *Wolbachia wAu*, un variant n'induisant pas d'IC (phénotype [mod-]), ne protège pas les embryons de l'IC induite par *wMel* (Poinso et al. 1998) ou *wCer2*, suggérant que sa fonction *resc* est différente ou simplement manquante. Les variants *wCer2* et *wMel* apparaissent presque totalement incompatibles, démontrant une évolution rapide des types de compatibilité. Les relations entre *wCer2* et *wRi*, dont la compatibilité partielle avec *wMel* a été établie par une étude antérieure (Poinso et al. 1998), sont également explorées. Les résultats montrent que *wRi* et *wCer2* ne sont pas totalement incompatibles. L'interprétation de ces observations implique des variations qualitatives et quantitatives des fonctions *mod* et *resc*, pour lesquelles nous proposons une formalisation.

INCIPIENT EVOLUTION OF *WOLBACHIA* COMPATIBILITY TYPES

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ABSTRACT

Cytoplasmic incompatibility (CI) is induced by the maternally inherited bacterium *Wolbachia*: when infected males mate with uninfected females, or with females bearing a different *Wolbachia* variant, paternal chromosomes behave abnormally and embryos die. This pattern can be interpreted as resulting from two bacterial effects: one (usually termed *mod*, for modification) would affect sperm, and induce embryo death, unless *Wolbachia* is also present in the egg, which implies the existence of a second effect, usually termed *resc*, for rescue. The fact that CI can occur in crosses between males and females infected by different *Wolbachia* shows that *mod* and *resc* interact in a specific manner. In other words, different compatibility types, or *mod* / *resc* pairs seem to have diverged from one (or a few) common ancestor(s), assuming that CI has not evolved many times independently. We are interested in the process allowing the evolution of compatibility types. Here this question is addressed experimentally after cytoplasmic injection, by investigating compatibility relationships between closely related *Wolbachia* variants naturally evolving in different dipteran hosts: *Drosophila simulans*, *D. melanogaster* and *Rhagoletis cerasi*. Our results suggest that closely related bacteria can be totally or partially incompatible. The compatibility relationships observed can be explained using a formal description of the *mod* and *resc* functions, implying both qualitative and quantitative variations.

Among the various known effects of the endocellular bacterium *Wolbachia* is cytoplasmic incompatibility (CI) (reviewed in Hoffmann & Turelli 1997; Charlat *et al.* 2002a). It becomes visible when males bearing the bacterium mate with uninfected females; a cross resulting in embryo death. On the contrary, hatching rates are normal if the female is also infected. This results in infected females being more fertile, in average, than uninfected ones, while infected males are in average less fertile than their uninfected counterpart. Because *Wolbachia* is transmitted by females only, through the egg cytoplasm, this finally ends in increasing infection frequency. Cytoplasmic incompatibility can thus be regarded as an invasion and maintenance strategy of the bacterium, based on the fact that infected females are immune from the partial sterility caused by infected males.

The phenomenon is well characterized cytogenetically (Breeuwer & Werren 1990; Callaini *et al.* 1996; Lassy & Karr 1996; Callaini *et al.* 1997; Tram & Sullivan 2002). In incompatible crosses, paternal chromosomes fail to condense normally, or at a sufficiently high speed, so that maternal chromosomes segregate on their own at the first mitosis. In diploid organisms, this typically results in developmental arrest. In haplo-diploids, where males naturally develop from unfertilized haploid eggs, CI-induced haploidy can result in male development from fertilized eggs, although embryo death might still be the rule, possibly due to imperfect haploidization (Vavre *et al.* 2000; Bordenstein *et al.* 2003).

The bacterial molecules involved are still unknown. The current framework is that of the modification / rescue model (Werren 1997), according to which two phenomena must be distinguished: one occurring in the male germline, before *Wolbachia* is shed from maturing sperm (termed *mod*, for modification) that disrupts paternal chromosomes behavior and one occurring in infected eggs (termed *resc*, for rescue) that restores normal development. Attempts have been made to translate *mod* and *resc* into more concrete factors, and it finally seems that a *Lock-and-Key* model, assuming that *mod* (the *Lock*) and *resc* (the *Key*) are controlled by different genetic determinants and directly interact with each other, is the most likely to be valid (Poinsot *et al.* 2003).

Aside from the incompatibility between infected males and uninfected females (often termed uni-directional, because the reverse cross is compatible), bi-directional incompatibility can also occur if males and females bear different *Wolbachia* variants. This more complex form of CI demonstrates that *mod* and *resc* interact in a specific manner. Assuming that CI did not evolve many times independently, which seems reasonable, this also means that compatibility types (or *mod* / *resc* pairs) can diverge. We are interested in the process behind this evolution. A theoretical analysis focusing on this issue has suggested that compatibility

types can readily evolve if *mod* and *resc* are controlled by different genetic determinants (Charlat *et al.* 2001). Empirically, this question has been investigated in *Drosophila simulans* and *Drosophila sechellia*, which are infected by closely related *Wolbachia* variants having evolved separately for not more than half a million years (Rousset & Solignac 1995). Relatedness between the bacteria of the two species is such that no molecular divergence is detectable, based on the 16S rRNA locus or the faster evolving *wsp* gene (Zhou *et al.* 1998; Charlat *et al.* 2002b). The compatibility relationships between the sisters *Wolbachia* strains were investigated by injecting the bacteria from *D. sechellia* into *D. simulans* (Charlat *et al.* 2002b). This allowed showing that compatibility types had not evolved during this brief period of isolation. The present study goes one step further in the empirical investigation of the evolution of compatibility types: closely related, but molecularly distinguishable *Wolbachia* were confronted in a single host genetic background, and their relationships tested.

The study involves three dipteran species and some of their symbionts: *Rhagoletis cerasi* (Tephritidae), *Drosophila melanogaster* and *D. simulans* (Drosophilidae). *R. cerasi* is infected by two *Wolbachia* variants (namely *wCer1* and *wCer2*). *wCer2* is known to induce strong CI in this species because males from doubly infected populations (with individuals bearing *wCer1* + *wCer2*) are incompatible with females from populations bearing only *wCer1* (Riegler & Stauffer 2002). After transfer into *D. simulans*, *wCer2* was found to induce low but significant levels of CI (about 40% embryonic mortality) (Riegler *et al.* in prep). *D. melanogaster* is infected by a *Wolbachia* called *wMel* that can induce CI in its original host, although at a low level (typically, only about 30% of embryos die in incompatible crosses in laboratory experiments, even less in the field) (Hoffmann 1988; Hoffmann *et al.* 1994; Solignac *et al.* 1994; Hoffmann *et al.* 1998; Olsen *et al.* 2001). After transfer into *D. simulans*, *wMel* was found to induce very strong CI (near 100% embryonic mortality) (Poinsot *et al.* 1998). *D. simulans* is naturally infected by 5 different *Wolbachia* (reviewed in Merçot & Charlat 2003). The one we are interested in here is called *wAu*. In the three populations where this has been investigated directly, *wAu* was not found to induce CI (Hoffmann *et al.* 1996; James & Ballard 2000; Charlat *et al.* 2003) (but let us mention the intriguing case of the “Lantana population” from Florida (Ballard *et al.* 1996); in two lines from this area, a *Wolbachia* infection was found to induce significant CI; later sequencing were performed on the same lines (James & Ballard 2000), and suggest that *wAu* was responsible for this phenotype). The *wCer2*, *wMel* and *wAu* triangle is of interest regarding the evolution of CI because these three *Wolbachia* are very closely related as indicated by the *wsp* gene, and confirmed by two other loci (Zhou *et al.* 1998; Riegler & Stauffer 2002;

Riegler *et al.* in prep). Specifically, based on *wsp* sequences, *wMel* and *wAu* differ by 5 substitutions, *wMel* and *wCer2* by 4 substitutions, and *wCer2* and *wAu* by one substitution. Figure 1 shows the most parsimonious phylogeny that can be inferred based on this limited variation.

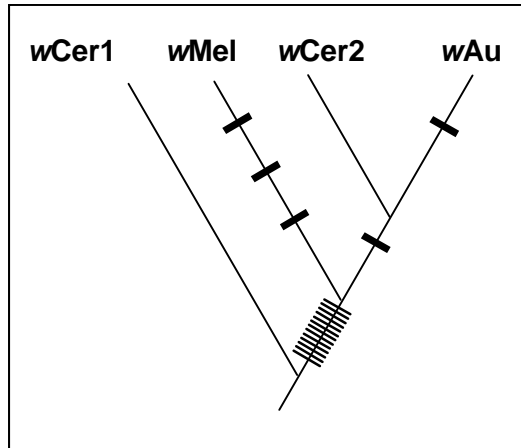


Figure 1.

Phylogenetic relationships between *wCer2*, *wMel*, and *wAu* based on *wsp* sequences. The gene region upon which is based this phylogeny is highly variable and thus cannot be aligned confidently with most *Wolbachia* sequences. The *wCer1* sequence (Riegler & Stauffer 2002), however, is sufficiently close to the *Mel* clade (the group including *wMel*, *wCer2* and *wAu*) for a good alignment to be obtained, and was here used as an outgroup. In this tree, the monophyly of the *Mel* clade is supported by 13 substitutions. Among the 5 substitutions that occurred within the *Mel* clade, 4 are non-informative (3 autapomorphies of *wMel* and 1 autapomorphy of *wAu*) but one supports the monophyly of the *wAu* + *wCer2* group. Thin ticks symbolize synapomorphies of the *Mel* clade, thick ticks symbolize substitutions within the *Mel* clade.

In addition to these three *Wolbachia*, *wRi* variant (a natural infection of *D. simulans* inducing high levels of CI) was included in this study. This was prompted by earlier results, having revealed intriguing compatibility relationships between *wRi* and *wMel* (Poinsot *et al.* 1998). Based on *wsp* sequences, the *wRi* variant clearly falls out of the *Mel* clade (the group including *wMel*, *wCer2* and *wAu*) (Zhou *et al.* 1998). Actually, *wRi* is even more distant from this group than is the *wCer1* variant, used as an outgroup in Figure 1.

The following conclusions can be drawn from our experiments: (i) *wAu* does not seem to rescue the CI induced by *wCer2*, in spite of their evolutionary closeness, (ii) *wMel* and *wCer2* appear to be incompatible in one direction of cross (male *wCer2* × female *wMel*), and nearly incompatible in the reverse cross, suggesting their compatibility types have evolved rapidly and finally (iii) *wCer2* and *wRi* are nearly incompatible in both directions of cross. To account for these complex patterns, we propose a formal model involving both qualitative and quantitative variations of the *mod* and *resc* functions.

MATERIAL AND METHODS

D. simulans lines

RC45 and RC50 are two lines infected by *wCer2*, obtained by cytoplasmic injection into the STC strain (Riegler *et al.* in prep). STC is an inbred stock from the Seychelles archipelago, originally infected by two *Wolbachia* (*wHa* and *wNo*) cured from infection following a

tetracycline treatment (Poinsot *et al.* 2000). Coffs Harbour S20 is an Australian strain founded using flies from a 1993 collection, infected by *wAu* (Hoffmann *et al.* 1996). Y6 is an isofemale lines from Yaounde (Cameroon), infected by *wAu* (Charlat *et al.* 2003). ME29 is infected by *wMel*, following cytoplasmic injection from *D. melanogaster* into a tetracycline treated *D. simulans* line from New Caledonia (Poinsot *et al.* 1998). ME29TC is an uninfected line, cured from infection following a tetracycline treatment on the ME29 line (this study). DSR is a Californian strain infected by *wRi* (Hoffmann *et al.* 1986). DSRTC is an uninfected line, cured from infection following a tetracycline treatment on the DSR line (this study).

***Wolbachia* detection**

In all experiments, detection of *Wolbachia* was done by PCR. DNA was obtained according to O' Neill *et al.* (1992). The *wsp* gene was amplified according to Zhou *et al.* (1998) the 16S gene according to O' Neill *et al.* (1992).

Rearing conditions

Flies were routinely maintained at 18°C, on axenic medium (David 1962). For two generations before each experiment, flies were maintained at 25°C at low larval density. One generation before each experiment, instead of rearing mass strains in bottles, 10 fertilized females of each line were left to lay in separate vials, so that their infection status could be controlled before choosing virgin flies for mating experiments. This was necessary for *wCer2* lines, where maternal transmission is low (about 50%) (Riegler *et al.* in prep) and generalized to all lines for homogeneity.

Compatibility relationships assays

Compatibility relationships were investigated by crossing males and females of different infection status in both directions. For example, consider one is studying compatibility between two CI inducing *Wolbachia* A and B. Comparing levels of embryonic mortality in the two following crosses: (1) male A × female B and (2) male A × female 0 (where 0 stands for uninfected), allows to test if *Wolbachia* B can rescue *Wolbachia* A. Under the *mod resc* model, this is to say: is *mod_A* compatible with *resc_B*? The level of compatibility can be

quantified as the percentage of embryos that are saved by the presence of *Wolbachia* B in females. The opposite direction of cross allows you to test if *mod_B* is compatible with *resc_A*.

In order to avoid possible variations of genetic background effects, that could confuse interpretations, experiments involving different *Wolbachia* variants in different genetic backgrounds were performed using F1 hybrids. For example, if *Wolbachia* A infects line 1 and *Wolbachia* B infects line 2, crosses between lines 1 and 2 were performed before starting CI assays, so that, in average, the genetic background was the same in all the individuals that were to be compared (if one neglects possible variations of mitochondrial genomes, that are not homogenized by this method). F1 hybrids can be difficult to obtain when males bear a CI-inducing *Wolbachia* that is not present in the female. To circumvent this problem, males were then taken from uninfected lines bearing the same genetic background, obtained by antibiotic treatment.

Experiments were performed using virgin males aged 3 to 4 days and virgin females aged 4 to 7 days. Mating was controlled and crosses where copulation lasted for less than 15 minutes were discarded, in order to ensure insemination. Inseminated females were individually placed at 25°C, on axenic medium colored with neutral red, making egg counting easier. Females were removed after 48 hours of laying and eggs left for an additional 24 hours at 25°C to allow hatching of all viable embryos, and finally placed at 4°C until egg counting. Embryonic mortality was then determined as the percentage of unhatched eggs. Samples with less than 20 eggs were discarded. For crosses showing 0% hatching, a fertility test was performed by crossing each parent with individuals of compatible infection status to distinguish between crosses where CI is 100% and crosses involving intrinsically sterile individuals, which were excluded from analysis. Finally, the infection status of parents was checked by PCR. It must be noted that experiments involving the *wCer2* bacterium require double sampling effort in comparison with classic CI assays. Indeed, because this variant has a very low transmission rate in its foreign host *D. simulans* (about 50%) (Riegler *et al.* in prep), $2n$ crosses have to be performed for n final results to be obtained.

Statistical analysis

Data was analyzed using non-parametrical Kruskal-Wallis and Wilcoxon tests.

RESULTS

The *wAu* / *wCer2* relationship

Although *wAu* does not appear to induce CI (Hoffmann *et al.* 1996; James & Ballard 2000; Charlat *et al.* 2003) (but see Ballard *et al.* 1996), it has been hypothesized that it could rescue the CI induced by another *Wolbachia*, if the two variants were sufficiently closely related (Bourtzis *et al.* 1998). Indeed, in *D. simulans*, another non CI inducing *Wolbachia* has been found to express such a [*mod- resc+*] phenotype (Merçot & Poinso 1998). Earlier experiments have revealed that *wAu* cannot rescue the CI induced by *wMel* (Poinso *et al.* 1998). We were interested in testing if *wAu* could rescue the CI induced by *wCer2*, its closest known relative. To do so, females from two *wAu* lines (Coffs, from Australia, and Y6, from Cameroon), as well as uninfected females, were crossed with *wCer2*-infected males (lines RC45 and RC50). As showed in table 1, the female infection status was not found to affect embryonic mortality significantly. Thus, *wAu* does not appear to rescue the *wCer2 mod* function.

Table 1.
Does *wAu* rescue *wCer2* ?

Male		Female		Ne	Nc	EM (%)	SE	W	P
mother (father)	Wolb	mother (father)	Wolb						
RC45 (Coffs)	<i>wCer2</i>	STC (Coffs)	0	1388	12	37.8	3.7		
RC45 (Coffs)	<i>wCer2</i>	Coffs (STC)	<i>wAu</i>	1517	13	30.3	6.8	1.19	>0.2
RC50 (Coffs)	<i>wCer2</i>	STC (Coffs)	0	1177	9	27.0	5.6		
RC50 (Coffs)	<i>wCer2</i>	Coffs (STC)	<i>wAu</i>	1026	7	29.0	6.1	0.37	>0.7
RC45 (Y6)	<i>wCer2</i>	STC (Y6)	0	1202	13	49.0	6.1		
RC45 (Y6)	<i>wCer2</i>	Y6 (STC)	<i>wAu</i>	1290	14	45.3	5.2	0.78	>0.4
RC50 (Y6)	<i>wCer2</i>	STC (Y6)	0	1446	16	46.3	7.3		
RC50 (Y6)	<i>wCer2</i>	Y6 (STC)	<i>wAu</i>	1327	15	44.8	7.2	0.20	>0.8

In order to avoid variations of genetic background effects, experiments were performed using F1 hybrids between lines. We thus mention mother's line (which determines offspring's infection status) and father's line (which contributes only to the nuclear genome) in parenthesis. Note RC45 and RC50 have the same genetic background as STC (see materials and methods). Abbreviations: Wolb (Wolbachia; 0: uninfected), Ne (total number of eggs counted), Nc (number of crosses), EM (mean embryonic mortality), SE (standard error), W (result of the Wilcoxon), P (associated α probability). The Wilcoxon tests were performed by comparing each cross involving infected females with the corresponding control cross, where the female is not infected.

The *wMel* / *wCer2* relationship

To test if *wMel* can rescue the CI induced by *wCer2*, females from the ME29 line, infected by *wMel*, as well as uninfected females, were crossed with *wCer2*-infected males (lines RC45

and RC50). As showed in table 2, embryonic mortality was not found significantly reduced by the presence of *wMel* in females. Thus, *wMel* does not appear to rescue the *wCer2 mod* function.

Table 2.
Does *wMel* rescue *wCer2* ?

Male		Female		Ne	Nc	EM (%)	SE	W	P
mother (father)	Wolb	mother (father)	Wolb						
RC45 (ME29)	<i>wCer2</i>	STC (ME29)	0	1296	12	40.2	6.9		
RC45 (ME29)	<i>wCer2</i>	ME29 (STC)	<i>wMel</i>	910	10	46.3	10.3	0.07	>0.9
RC50 (ME29)	<i>wCer2</i>	STC (ME29)	0	1120	11	39.2	9.3		
RC50 (ME29)	<i>wCer2</i>	ME29 (STC)	<i>wMel</i>	1156	13	41.0	4.5	0.61	>0.5

Same legend as table 1.

To test if *wCer2* can rescue the CI induced by *wMel*, RC45 and RC50 females (bearing *wCer2*), as well as uninfected females, were crossed with ME29 males, bearing *wMel*. As showed in table 3, embryonic mortality was significantly reduced by the presence of *wCer2* in females. However, the difference was quantitatively very small (7.2% with RC45 females, 2.9% with RC50 females). Thus, *wCer2* can rescue a very small proportion of the embryos when faced with the *wMel mod* function.

Table 3.
Does *wCer2* rescue *wMel* ?

Male		Female		Ne	Nc	EM (%)	SE	W	P
mother (father)	Wolb	mother (father)	Wolb						
ME29 (STC)	<i>wMel</i>	STC (ME29)	0	1708	17	99.6	0.1		
ME29 (STC)	<i>wMel</i>	RC45 (ME29)	<i>wCer2</i>	1642	16	92.8	2.5	2.45	<0.02
ME29 (STC)	<i>wMel</i>	RC50 (ME29)	<i>wCer2</i>	1713	16	96.7	1.1	2.25	<0.03
ME29 (STC)	<i>wMel</i>	ME29 (STC)	<i>wMel</i>	1071	11	23.3	3.8	3.29	<10 ⁻³

Same legend as table 1.

In this experiment, males bearing *wMel* were also mated with females bearing *wMel*. As expected, *wMel* was found able to rescue its own *mod* function, much more efficiently so than *wCer2*.

The *wRi* / *wMel* relationship (verification)

Earlier studies reported an unexpected and complex pattern of compatibility between *wRi* (a strong CI inducer, naturally infecting *D. simulans*), and the *wMel* variant injected from *D. melanogaster*: *wRi* was found fully efficient at rescuing the *wMel mod* function, while

wMel was found only partially efficient at rescuing the *wRi mod* function (Poinsot *et al.* 1998). These results made the *wCer2 / wRi* relationships worth investigating. Before doing so, we tested whether these initial observations could be retrieved.

To test if *wRi* can rescue the CI induced by *wMel*, males bearing *wMel* were crossed with females bearing *wRi* as well as uninfected females. As expected, a significant reduction of embryonic mortality was observed when females carried *wRi* (table 4). In this experiment, *wMel* males were also mated with *wMel* females. As expected, *wMel* was found to rescue its own *mod* function, but notably, embryonic mortality was still higher than in crosses with *wRi* females. Comparing these two crosses allows to show that, in this experiment, females bearing *wRi* were better protected from the *wMel mod* function than females bearing *wMel* itself (Wilcoxon, $W = 3.65$; $P < 10^{-3}$).

Table 4.

Does *wRi* rescue *wMel* (verification)?

Male		Female		Ne	Nc	EM (%)	SE	W	P
mother (father)	Wolb	mother (father)	Wolb						
ME29 (DSRTC)	<i>wMel</i>	DSRTC (ME29TC)	0	1245	12	99.5	0.3		
ME29 (DSRTC)	<i>wMel</i>	DSR (ME29TC)	<i>wRi</i>	1297	14	7.2	1.1	4.32	$<10^{-5}$
ME29 (DSRTC)	<i>wMel</i>	ME29 (DSRTC)	<i>wMel</i>	337	7	34.5	7.9	3.54	$<10^{-3}$

Same legend as table 1.

To test if *wMel* can rescue the CI induced by *wRi*, males bearing *wRi* were crossed to females bearing *wMel*, as well as uninfected females. As showed in table 5, the presence of *wMel* in females was found to reduce embryonic mortality significantly, although it was still high (near 70%). A comparison with crosses between *wRi* males and *wRi* females shows that females bearing *wRi* are more efficiently protected from the *wRi mod* function than females bearing *wMel* (Wilcoxon, $W = 3.9$; $P < 10^{-4}$). Thus, as observed earlier, *wMel* can rescue the *wRi mod* function, but only partially so.

Table 5.

Does *wMel* rescue *wRi* (verification)?

Male		Female		Ne	Nc	EM (%)	SE	W	P
mother (father)	Wolb	mother (father)	Wolb						
DSR (ME29TC)	<i>wRi</i>	ME29TC (DSRTC)	0	1306	11	97.4	1.0		
DSR (ME29TC)	<i>wRi</i>	ME29 (DSRTC)	<i>wMel</i>	708	13	69.3	3.7	4.14	$<10^{-4}$
DSR (ME29TC)	<i>wRi</i>	DSR (ME29TC)	<i>wRi</i>	974	9	5.3	0.8	3.73	$<10^{-3}$

Same legend as table 1.

The *w*Ri / *w*Cer2 relationship

To test if *w*Ri can rescue the CI induced by *w*Cer2, males bearing *w*Cer2 (lines RC45 and RC50) were crossed with females bearing *w*Ri as well as uninfected females. Unexpectedly, the two different *w*Cer2 lines lead here to different conclusions, as showed in table 6. In crosses involving RC45 males, the presence of *w*Ri in females was found to reduce embryonic mortality significantly, while this was not the case in crosses involving RC50 males.

Table 6.
Does *w*Ri rescue *w*Cer2 ?

Male		Female		Ne	Nc	EM (%)	SE	W	P
mother (father)	Wolb	mother (father)	Wolb						
RC45 (DSRTC)	<i>w</i> Cer2	STC (DSRTC)	0	1706	15	50.3	5.5		
RC45 (DSRTC)	<i>w</i> Cer2	DSR (STC)	<i>w</i> Ri	2411	22	31.7	6.0	2.45	<0.02
RC50 (DSRTC)	<i>w</i> Cer2	STC (DSRTC)	0	1420	14	19.5	2.9		
RC50 (DSRTC)	<i>w</i> Cer2	DSR (STC)	<i>w</i> Ri	1248	12	26.9	8.9	0.31	>0.4

Same legend as table 1.

To test if *w*Cer2 can rescue the CI induced by *w*Ri, males bearing *w*Ri were crossed with females bearing *w*Cer2 (RC45 and RC50) as well as uninfected females. The results are presented in table 7. Here the rescue capabilities of the RC45 and RC50 lines were analyzed in two different experiments, realized one month apart (explaining why the control cross “male *w*Ri × female 0” is presented twice). A similar pattern as in the reciprocal experiment was observed: the presence of *w*Cer2 was found to reduce hatching rates significantly in crosses involving RC45 females, but not RC50 females.

Table 7.
Does *w*Cer2 rescue *w*Ri ?

Male		Female		Ne	Nc	EM (%)	SE	W	P
mother (father)	Wolb	mother (father)	Wolb						
DSR (STC)	<i>w</i> Ri	STC (DSRTC)	0	3416	24	96.6	1.0		
DSR (STC)	<i>w</i> Ri	RC45 (DSRTC)	<i>w</i> Cer2	2400	19	90.9	1.7	2.93	<10 ⁻²
DSR (STC)	<i>w</i> Ri	STC (DSRTC)	0	2617	20	83.4	2.8		
DSR (STC)	<i>w</i> Ri	RC50 (DSRTC)	<i>w</i> Cer2	798	7	84.1	12.9	1.88	>0.05

Same legend as table 1.

DISCUSSION

Did *wAu* lose its *resc* function?

Theoretical investigations have revealed that CI levels are not directly subject to selection (Prout 1994; Turelli 1994; Hurst & McVean 1996), as long as population structure is not too strong (Frank 1998). In other words, although high levels of CI facilitate the initial invasion of uninfected populations, there is no selective pressure among compatible *Wolbachia* variants in favor of higher embryonic mortality in crosses between infected males and uninfected females. This non-intuitive conclusion can be simply understood within the framework of the *mod resc* model, by noting that since (i) *mod* is expressed only in males, and (ii) *Wolbachia* is transmitted only by females, variations affecting the *mod* function are neutral. This rationale has led to the prediction that non-CI inducing *Wolbachia* (the [*mod-resc+*] phenotype) could arise and invade infected populations, either by drift, or with the help of selection if the ancestral [*mod+*] phenotype was selected against through pleiotropic effects (Turelli 1994; Hurst & McVean 1996). Validating this view, a non CI inducing *Wolbachia* naturally infecting *D. simulans* (namely *wMa*, also called *wKi* in some publications) has been found to rescue the CI induced by the closely related strain *wNo* (Poinsot & Mercot 1999; Mercot & Charlat 2003). Once a [*mod-resc+*] *Wolbachia* has reached fixation, thus eliminating CI-inducing variants, the next predicted evolutionary change is the loss of its *resc* function, which has become useless. Indeed, if no CI is expressed in the population, maintaining a functional rescue is not of any help. The [*mod-resc+*] *Wolbachia* can then be gradually replaced by a [*mod-resc-*] phenotype, either by drift, or with the help of selection if the [*resc+*] phenotype is selected against through pleiotropic effects.

Which of these two steps does *wAu* illustrate? When faced with other CI inducing *Wolbachia*, (including the close relative *wMel* found in *D. melanogaster*), *wAu* is not found to rescue embryos (Poinsot *et al.* 1998). Here we challenged this [*resc-*] status by testing if *wAu* could rescue the CI induced by *wCer2*, its closest known relative. Our results suggest it cannot, consistent with the view that *wAu* has lost its rescue ability, or that *resc* is specifically repressed by the host. However, it must be noted that a minute level of rescue, of the kind expressed by *wCer2* when faced with the *wMel mod* function, cannot be excluded. Indeed, as visible in table 1, standard errors are such that small differences could remain undetected.

Compatibility relationships between CI inducing variants

We investigated the relationship between *wMel* and *wCer2*, two closely related CI inducing *Wolbachia*, after injection into *D. simulans*. At first sight, this relationship appears asymmetrical. Indeed, *wMel* was found unable to rescue the *wCer2 mod* function, while *wCer2* rescued a tiny proportion of embryos when faced with the *wMel mod* function. It should be noted however that the levels of CI induced by *wCer2* and *wMel* are such that rescue of *wMel* by *wCer2* is more easily detected than the reverse. Indeed, *wMel* induces very high CI, almost 100%, with very low variability (standard error: 0.1%). Thus, even a tiny rescue can be detected here using a non-parametric test. On the contrary, *wCer2* induces low and variable CI, so that a small rescue of *wCer2* by *wMel* could remain hidden unless very large samples are used.

In a previous experiment, *wCer2* was found to rescue its own *mod* function (Riegler *et al.* in prep). However, this self rescue was incomplete, most likely because *wCer2* in *D. simulans* is only transmitted to a little more than half of the offspring, so that half of the embryos are not protected from CI (Riegler *et al.* in prep). Inefficient transmission is likely here to have the same effect: if *wCer2* was more efficiently transmitted, rescue of *wMel*-induced CI would probably be higher. But still, it would be far from complete. In our experiment, if one assumes that transmission efficiency was 50%, then *wCer2* should rescue 10% of the embryos affected by the *wMel mod* function, instead of the 5% observed in average.

Earlier reports on the compatibility relationships between *wMel* and *wRi* (Poinsot *et al.* 1998) prompted us to include *wRi* in the present study, although this variant is, on the basis of *wsp* sequences, much less closely related to *wCer2* than is *wMel* (Zhou *et al.* 1998; Riegler & Stauffer 2002). We confirmed that *wRi* can fully rescue the *wMel mod* function, while *wMel* can rescue the *wRi mod* function only partially. Surprisingly, we found that females bearing *wRi* were even *more* compatible with males bearing *wMel* than females bearing *wMel* itself, which had not been observed previously (Poinsot *et al.* 1998). In other words, in this experiment, *wMel* was found partially “suicidal”, that is, imperfectly rescuing its own CI. This pattern reminds the above-mentioned *wCer2* imperfect self-rescue, and a similar cause (that is, imperfect maternal transmission) is not ruled out. However, from the PCR results obtained during CI assays, *wMel* appears efficiently transmitted; the “inefficient transmission” is however not ruled out, and would deserve more focused experiments. Other interpretations can also be proposed. Imperfect self-rescue might result from insufficient

bacterial density in the eggs, or an insufficient production of the “*resc* factors” as previously suggested (Breeuwer & Werren 1993). Finally, the *wMel* clone used in this experiment might represent the intermediate *mod_Bresc_A* stage predicted by theory for the evolution of compatibility types (Charlat *et al.* 2001).

After this replication of earlier results, we investigated the *wCer2* / *wRi* relationships. We found that *wRi* can partially rescue the *wCer2 mod* function of the RC45 line, but not the RC50 line. This discrepancy might result from the very low CI expression of RC50 in this experiment (19% embryonic mortality): obviously, if CI expression is low, rescue is difficult to detect because of natural background mortality. Similarly, *wCer2* was found to rescue the *wRi mod* function in the RC45 but not the RC50 line. This parallel makes it tempting to assume similar causes, that is, a low density of *wCer2* in both male and female germlines in RC50. Overall, our results suggest that *wRi* and *wCer2* are not totally incompatible in both directions of cross.

Attempting a synthesis...

The molecular bases of CI are currently unknown, but several models have been proposed in the literature. When critically confronting them with all the CI patterns known to date, it appears that a lock-and-key model (where *mod* and *resc* are determined by different bacterial genes and the rescue of embryos resulting from a physical interaction between their products) is the most parsimonious (Poinsot *et al.* 2003). We will try here to interpret our observations within this framework, using a symbolism modified from an earlier model (Charlat *et al.* 2001).

We will describe the “male side” of CI, using three parameters:

- (i) *mod_I* (*mod* intensity) often referred to as “CI level”, i.e. the percentage of embryonic mortality in crosses between infected males and uninfected females. Physically, *mod_I* equals the proportion of sperm where the bacterium is still present at the stage where “modification” takes place, multiplied by the probability that a modified sperm will fail unless rescued
- (ii) *Lock*, the “identity” of the *mod* function (equivalent to the *mod_C* parameter in Charlat *et al.* 2001, but hopefully more explicit) is a qualitative trait, symbolized here as a linear sequence of 10 characters, with *n* possible states for each character (1, 2, ..., *n*)

- (iii) N_{Locks} is the “number of *Locks*” deposited in sperm that will have to be inhibited by the “*Key*” for development to proceed; here we arbitrarily define that N_{Locks} varies between 0 and 100.

We will describe the “female side” of CI using also three parameters:

- (i) TE (maternal Transmission Efficiency), which is the proportion of eggs bearing *Wolbachia*.
- (ii) *Key* (equivalent to the $resc_C$ parameter in Charlat *et al.* 2001) is the female counterpart of the *Lock* parameter. Aligning the *Lock* and *Key* sequences allows to calculate a compatibility score varying from 0 to 100% and equal to the percentage of identity between the two sequences. In the present simplified model (10 sites only) each identical site translates into a 10% increase in the compatibility score.
- (iii) N_{Keys} is the “number of *Keys*” available in an infected egg. If $TE = 100\%$ and compatibility between *Lock* and *Key* is complete (identical sequences), all embryos develop normally as long as $N_{Keys} \geq N_{Locks}$. If $N_{Locks} > N_{Keys}$, then a proportion N_{Keys} / N_{Locks} is rescued.

With these parameters in mind, let us try and characterize the four *Wolbachia* variants under study (wAu , $wCer2$, $wMel$ and wRi) by filling in table 8, step by step.

Table 8.

A possible combination of *mod* and *resc* properties inferred from our experiments.

	wAu	wMel	wCer2	wRi
<i>mod</i> ₁	0	99%	40%	95%
<i>Lock</i>	?	1111111111	2222222222 / 1112222222	1111111111
N_{Locks}	?	40	20	100
<i>resc</i> ₁	1	70% / 100%	50%	100%
<i>Key</i>	?	1111111111 / 2221111111	2222222222 / 1112222222	1111111111
N_{Keys}	?	40	20	100

mod_1 can be directly measured by crossing infected males with uninfected females. This allows to fill in the first line of table 8. Let us continue, by considering wAu properties. Aside from mod_1 , other traits cannot be clearly inferred from our data. $Lock_{(wAu)}$ can be anything, including a total absence of “*Lock* sequence”; $N_{Locks(wAu)}$ can have any value between 0 and 100; from previous estimates (Hoffmann *et al.* 1996), it appears that $TE_{(wAu)} = 100\%$; however, we do not know if the apparent [*resc*-] phenotype of wAu is due to (i) a total absence of the *Key* sequence, (ii) the *Key* sequence being incompatible with all the *Locks* tested so far or (iii) a very low value of the N_{Keys} parameter.

Consider now $wMel$. Let us arbitrarily define $Lock_{(wMel)}$ as 1111111111. As a first hypothesis, (symbolized in bold in table 8), we will assume that the imperfect self rescue of $wMel$ (30% mortality in the intra-strain cross) is simply caused by imperfect maternal transmission (i.e. $TE_{(wMel)} = 70\%$), and not by a difference between $Lock_{(wMel)}$ and $Key_{(wMel)}$. We thus assume that $Key_{(wMel)} = 1111111111$.

We now turn to $wCer2$. Since $wMel$ does not appear to rescue $wCer2$, $Lock_{(wCer2)}$ has to be 100% different from $Key_{(wMel)}$. Thus $Lock_{(wCer2)}$ can be coded for example 2222222222. $TE_{(wCer2)}$ is known to be approximately 50% (Riegler *et al.* in prep). Since the level of imperfect self-rescue of $wCer2$ is in line with this low maternal transmission, $Key_{(wCer2)}$ has to be perfectly identical to $Lock_{(wCer2)}$, i.e. $Key_{(wCer2)} = 2222222222$. Here we meet a difficulty: because $wCer2$ is known to rescue $wMel$ partially, $Key_{(wCer2)}$ cannot be completely different from $Lock_{(wMel)}$. To circumvent this problem, we can reconsider a previous assumption, which was the value of $TE_{(wMel)}$. Until now, we have assumed that the imperfect self rescue of $wMel$ was caused by imperfect maternal transmission in $wMel$ ($TE_{(wMel)} = 70\%$) (hypothesis in bold in table 8). We will now consider another possibility (the italics hypothesis in table 8), where $TE_{(wMel)} = 100\%$ but $Key_{(wMel)} = 2221111111$ (it could be anything with three 2 instead of three 1) i.e. 30% of the sites are different between the *Key* and the *Lock*. If $Key_{(wMel)} = 2221111111$, then $Lock_{(wCer2)}$ must be totally different (for example 1112222222) because $wMel$ cannot rescue $wCer2$ at all. To insure self compatibility in $wCer2$, $Key_{(wCer2)}$ must also be 1112222222. However, a 30% similarity between $Lock_{(wMel)}$ and $Key_{(wCer2)}$ together with the 50% maternal transmission of $wCer2$ imply that $wCer2$ should rescue $30\% \times 0.5 = 15\%$ of the embryos when faced with the $wMel$ *mod* function, while we observe it rescues no more than 7.5%. Here comes the relevance of the N_{Locks} and N_{Keys} parameters. If we assume, $N_{Locks(wMel)} = N_{Keys(wMel)} = 40$ and $N_{Locks(wCer2)} = N_{Keys(wCer2)} = 20$, we approximately get back on our feet.

Now consider wRi . Since wRi can totally rescue $wMel$, then $Key_{(wRi)}$ must be identical to $Lock_{(wMel)}$, i.e. $Key_{(wRi)} = 1111111111$. Since wRi totally rescues its own CI, we also have $Lock_{(wRi)} = 1111111111$. Now we need to explain why $wMel$ does not rescue 70% of the embryos when faced with the wRi *mod* function, which would be expected since $Key_{(wMel)}$ (222111111111) would share 70% similarity with $Lock_{(wRi)}$. We must then assume that $N_{Locks(wRi)}$ is higher than $N_{Keys(wMel)}$. If $N_{Locks(wRi)} = 100$, then the model fits the data: the expected proportion of rescued embryos is

$$(1 - mod_{I(wRi)}) + mod_{I(wRi)} \times 0.7 \times (40 / 100) = 0.32$$

We thus end up with a plausible interpretation. Of course, we do not claim that the picture is complete or accurate, but the kind of data processing we propose is explicit, and can be falsified or improved by additional experiments. To terminate, let us try and figure out how compatibility types have evolved following the divergence of *wRi*, *wMel* and *wCer2*.

On figure 2 (based on the phylogenetic tree inferred from *wsp* sequences, Zhou *et al.* 1998),

we have represented the most parsimonious distribution of character changes and ancestral character states for the *Lock* and *Key* sequences deduced from our hypotheses. The figure would suggest that most changes have occurred within the *wCer2* lineage. This can be put in relation with the fact that the natural host of *wCer2* is a Tephritidae fruit fly and not a Drosophilid. In other words, host traits might constrain the evolution of compatibility types.

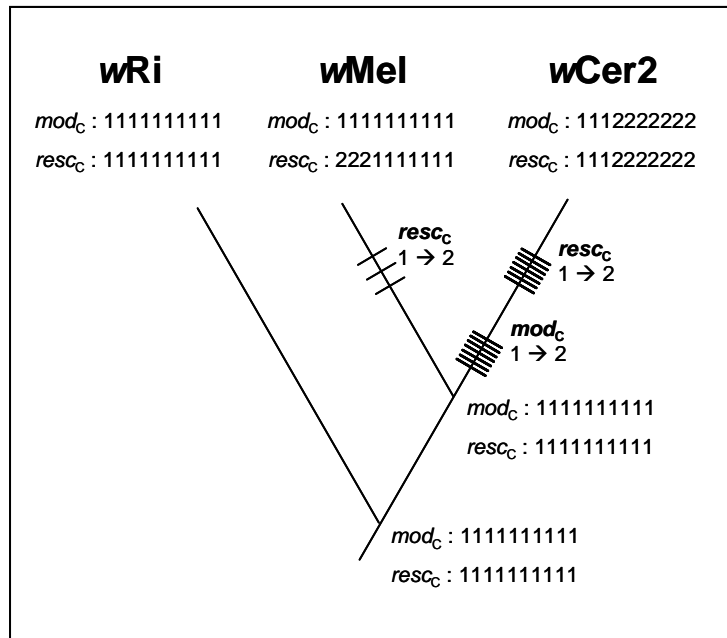


Figure 2. Most parsimonious distribution of character changes and ancestral character states within the *Lock* and *Key* sequences. Tics symbolize character changes.

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Chapitre 3.

Le phénotype [*mod*]

Chapitre 3.

Le phénotype [*mod*-]

Des *Wolbachia* n'induisant pas d'IC (phénotype [*mod*-]) ont été observées dans plusieurs espèces hôtes (Rousset & Solignac 1995; Hoffmann *et al.* 1996). Leur apparentement étroit avec des *Wolbachia* de type [*mod*+] est en accord avec une perte secondaire de l'IC.

Dans ce chapitre sont présentées deux études portant sur de telles bactéries. Les résultats suggèrent que *Wolbachia* peut être maintenue à long terme dans les populations naturelles malgré la perte de l'IC.

Article N°5.

Characterization of non-Cytoplasmic Incompatibility inducing *Wolbachia* in two continental African populations of *Drosophila simulans*

Charlat, S., Le Chat, L. & Merçot, H. 2003

Heredity, **90** : 49-55.

En bref...

Des cinq différentes Wolbachia observées à l'état naturel chez Drosophila simulans, deux n'expriment aucune IC détectable. Nous nous intéressons ici à deux populations Africaines porteuses de telles infections : l'une originaire de Yaoundé (Cameroun), l'autre des pentes du mont Kilimandjaro (Tanzanie). Dans ces deux populations, les infections ont été caractérisées sur la base de leur phénotype d'IC, des séquences du gène wsp, et des haplotypes mitochondriaux associés. La population du Cameroun s'est révélée infectée par une bactérie identique en tous points à wAu, précédemment décrite en Australie, suggérant que cette infection est antérieure aux vagues de migrations récentes. La population Tanzanienne est infectée par la Wolbachia wKi (synonyme : wMa), dont le phénotype [mod- resc+] a déjà été démontré. Les données de séquences révèlent un apparentement étroit avec la bactérie wNo, comme précédemment suspecté sur la base de leurs relations de compatibilité. En revanche, les types mitochondriaux associés suggèrent des histoires évolutives bien distinctes pour ces deux infections.

Characterization of non-cytoplasmic incompatibility inducing *Wolbachia* in two continental African populations of *Drosophila simulans*

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Wolbachia is an endocellular bacterium infecting arthropods and nematodes. In arthropods, it invades host populations through various mechanisms, affecting host reproduction, the most common of which being cytoplasmic incompatibility (CI). CI is an embryonic mortality occurring when infected males mate with uninfected females or females infected by a different *Wolbachia* strain. This phenomenon is observed in *Drosophila simulans*, an intensively studied *Wolbachia* host, harbouring at least five distinct bacterial strains. In this study, we investigate various aspects of the *Wolbachia* infections occurring in two continental African populations of *D. simulans*: CI phenotype, phylogenetic position based on the *wsp* gene and associated mitochondrial haplotype. From the East African population (Tanzania), we show that (i) the

sIII mitochondrial haplotype occurs in continental populations, which was unexpected based on the current views of *D. simulans* biogeography, (ii) the *wKi* strain (that rescues from CI while being unable to induce it) is very closely related to the CI-inducing strain *wNo*, (iii) *wKi* and *wNo* might not derive from a unique infection event, and (iv) *wKi* is likely to represent the same entity as the previously described *wMa* variant. In the West African population (Cameroon), the *Wolbachia* infection was found identical to the previously described *wAu*, which does not induce CI. This finding supports the view that *wAu* might be an ancient infection in *D. simulans*.

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Keywords: *Wolbachia*; cytoplasmic incompatibility; *Drosophila simulans*; symbiosis; endocellular bacteria; mitochondrial haplotypes

Introduction

Wolbachia are maternally transmitted endocellular bacteria infecting arthropods and nematodes (reviewed in Stouthamer *et al.*, 1999; Stevens *et al.*, 2001). In arthropods, the infection can result in various alterations of sexuality and reproduction such as feminization (Rigaud, 1997), thelytokous parthenogenesis (Stouthamer, 1997), male killing (Hurst *et al.*, 1999) and cytoplasmic incompatibility (Hoffmann and Turelli, 1997; Charlat *et al.*, 2001). All these phenomena drive infected females to produce more females than uninfected ones, allowing *Wolbachia* to spread and maintain themselves in hosts' populations. The most common phenomenon, cytoplasmic incompatibility (CI), is observed when infected males mate with uninfected females or with females infected by a different, incompatible *Wolbachia* strain. In such crosses, fertilization is apparently normal but subsequent mitoses are disrupted, leading to the death of the zygote (Reed and Werren, 1995; Callaini *et al.*, 1996, 1997; Lassy and Karr, 1996). Basically, infected cytoplasm is selected for because the eggs laid by infected females are protected from CI, while those laid by uninfected females are not.

The mechanism involved is presently unknown, but CI can be interpreted using the *mod/resc* model (Werren, 1997), which implies the existence of two bacterial functions: modification (*mod*) and rescue (*resc*). The *mod* function would somehow modify the male pronuclei (Presgraves, 2000), before *Wolbachia* are shed from maturing sperm, and the *resc* function would rescue the embryo through an interaction with modified sperm. An egg fertilized with a modified sperm will not develop normally unless a specific *resc* function is expressed in the egg.

CI is observed in *Drosophila simulans*, an extensively studied *Wolbachia* host (reviewed in Merçot and Charlat, 2002), harbouring several different bacterial variants. Three variants have been shown to induce CI when present in males and to rescue from their own effect when present in females: *wRi* (Hoffmann *et al.*, 1986), *wHa* (O'Neill and Karr, 1990) and *wNo* (Merçot *et al.*, 1995). Three other variants have been described that do not seem to induce CI when present in males: *wMa* (Rousset and Solignac, 1995), *wAu* (Hoffmann *et al.*, 1996) and *wKi* (Merçot and Poinso, 1998a; Poinso and Merçot, 1999). Furthermore, *wKi* has been demonstrated to possess a functional *resc*: eggs infected by *wKi* are rescued in crosses with *wNo*-infected males (Merçot and Poinso, 1998a; Poinso and Merçot, 1999). Indirect arguments suggest that *wMa* would show the same phenotype (Bourtzis *et al.*, 1998).

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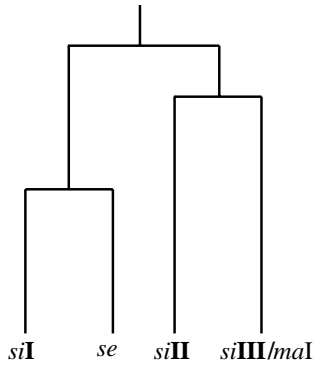


Figure 1 Cytoplasmic history in *D. simulans*. Phylogenetic relationships between mitochondrial haplotypes harboured by *D. simulans* (*siI*, *siII*, *siIII*), *D. sechellia* (*se*) and the *mal* haplotype of *D. mauritiana* (Satta and Takahata, 1990; Ballard, 2000a,b). *se* and *siI* form a monophyletic group, which might be because of the persistence of an ancestral polymorphism in *D. simulans*, or to an introgression event. *mal* and *siIII* are virtually identical, a pattern most likely because of a well-accepted recent introgression (Solignac and Monnerot, 1986; Ballard, 2000c).

These different variants are not randomly associated with the three very distinct mitochondrial haplotypes that have been described in *D. simulans* (*siI*, *siII* and *siIII*; Figure 1). This is expected because *Wolbachia* and mitochondria are transmitted together through the egg cytoplasm so that they should remain associated over time (provided that horizontal and/or paternal transmission of *Wolbachia* and/or mitochondria are not too frequent). Thus, the *wRi* and *wAu* variants are associated with the *siII* haplotype (Hale and Hoffmann, 1990; James and Ballard, 2000), the *wHa* and *wNo* variants are associated with the *siI* haplotype (Montchamp-Moreau *et al*, 1991; Rousset and Solignac, 1995), and the *wMa* variant is associated with the *siIII* haplotype (Rousset *et al*, 1992). As shown in the present article, *wKi* is also associated with *siIII*.

In the present study, two *D. simulans* populations from the African continent were investigated: one from East Africa (Kilimanjaro, Tanzania), known to be infected by *wKi* (Merçot and Poinot, 1998a) and one from West Africa (Yaounde, Cameroon). Three different traits were considered: (i) CI phenotype (the *Wolbachia* ability to induce CI or to rescue from it), (ii) sequences of the *Wolbachia Surface Protein* gene and (iii) associated mitochondrial haplotype. Our main conclusions are the following. From the East African population, we show that (i) the *siIII* mitochondrial haplotype occurs in continental Africa, which was unexpected based on the current views of *D. simulans* biogeography, (ii) the *wKi* strain is very closely related to *wNo*, (iii) *wKi* and *wNo* might not derive from a unique infection event, and (iv) *wKi* is likely to represent the same entity as the previously described *wMa* variant. In the West African population, the *Wolbachia* infection was found to be identical to the previously described *wAu* strain, based on all the traits under study. This finding supports the view that the *wAu* infection in *D. simulans* might be ancient, and raises the question of how non-CI-inducing *Wolbachia* maintain themselves in natural populations.

Materials and methods

Drosophila simulans strains

Reference lines: Agadir is a strain captured in Morocco in 1996, infected by *wRi* (Poinot and Merçot, 1999). NHa originates from the Noumea 89 strain, bi-infected by *wHa* and *wNo*. Following segregation of the two variants, NHa only bears *wHa* (Poinot and Merçot, 1997). N7No also originates from Noumea 89. Following segregation of the two variants, N7No only bears *wNo* (Merçot and Poinot, 1998b). Coffs Harbour S20 is an Australian strain founded using flies from a 1993 collection, infected by *wAu* (Hoffmann *et al*, 1996). SimO is a naturally uninfected strain from Nasr'allah (Tunisia) (Merçot *et al*, 1995). STC is an inbred stock from the Seychelles strain (Seychelles archipelago), originally bi-infected by *wHa* + *wNo*, cured from its *Wolbachia* following a tetracycline treatment (Poinot *et al*, 2000). ME29 is a *D. simulans* line transinfected with the *Wolbachia wMel*, naturally infecting the *D. melanogaster* Wien5 isofemale line (Poinot *et al*, 1998).

Studied lines: Yaounde: 19 isofemale lines have been studied, originating from females captured in Yaounde (Cameroon) in 1997 by B Riera. Kilimanjaro: KC9, K45 and K39 are isofemale lines infected, or originally infected, by *wKi*. K60 is an isofemale line naturally uninfected. K10P is a mass strain founded using a pool of 10 uninfected isofemale lines. All lines originated from flies captured in 1996 in Tanzania by D Lachaise (Poinot and Merçot, 1999).

Rearing conditions

During the experiment, all lines were maintained at 25°C in bottles with axenic medium (David, 1962) at low larval competition. For three generations at least before the beginning of CI experiments, all lines concerned were maintained by crossing 20 virgin females aged 4–6 days and 25 virgin males aged 3–4 days in bottles with axenic medium. After 24 h of egg laying, individuals were transferred to a second bottle for another 24 h, before the adults were discarded. Given the laying rates on the strains used, this protocol ensures a low larval competition and (when flies are infected) the maximum expression of CI.

Cytoplasmic incompatibility tests

Individual crosses were carried out using 3-day-old virgin males and 4 to 5-day-old virgin females. Each cross was initiated by placing one male and one female in a vial with axenic medium until mating was observed. The male was then removed and the female was supplied with a laying plate for 48 or 72 h. Upon removal of the female, the eggs were placed at 25°C for 24 h before egg hatch was measured using all eggs. Laying plates containing less than 20 eggs were discarded. All individuals from infected strains were checked by PCR for the presence of *Wolbachia* using 16S primers (O'Neill *et al*, 1992) or general *wsp* primers: 81F and 691R (Braig *et al*, 1998).

wsp sequencing

DNA was extracted from individually crushed flies, using the crude STE boiling method (O'Neill *et al*, 1992).

The *wsp* gene was then amplified by PCR using general primers 81F and 691R (Braig *et al*, 1998). PCR was performed in a 25 μ l reaction volume, using 1.25 units of *Taq* DNA polymerase (Perkin Elmer) and 1 μ l of DNA template, in the following conditions: 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1 μ M reverse and forward primers. Thermal cycles were as follows: 94°C for 1 min, (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) 34 times and 72°C for 5 min. A second PCR was performed in 50 μ l reaction volumes with the same concentrations as above, using 2 μ l of the first PCR product as template.

In all 50 μ l of the second PCR product was run on a 1% agarose gel. Amplified DNA was then purified using 'Quiaquick Gel Extraction Kit' (Quiagen). Automatic sequencing was done using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems). Each sequence was obtained twice with each primer, making a total of four sequences obtained independently from one DNA extract, from which a consensus was derived. Each base from the final consensus sequence was present in at least three out of the four sequences for every site. Alignment of our sequences with databank sequences was performed using CLUSTALW.

Mitochondrial haplotype determination

Mitochondrial haplotypes (*siI*, *siII* or *siIII*) were determined by restriction fragment length polymorphism. DNA was extracted as in Baba-Aïssa *et al* (1988) and digested with restriction enzymes *Hpa* I and *Acc* I, allowing a double-checking of the haplotypes. *siI*, *siII* and *siIII* were distinguished using restriction maps from Baba-Aïssa *et al* (1988).

Results

Prevalence and CI assays

The prevalence and CI phenotype in the East African population (Kilimanjaro, Tanzania) are known from a previous study (Merçot and Poinot, 1998a): 9 lines out of 49 were found infected (prevalence 18.4%) and the *Wolbachia* variant present in this population, baptized *wKi*, does not induce CI but is capable of rescuing the CI induced by *wNo*.

Isofemale lines from the West African population (Yaounde, Cameroon) were screened by PCR for the presence of *Wolbachia*. Six out of 19 were found to be infected (prevalence: 32%). The CI assays described below were performed using these infected lines.

In order to detect the possible expression of CI in Yaounde lines (*mod* test), males from five infected and five uninfected isofemale lines were individually crossed with two types of uninfected females: from an uninfected reference strain (SimO) and from a pool of Yaounde uninfected lines (massY-). The results, presented in Figure 2a, were analysed using a nonparametrical Wilcoxon test. As presented in Table 1A, no expression of CI was detected: with both SimO and mass Y- females, infected males are not significantly less fertile than uninfected ones.

In order to determine if the *Wolbachia* present in Yaounde was able to rescue the CI induced by other strains (*resc* test), females from five infected and five uninfected isofemale lines from Yaounde were

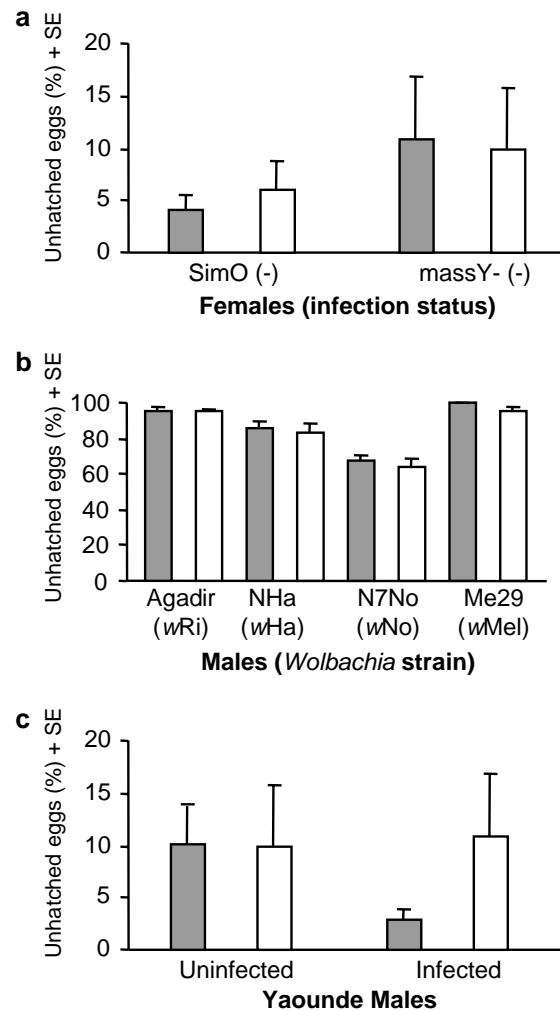


Figure 2 Results of crosses realized with the Yaounde population. A total of 15 replicates were obtained for each category of cross. (a) *mod* test, involving Yaounde males, infected (grey) and uninfected (white); (b) *resc* test, involving Yaounde females, infected (grey) and uninfected (white); (c) fertility test, involving Yaounde females, infected (grey) and uninfected (white).

crossed with males infected by each of the three CI-inducing *Wolbachia* variants naturally infecting *D. simulans* (*wRi*, *wHa* and *wNo*) and with males from the ME29 line (a *D. simulans* line transinfected with *wMel*, the *Wolbachia* naturally infecting *D. melanogaster*; Poinot *et al*, 1998). The results, shown in Figure 2b, were analysed using a Wilcoxon test. As presented in Table 1B, no rescue was detected: with all types of males (infected by *wRi*, *wHa*, *wNo* or *wMel*), infected females are not significantly more fertile than uninfected ones.

We finally tested whether the presence of *Wolbachia* in the Yaounde population affected female fertility. Females from the massY- (uninfected) and massY+ (infected) were crossed with uninfected and infected males. The results, presented in Figure 2c, were analysed using a Wilcoxon test. As shown in Table 1C, no effect on fertility was detected: with both infected and uninfected males, infected females are not significantly more or less fertile than uninfected ones.

Table 1 Results of Wilcoxon tests

	Question addressed	Comparison ^a	W	P		
		Male × Fem	versus	Male × Fem		
A	Mod?	Y+ × SimO	vs	Y- × SimO	0.249	<0.81
		Y+ × massY-	vs	Y- × massY-	0.124	<0.91
B	Resc?	Agadir × massY-	vs	Agadir × massY+	0.933	<0.36
		NHa × massY-	vs	NHa × massY+	0.601	<0.55
		N7No × massY-	vs	N7No × massY+	0.27	<0.79
		Me29 × massY-	vs	Me29 × massY+	1.327	<0.19
C	Effect on female fertility?	Y- × massY-	vs	Y- × massY+	0.85	<0.4
		Y+ × mass Y-	vs	Y+ × mass Y+	1.203	<0.24

^aY+ and Y- are males from the Yaounde population, infected and uninfected, respectively. Fem: female.

wsp sequences

wsp gene sequences were determined from two West African lines (Y6 and Y12), one East African line (KC9, infected by *wKi*), as well as in ME29 (infected by *wMel*) and Coffs Harbour S20 (infected by *wAu*).

The sequence length was 598 bp for Y6, Y12, Coffs Harbour S20 and ME29. The Coffs Harbour S20 sequence was, as expected, identical to the one obtained by Zhou *et al* (1998) using the same line (GenBank AF020067). The Y6 and Y12 sequences (GenBank AF290890) were identical to the Coffs Harbour S20 sequence. The ME29 sequence (GenBank AF290891) was identical to some of the previously determined *wsp* sequences obtained from *D. melanogaster* by Zhou *et al* (1998) (GenBank AF020063, AF020064, AF020065, AF020072). The Y6, Y12 and Coffs Harbour S20 sequences differed by five substitutions from the ME29 sequence.

The sequence length was 566 bp for KC9. The KC9 sequence (GenBank AF290889) was identical to the *wNo* and *wMau* sequences previously obtained by Zhou *et al* (1998) (GenBank AF020074 and AF020069). Let us note here that the AF020069 sequence (Zhou *et al*, 1998) was obtained using a *D. simulans* line artificially infected by *wMau* (Giordano *et al*, 1995), the *Wolbachia* strain naturally infecting *D. mauritiana*. Since *wMau* and *wMa* are closely related (Rousset and Solignac, 1995), Zhou *et al* (1998) term this strain *wMa*.

Mitochondrial haplotypes

Mitochondrial haplotypes were determined in four West African isofemale lines (Y6 and Y12, infected; Y4 and Y5, uninfected), in four East African isofemale lines (K45, KC9, K39, originally infected; K60, originally uninfected), in one East African mass strain (K10P, originally uninfected), as well as in Coffs Harbour S20 and SimO (*siII* references) and STC (*siI* reference).

As expected from previous typing (Montchamp-Moreau *et al*, 1991; James and Ballard, 2000), we found that SimO, Coffs Harbour S20 and STC harboured, respectively, *siII*, *siII* and *siI*. West African lines harboured *siII*, regardless of their original infection status. East African isofemale lines harboured *siIII*, regardless of their original infection status. The East African mass strain, originally uninfected, was heterogeneous, harbouring *siIII* as well as *siII* cytoplasm.

Discussion

siIII mitochondrial haplotype occurs in continental populations

The three distinct mitochondrial haplotypes of *D. simulans* show a very strong geographic structuration, on the basis of which biogeographical inferences have been made (Lachaise *et al*, 1988). The classical view is that (i) *siI* is restricted to the Seychelles archipelago and Indo-Pacific islands, (ii) *siII* is much more widely distributed, occurring in all continental populations, as well as in Madagascar and La Reunion islands, and (iii) *siIII* is restricted to Madagascar and La Reunion islands (Solignac and Monnerot, 1986; Baba-Aïssa *et al*, 1988; Montchamp-Moreau *et al*, 1991; Ballard, 2000b).

We have determined the mitochondrial haplotype of several lines from the Kilimanjaro population (Tanzania). The four isofemale lines were found to harbour the *siIII* haplotype, while a pool from the same area was found to be polymorphic, with *siII* and *siIII* cytoplasm. This is not the first report that the *siII* and *siIII* cytoplasm can be found in sympatry: this situation occurs in Madagascar and La Reunion (Baba-Aïssa *et al*, 1988; Ballard, 2000b). However, the *siIII* haplotype had never been observed in continental populations. This finding suggests that, at least at the mitochondrial level, continental and insular populations are not differentiated. Consistent with our finding are some recent results based on the *vermillion* locus suggesting that continental and island populations from East Africa are also similar at the nuclear level (N. Derome, personal communication). A more systematic screening of mitochondrial haplotypes in continental East African populations could show whether the pattern we report here reflects a very general or only localized situation. Let us finally mention here that in an earlier paper (Nigro, 1994), the SimO strain (Nasr'allah, Tunisia) was mistakenly reported to harbour the *siIII* mitochondrial variant (instead of *siII*), owing to an unfortunate confusion between strain names.

wKi and *wNo* are closely related, but might not derive from a unique infection event

The [*mod- resc+*] phenotype, where *Wolbachia* does not induce CI but is capable of rescuing it, was initially described in the Kilimanjaro population (Merçot and

Poinsot, 1998a; Poinsot and Merçot, 1999) using the lines investigated in the present study. The *Wolbachia* strain responsible for this phenotype was baptized *wKi*. In these studies, it was shown that *wKi*, when present in males, does not induce CI, but, when present in females, rescues the embryonic mortality induced by *wNo*, a relationship that was confirmed in additional experiments (Charlat *et al*, 2002). The authors thus characterized *wKi* through CI assays, the results of which suggested that *wKi* and *wNo* might be closely related. However, neither the precise phylogenetic position of *wKi* nor the mitochondrial haplotype associated with this infection were determined. Our sequence results show that *wKi* and *wNo* bear the same *wsp* sequence. Thus, as expected from their CI relationships, and provided that recombination is not misleading us (Werren and Bartos, 2001; Jiggins *et al*, 2001; Charlat and Merçot, 2001), these two *Wolbachia* strains are very closely related.

We found that all the infected (or originally infected) lines from Kilimanjaro harbour the *siIII* mitochondrial haplotype. As mentioned above, such a result was unexpected based on the geographical origin of this strain. It was also unexpected on the basis of the molecular resemblance between *wNo* and *wKi*: since *wNo* is associated with the *siI* mitochondrial haplotype, a reasonable prediction was that the same would be true for *wKi*. The hypothesis that *wKi* and *wNo* could derive from a unique infection event, having occurred within the *siI* lineage, is ruled out.

Based on our results, could *wNo* and *wKi* result from a divergence associated with the *siI/siIII* split? In other words, could these two bacterial variants derive from a unique and ancestral infection event, having occurred prior to the coalescence between the three mitochondrial haplotypes? Under such a view, the *wNo/wKi* strain would have been subsequently lost from the *siII* lineage, since the *siII* and *siIII* haplotypes form together a monophyletic group (Figure 1). We think this scenario is somewhat unlikely. Indeed, *wNo* and *wKi* are very closely related: identical *wsp* sequences and one substitution over 800 bp on the 16S rRNA sequence (A James and J Ballard, personal communication). By contrast, the *siI* and *siIII* haplotype are significantly divergent: 355 nucleotide substitutions over 14 959 bp (2.4% divergence) (Ballard, 2000a). In *Drosophila*, mitochondria seem to evolve at a faster rate than nuclear genes (Moriyama and Powell, 1997), but it has also been suggested that endocellular bacteria have increased substitution rates (Clark *et al*, 1999). The discrepancy between *Wolbachia* and mitochondrial divergence would thus make more likely the hypothesis of a recent horizontal transfer. This interpretation must however be considered cautiously, as it does not rely on well-calibrated molecular clocks.

Theory suggests that nothing opposes the decrease of CI intensity within a population of CI-inducing *Wolbachia*. Indeed, although CI allows *Wolbachia* to invade host populations, any mutant clone inducing a lower CI, or no CI at all, would not be selected against, as long as the *resc* function is maintained (Prout, 1994; Turelli, 1994; Hurst and McVean, 1996). Accordingly, it has been suggested that non-CI-inducing *Wolbachia* could derive from CI-inducing ones. The fact that *wNo* and *wKi* are so closely related suggests that a shift between the [*mod+*] and [*mod-*] phenotypes can occur within a relatively brief period of time.

wKi and *wMa* represent the same entity

The *wMa* *Wolbachia* strain was initially described from Madagascar (Rousset *et al*, 1992) as a non-CI-inducing strain (Rousset and Solignac, 1995). Based on 16S rRNA sequences, a slowly evolving marker, these authors showed that *wMa* and *wNo* are closely related. However, the CI relationships between *wMa* and *wNo* were not investigated. In fact, lines singly infected by *wNo* were not available until this variant was isolated by segregation from doubly infected lines (Merçot *et al*, 1995).

Let us consider the following list of arguments, strongly suggesting that *wKi* and *wMa* represent the same entity. (i) *wMa* and *wKi* show identical *wsp* sequences (Zhou *et al*, 1998; this study), as well as identical 16S sequences (Rousset *et al*, 1992; A James and J Ballard, personal communication). (ii) *wMa* and *wKi* are both associated with the *siIII* mitochondrial haplotype (Rousset and Solignac, 1995; James and Ballard, 2000; this study). (iii) *wMa* and *wKi* are both non-CI-inducing strains (Rousset and Solignac, 1995; Merçot and Poinsot, 1998a). (iv) On the basis of mitochondrial haplotypes, it is well accepted that a recent introgression took place between *D. simulans* and *D. mauritiana* (Solignac and Monnerot, 1986; Ballard, 2000c), allowing the *siIII* haplotype, together with the *wMa* *Wolbachia* strain, to invade *D. mauritiana*. Accordingly, the *Wolbachia* strain occurring in *D. mauritiana*, usually referred to as *wMau*, is identical to *wMa*, on the basis of the 16S rRNA (Rousset and Solignac, 1995). Just as *wMa*, *wMau* does not induce CI (Giordano *et al*, 1995; Rousset and Solignac, 1995). However, it appears that *wMau*, when injected into *D. simulans*, is able to rescue the CI induced by *wNo* (Bourtzis *et al*, 1998). Thus, *wMau* and *wKi* show the same CI phenotype, indirectly suggesting that the same could be true for *wMa* and *wKi*. Based on these different arguments, we believe that *wKi* and *wMa* represent the same entity. Since the *wMa* name was published first, we recommend referring to *wKi* as '*wMa*' in future publications.

wAu is in West Africa

The *wAu* infection was originally reported in Australia (Hoffmann *et al*, 1996) and more recently in Madagascar and Florida, USA (James and Ballard, 2000). Its presence is also suspected, although not clearly demonstrated, in Ecuador (Turelli and Hoffmann, 1995). Based on this geographical distribution, the *siII* mitochondrial haplotype was expected, and indeed observed by James and Ballard (2000). We found the *siII* haplotype in the Coffs Harbour S20 line, confirming this result.

The *Wolbachia* strain that we found in populations from Cameroon seems identical to *wAu*: (i) the two strains harbour the same *wsp* sequence, (ii) they are both associated with the *siII* haplotype and (iii) they do not induce CI (Hoffmann *et al*, 1996), nor are they able to rescue CI from any of the CI-inducing *Wolbachia* naturally infecting *D. simulans* (Merçot and Poinsot, 1998b), or *wMel*, injected from *D. melanogaster* into *D. simulans* (Poinsot *et al*, 1998).

D. simulans non-African populations are thought to result from a recent expansion of the species (Lachaise *et al*, 1988). In other words, the Yaounde population, where we observed *wAu*, is probably older than Australian or American populations that have pre-

viously been found infected by this variant. Supporting this view are some results based on the *vermillion* nuclear gene, confirming that flies from the Yaounde population are probably not reintroduced from recently colonized areas (Hamblin and Veuille, 1999). The presence of *wAu*, a non-CI-inducing strain, in ancient populations – Cameroon (this study), but also Madagascar (James and Ballard, 2000) – is consistent with current views on CI evolution. Indeed, since the [*mod*–] phenotype is expected to derive from the [*mod*+] phenotype (Prout, 1994; Turelli, 1994; Hurst and McVean, 1996), [*mod*–] strains should, in general, be more ancient than [*mod*+] ones. If, as we suspect, *wAu* has been present for a long time in *D. simulans*, this infection should be associated with a high diversity of mitochondrial haplotypes, unless recent selective sweeps occurred. A study including *wAu*-infected flies from Madagascar does not support this prediction: mitochondrial genomes associated with *wAu* cluster together in a narrow monophyletic group (Ballard, 2000b). Including flies from Australia, America and West Africa in such an analysis might clarify this issue.

Non-CI-inducing *Wolbachia* are widespread in *D. simulans*

The role played by CI in the spread of *Wolbachia* has been extensively modelled (reviewed in Hoffmann and Turelli, 1997) and witnessed in real time in the wild (Turelli and Hoffmann, 1995). However, it appears that non-CI-inducing strains can also be maintained in natural populations, as suggested by the widespread occurrence of *wAu* and *wKi/wMa*. This apparently paradoxical situation might not be so if the transmission from mothers to offspring is perfect, as observed in Australian populations, in which case *Wolbachia* infection would simply behave as a neutral variant (Hoffmann et al, 1996). In West African lines, however, it seems that *wAu* is not perfectly transmitted: uninfected individuals are often collected from initially infected isofemale lines (unpublished results), and the same is true from *wKi/wMa*. If, as we suspect, transmission is not perfect, other factors, such as positive effects on host fitness, high rates of horizontal transmission, or other reproductive phenotypes, might have to be hypothesized and tested.

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What maintains non cytoplasmic incompatibility inducing *Wolbachia* in their hosts : a field study in *Drosophila yakuba*

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En bref...

En théorie, une Wolbachia n'induisant pas d'IC doit rapidement disparaître des populations hôtes. En effet, à moins que la bactérie ne soit parfaitement transmise au cours des générations, ou n'ait des effets positifs sur la survie ou la reproduction de l'hôte, l'IC est un élément indispensable au maintien de l'infection. Nous considérons ici cette question par l'étude d'une population naturelle de Drosophila yakuba. Les paramètres d'importance (intensité de mod, taux de transmission, effets sur la fécondité) sont mesurés dans les toutes premières générations suivant la collecte, afin d'éviter les biais potentiels liés aux conditions de vie en laboratoire. Nos résultats suggèrent une absence totale d'expression d'IC, confirmant les résultats d'études antérieures non publiées menées sur des lignées de laboratoire. Le taux de transmission maternelle de l'infection est estimé à 100%. D'autre part, un effet positif de l'infection sur la fécondité est observé en première génération, mais n'est pas répété cinq générations plus tard. Si, l'on exclu cet effet positif douteux sur la fécondité des femelles, Wolbachia semble ici maintenue comme un caractère neutre, comme suggéré précédemment chez Drosophila simulans pour un variant étroitement apparenté. En accord avec cette interprétation, les fréquences d'infections dans les populations naturelles sont faibles, suggérant que de nombreuses espèces pourraient héberger ce type de bactéries, difficilement détectables sans un effort d'échantillonnage important.

**WHAT MAINTAINS NON CYTOPLASMIC INCOMPATIBILITY INDUCING
WOLBACHIA IN THEIR HOSTS : A FIELD STUDY IN *DROSOPHILA YAKUBA*.**

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ABSTRACT

Cytoplasmic incompatibility (CI) allows *Wolbachia*, a maternally inherited endocellular bacterium, to invade hosts populations: by specifically inducing sterility in crosses between infected males and uninfected females, *Wolbachia* indirectly enhances infected females' relative fitness, so that infection frequency increases. Population models, as well as empirical data, highlight that the spreading and maintenance of *Wolbachia* in host populations is strongly influenced by the level of CI (the proportion of embryos that are killed in incompatible crosses), but also by two other parameters: (i) the efficiency of bacterial transmission from infected females to their offspring and (ii) any positive or negative fitness effects of infection, apart from CI itself. In some species, non-CI inducing *Wolbachia*, that are thought to derive from CI-inducing ancestors, are common. In theory, the maintenance of such infections is not possible unless the bacterium is perfectly transmitted to offspring, or provide a fitness benefit to infected females. The present study aims to test this view by investigating a population of *Drosophila yakuba* from Gabon, West-Africa. We did not find any evidence for CI using wild caught females. Infected females from the field transmitted the infection to 100% of their offspring. A positive effect on female fecundity was observed one generation after collecting, but this was not retrieved five generations later, using additional lines. Similarly, the presence of *Wolbachia* was found to affect mating behavior, but the results of two experiments realized five generations apart were not consistent. Finally, *Wolbachia* was not found to affect sex-ratio. Overall, our results would suggest that *Wolbachia* behaves like a neutral or nearly neutral trait in this species, maintained in the host owing to perfect maternal transmission.

Wolbachia is an endocellular bacterium, widespread in Arthropods and Nematodes (reviewed in O'Neill *et al.* 1997; Stouthamer *et al.* 1999). Because it is transmitted by females only, it can invade host populations by pushing infected females to produce more daughters than uninfected ones, or daughters that survive better, regardless of possible detrimental effects to sons. In its arthropod hosts, *Wolbachia* has evolved a number of “reproductive manipulations” that can be interpreted within this frame: it can kill infected females sons (reviewed in Hurst & Jiggins 2000), feminize males (reviewed in Rigaud 1997; Stouthamer 1997) or reduce uninfected females’ fertility through cytoplasmic incompatibility (CI) (reviewed in Hoffmann & Turelli 1997; Charlat *et al.* 2002). Based on current data, CI seems to be the most widespread phenomenon, and also the only one that no other symbionts have been found to achieve. In its simplest form, CI shows itself when males bearing the bacterium mate with uninfected females. Embryos resulting from such crosses die because chromosomes of paternal origin behave abnormally at the first mitosis (Callaini *et al.* 1996; Lassy & Karr 1996; Callaini *et al.* 1997; Tram & Sullivan 2002). By contrast, crosses involving infected females (male uninfected \times female infected and male infected \times female infected) are normally fertile, as well as that between uninfected males and uninfected females. In other words, infected females are immune from the sterility caused by infected males, so that infection frequency increases. Based on theoretical and empirical work (Caspari & Watson 1959; Fine 1978; Turelli & Hoffmann 1995; Hoffmann & Turelli 1997), the spreading of *Wolbachia* in uninfected populations, as well as its maintenance after invasion, are best understood by considering three factors: (i) the level of CI (the percentage of embryos that do not hatch because of CI in crosses between infected males and uninfected females), (ii) the efficiency of transmission from infected mothers to their offspring and finally, (iii) any positive or negative effects on host fitness, apart from CI itself. Basically, high levels of CI, efficient maternal transmission and low fitness costs will facilitate invasion of and maintenance in host populations. Understanding the long term stability of *Wolbachia*-host associations thus requires to determine the evolutionary trajectories of these different parameters. Turelli (1994) investigated this issue by delineating the selective pressures acting on CI-*Wolbachia* and their hosts. He concluded that both host and symbiont factors are selected for increasing transmission rates and decreasing negative fitness effects. Predictions regarding CI levels are less straightforward. If infection is not fixed, host factors that can decrease CI are supposed to invade, because CI reduces hatching rates in crosses between infected males and uninfected females. On the contrary, from the bacterial point of view, CI levels are selectively neutral within infected populations, as long as population structure is not too strong (Frank 1998).

This non-intuitive conclusion, also reached by Prout (1994) can be understood by noting that the bacterial factors determining CI levels are expressed only in males. Because *Wolbachia* is transmitted by females only, any variations affecting these determinants are neutral.

It was hence hypothesized that long term *Wolbachia*-host co-evolution would lead to reduced CI levels, due to selection on host factors decreasing CI and drift on bacterial factors (as suggested by Turelli, 1994), this reduction might also occur through selection on bacterial factors if (i) elevated CI levels result from high bacterial densities, and if (ii) high bacterial densities are costly to the host). Consistently, low CI levels (Hoffmann 1988), or even a total absence of CI expression, have been observed in several species (Giordano *et al.* 1995; Rousset & Solignac 1995; Hoffmann *et al.* 1996; Merçot & Poinso 1998). Simple population models predict that such variants should be lost from their host unless transmission from mothers to offspring is perfect, or *Wolbachia* increases host fitness in any manner; a prediction that has previously been tested in two *Drosophila* species: *D. melanogaster* and *D. simulans*.

In *D. melanogaster*, infected populations can be found throughout the whole species distribution (Solignac *et al.* 1994). Yet, in cases where this was investigated, CI was not expressed in the field, although CI can be detected in the laboratory if very young males are used (Reynolds & Hoffmann 2002; Weeks *et al.* 2002); transmission rates were not perfect (95% confidence intervals ranging from 83% to 99.2%) and positive fitness effects were not apparent (Hoffmann *et al.* 1998; Olsen *et al.* 2001). Infection maintenance thus represents here something of a paradox, the solution of which might lie in yet unidentified positive fitness effects.

In *D. simulans*, some Australian populations, infected by the *wAu* variant, have been investigated (Hoffmann *et al.* 1996). Here, the infection was not found to cause CI. However, its maintenance in these populations is not confounding, because maternal transmission appears to be perfect. No positive or negative effects on host fitness have been detected, suggesting that the *wAu* infection is maintained as a neutral trait. Intriguingly, a very closely related variant (identical to *wAu* based on sequence data) has been detected in the 3 species forming the Yakuba complex: *Drosophila yakuba*, *D. teissieri* and *D. santomea* (Lachaise *et al.* 2000). Here we report on a field study realized on a *D. yakuba* population from Gabon (West Africa). In an attempt to test if the infection frequency in this species was satisfactorily explained by current models, we addressed the following questions: (i) what is the infection frequency, (ii) how efficient is maternal transmission, (iii) is there any evidence for CI and (iv) can we identify other effects that could select for *Wolbachia* maintenance.

MATERIAL AND METHODS

Line collection

D. yakuba females were collected in Gabon in March 2002 on the border of the Ogoue river, about 250 km east from Libreville (GPS position: Latitude: S 00°06.073'; Longitude: E 011°35.594'). Females were transferred individually in plastic vials with instant medium (formula 4-2, Carolina biological supply company, Burlington, North Carolina 27215, USA) until arrival in the laboratory (6 days later).

***Wolbachia* detection and identification**

DNA was extracted according to O' Neill *et al.* (1992). The presence or absence of *Wolbachia* was determined by PCR amplification using 16S general primers (76F, 994R) (O'Neill *et al.* 1992). When infection was not detected, quality of DNA extracts was checked by amplifying mitochondrial DNA using primers Dick and Pat from Simon *et al.* (1994).

The identity of the *Wolbachia* variant in our sample was checked by amplifying the a fragment of the *wsp* gene with primers specifically designed for the Mel *Wolbachia* clade (Zhou *et al.* 1998; Riegler & Stauffer 2002), to which *wAu* belongs.

Transmission rates

Transmission rates were measured from G0 to G1 and from G1 to G5, as the number of infected individuals over the total number of adult offspring tested. Because there is no expression of CI (see Results section), this estimate is not biased: uninfected embryos do not have lower probability of survival than infected ones. If CI had been detected, it would have been necessary to cross infected females with uninfected males in order to measure transmission efficiency.

CI assays

Upon arrival in the laboratory in Paris, wild caught females (generation 0: G0) were left to oviposit for 48 hours at 25°C on petri dishes filled with axenic medium (David 1962) colored

with neutral red (making egg counting easier) and thinly layered with yeast. Eggs were left to hatch for 24 hours and the number of hatched and unhatched eggs were counted. Embryonic mortality was determined as the percentage of unhatched eggs. The infection status of G0 females was then checked by PCR.

G1 flies were obtained from vials where G0 had laid before arrival in the lab, allowing an experiment with bigger sample size, and with more controlled conditions to be performed. Virgin males and females were collected from 2 to 6 days prior to the experiment, that was performed as follows. Mating was controlled and crosses where copulation lasted for less than 15 minutes were discarded, in order to ensure insemination. Inseminated females were left to lay and embryonic mortality was measured as described above. Finally, the infection status of all flies was checked by PCR. A similar experiment was realized at generation 5.

Fecundity and fertility assays

Fertility and fecundity data were obtained during the CI experiment. Fecundity was estimated as the total number of eggs laid during 48 hours. Fertility was estimated as the hatching rates in crosses with uninfected males.

Behavioral assays

Two aspects of mating behavior were monitored: the time separating contact from copulation (time before copulation: TBC), and the duration of copulation (copulation duration: CD). These measures were performed during CI assays, with one male and one female in each vial.

Sex-ratio assays

In generation 5, females used in CI assays were left to lay in vials filled with axenic medium for 48 hours at 25°C. Sex ratio was estimated by counting males and females emerging from these vials.

Statistical analysis

Embryonic mortality was often close to 0%, so that data was not normally distributed. We therefore used non parametrical tests (Wilcoxon or Kruskal-Wallis) to analyze fertility and

CI data. Fecundity and behavioral data were analyzed by ANOVA. Sex-ratio results were analyzed by ANOVA after arcsine root transformation.

RESULTS

Infection frequencies

From the wild females having survived the trip back from Gabon to France, 14 isofemale lines were established. Among these, 5 were found infected and 9 uninfected; infections status that were confirmed in the following generations (until generation 5). Infection frequency was better estimated using ethanol stored material. 98 individuals were analyzed and 32 were found infected using 16S general primers. Three negative samples were found not to amplify mitochondrial DNA, and were thus discarded. Using a pool from isofemale lines and ethanol stored samples, the estimated infection frequency was thus 33.9% (95% confidence interval: 25.0%-42.8%).

Fifteen ethanol stored individuals from another site (Franceville, about 250 km east) were also analyzed. Four were found infected and one negative sample did not amplify mitochondrial DNA, making the estimated infection frequency 28.6% (95% confidence interval 4.9%-52.3%). Finally, 3 ethanol stored flies from the Atlantic ocean coast (Libreville) were analyzed, two of which were positive and one a true negative.

Transmission efficiency

To measure the ability of females from the field to transmit infection to their offspring, a total of 208 G1 flies (110 males and 98 females) from four different infected G0 females were tested for the presence of *Wolbachia*. All of them were found infected, making the estimated transmission efficiency 100%. On the contrary, the 64 G1 flies from lines that had been found uninfected in G0 were all uninfected, as expected.

The efficiency of transmission was measured again from G4 to G5 using four lines derived from the same four infected G0 females. A total of 111 flies from infected mothers (57 males and 54 females) were tested for infection. All of them were found positive.

CI assays

The experiment using G0 flies was done totally blindly, as the infection status of the females from the field were unknown. Based on previous estimates of infection frequency (Lachaise *et al.* 2000), we were however expecting a polytypic situation (with both infected and uninfected individuals), that would allow to compare embryonic mortality in offspring from infected versus uninfected mothers. This was the case. However, among the 14 G0 females, only nine laid more than 10 eggs (three infected females and six uninfected). The low number of eggs laid might have been caused by female age, hard conditions in the field, and a long trip from Africa to Europe. Embryonic mortality was very low in all crosses: only one egg over 286 did not hatch (from one uninfected female). This is clearly different from what is often seen in laboratory lines, where inbreeding depression usually causes at least 10% embryonic mortality in the absence of *Wolbachia* (see below for an example). Clearly, uninfected females did not have a higher embryonic mortality than infected ones, suggesting that there was no CI in the field.

To corroborate the absence of incompatibility, we repeated the CI tests in G1. Virgin G1 flies were collected in two infected lines (GN40-W and GN43-W; GN standing for Gabon and W standing for “*Wolbachia*”) and two uninfected lines (GN50-0 and GN65-0; 0 standing for uninfected). In order to test if CI was expressed, infected males and uninfected males were crossed with uninfected females. Results are presented in table 1a. Here again, embryonic mortality was very low (36 unhatched eggs over 3456). No significant difference was found between the four data sets (Kruskal-Wallis, $H = 3.13$, 3 df, $P > 0.3$), and no effect of male infection status was detected (Wilcoxon, $W = 1.04$, $P > 0.2$). These data support the results obtained from field collected flies.

The experiment was repeated in G5, using the same lines as in G1, together with two additional infected lines (GN42-W and GN45-W) and two additional uninfected lines (GN52-0 and GN67-0). Results are presented in table 1b. In general, embryonic mortality was much higher than in G1, presumably due to inbreeding depression (these are isofemale lines). No significant difference was found between the 8 data sets (Kruskal-Wallis, $H = 10.54$, 7 df, $P > 0.1$), and no effect of male infection status was detected (Wilcoxon, $W = 0.54$, $P > 0.5$). Thus, there was no evidence for CI, nor for variations between lines.

Table 1

CI assay in G1 and G5: descriptive statistics

a. G1 experiment				
Male	N cross	Mean EM	SE	N eggs
GN50-0	10	2.2%	0.8%	600
GN65-0	13	0.9%	0.3%	993
GN40-W	10	0.9%	0.3%	706
GN43-W	15	0.8%	0.2%	1157
b. G5 experiment				
Male	N cross	Mean EM	SE	N eggs
GN49-0	8	23.9%	14.8%	577
GN50-0	8	9.5%	6.3%	744
GN65-0	8	15.7%	12.7%	711
GN67-0	8	11.9%	8.7%	711
GN40-W	8	50.6%	18.3%	583
GN42-W	6	4.3%	1.7%	458
GN43-W	8	2.7%	1.7%	686
GN45-W	7	17.3%	15.0%	587

*All males were crossed with uninfected females.**EM: Embryonic Mortality.*

Fertility

In generation 1, we tested whether *Wolbachia* could affect female fertility by crossing uninfected males with females from two infected and two uninfected lines (GN40-W, GN43-W, GN50-0 and GN65-0). The results are presented in table 2a. Fertility was very high in all crosses. No significant difference was found between the four data sets (Kruskal-Wallis, $H = 6.07$, 3 df, $P > 0.1$), and no effect of female infection status was detected (Wilcoxon, $W = 0.66$, $P > 0.5$).

The experiment was repeated in G5 using the same four lines as in G1, with two infected and two uninfected lines added (GN42-W, GN45-W, GN52-0 and GN67-0). As showed in table 2b, fertility was much lower and more variable. Significant heterogeneity was found among lines (Kruskal-Wallis, $H = 19.9$, 7 df, $P < 0.01$), but there was no effect of female infection status (Wilcoxon, $W = 0.86$, $P > 0.3$). Clearly, some lines were suffering from strong inbreeding depression, especially GN49-0 and GN40-W.

Table 2

CI assay in G1 and G5: descriptive statistics

a. G1 experiment				
Female	N cross	Mean EM	SE	N eggs
GN50-0	11	97.7%	0.7%	725
GN65-0	12	99.2%	0.4%	868
GN40-W	13	99.3%	0.3%	1121
GN43-W	12	97.6%	1.5%	1164
b. G1 experiment				
Female	N cross	Mean EM	SE	N eggs
GN49-0	8	40.6%	14.3%	815
GN50-0	8	4.4%	1.5%	632
GN65-0	8	1.5%	1.0%	714
GN67-0	8	14.6%	12.3%	582
GN40-W	6	1.6%	1.2%	500
GN42-W	8	20.8%	5.2%	710
GN43-W	5	12.3%	9.3%	500
GN45-W	7	29.8%	19.2%	649

*All females were crossed with uninfected males.**EM: Embryonic Mortality.*

Fecundity

Infected G0 females laid more eggs than uninfected ones (40 eggs in average versus 27.7), but the sample was very small (six uninfected females and three infected females) and the difference was not significant (t test, $P = 0.56$).

Data from the CI experiment allowed to investigate this question with bigger samples in G1. Effects on female fecundity were tested by comparing the number of eggs laid by infected and uninfected females (lines GN40-W, GN43-W, GN50-0 and GN65-0), in crosses involving infected and uninfected males. The results, shown in table 3a, were analyzed by ANOVA (table 4a). A significant effect of female infection status was found, with infected females laying more eggs in average than uninfected ones (88.3 versus 72). However, a possible bias in the fecundity data must be noted: because CI assays require many G1 males and females, the infected and uninfected lines used (GN40-W, GN43-W, GN50-0 and GN65-0) derived from the most fecund G0 females in each category, having laid respectively 39, 71, 23 and 40 eggs. The effect observed might result from this non-random sampling, or also from small sample size giving lines specific effects.

The G5 experiment allowed to further investigate this issue. The two additional infected and uninfected lines (GN42-W, GN45-W, GN52-0 and GN67-0) were randomly chosen. The results, shown in table 3b, were analyzed by ANOVA (table 4b). Here no effect of infection status was detected, but the *LF (Line Female)* effect was found significant at the 5% threshold, confirming that the effect observed in G1 might result from an experimental bias.

Table 3

Fecundity in G1 and G5: descriptive statistics

a. G1 experiment				
WM	Female	N cross	Mean Nb eggs	SE
0	GN40-W	13	86.2	4.8
0	GN43-W	12	97.0	3.7
0	GN50-0	11	65.9	5.1
0	GN65-0	12	72.3	6.7
W+	GN40-W	13	89.8	3.7
W+	GN43-W	12	80.3	7.9
W+	GN50-0	12	76.2	4.2
W+	GN65-0	13	72.9	4.5
b. G5 experiment				
WM	Female	N cross	Mean Nb eggs	SE
W-	GN40-W	6	83.3	13.4
W-	GN42-W	8	88.7	6.9
W-	GN43-W	5	100.0	5.4
W-	GN45-W	7	92.7	10.0
W-	GN49-0	8	101.9	10.7
W-	GN50-0	8	79.0	7.1
W-	GN65-0	8	89.2	6.0
W-	GN67-0	8	72.7	11.0
W+	GN40-W	5	84.0	9.9
W+	GN42-W	7	91.3	6.9
W+	GN43-W	8	92.5	9.9
W+	GN45-W	8	87.5	8.6
W+	GN49-0	7	93.0	15.6
W+	GN50-0	8	79.9	10.0
W+	GN65-0	7	85.4	13.0
W+	GN67-0	7	60.9	15.2

WM: male infection status (W-: uninfected, W+: infected)

SE: standard error

Table 4

Fecundity in G1 and G5: ANOVAs

a. G1 experiment				
Source	df	Mean square	F	Pr>F
WM	1	19.4464	0.06	0.8003
WF	1	865.0619	22.75	0.0001
LM(WM)	2	662.9382	2.20	0.1177
LF(WF)	2	5.0645	0.02	0.9834
WM*WF	1	1121.3592	3.72	0.0574
LM(WM)*LF(WF)	8	383.5452	1.27	0.2702
Error	82	301.8226		
b. G5 experiment				
Source	df	Meansquare	F	Pr>F
WM	1	471.4922	0.69	0.4107
WF	1	1469.0703	2.14	0.1492
LM(WM)	6	614.0666	0.90	0.5050
LF(WF)	6	1721.4729	2.51	0.0327
WM*WF	1	88.7699	0.13	0.7204
LM(WM)*LF(WF)	46	599.0671	0.87	0.6786
Error	53	685.6698		

*WM = male infection status (infected/uninfected)**WF = female infection status (infected/uninfected)**LM = line male, LF = line female. LM and Lf are nested within WM and WF, respectively.*

Sexual behavior

We investigated potential effects of infection status on sexual behavior by measuring the time between contact and copulation (time before copulation: TBC) and copulation duration (CD) in single pair crosses. This experiment was performed in G1 using females from two infected and two uninfected lines (GN40-W, GN43-W, GN50-0 and GN65-0), mated with infected and uninfected males. The results, presented in table 5a, were analyzed by ANOVA (table 6a). No factor was found to affect TBC significantly. On the contrary, an effect of female infection status (infected / uninfected) on CD was detected. On average, copulation was longer with infected than with uninfected females (40.4 versus 36.1 minutes).

The experiment was repeated in G5 using the same four lines as in G1, with two infected and two uninfected lines added (GN42-W, GN45-W, GN52-0 and GN67-0). The results, presented in table 5b, were analyzed by ANOVA (table 6b). Here female infection status was found to affect TBC significantly, with infected females tending to mate earlier

than uninfected ones (49.8 versus 71.4 minutes). On the contrary, none of the factor was found to affect CD.

Table 5

Mating behavior in G1 and G5: descriptive statistics

a. G1 experiment						
WM	Female	N	TBC	SE	CD	SE
W-	GN40-W	6	65.7	13.4	44.3	4.6
W-	GN43-W	8	37.6	21.3	38.3	2.2
W-	GN50-0	10	48.3	10.6	34.7	2.6
W-	GN65-0	11	27.7	5.3	37.4	2.5
W+	GN40-W	10	63.1	16.6	43.6	3.4
W+	GN43-W	12	23.3	8.5	37.3	2.0
W+	GN50-0	11	44.6	9.8	40.4	2.8
W+	GN65-0	12	43.2	15.4	32.3	1.7
b. G5 experiment						
WM	Fem	N	TBC	SE	CD	SE
W-	GN40-W	7	52.6	12.1	35.4	3.1
W-	GN42-W	8	56.8	22.2	47.6	4.1
W-	GN43-W	8	17.0	4.1	36.8	3.3
W-	GN45-W	8	63.0	13.9	45.0	7.0
W-	GN49-0	8	90.0	37.2	39.8	4.2
W-	GN50-0	8	79.8	33.3	41.4	2.5
W-	GN65-0	8	47.1	14.1	35.9	1.9
W-	GN67-0	8	57.4	16.2	31.4	1.7
W+	GN40-W	7	64.9	14.7	38.3	3.1
W+	GN42-W	7	22.6	6.2	34.3	2.0
W+	GN43-W	8	53.3	11.7	37.6	3.3
W+	GN45-W	8	67.5	20.6	35.3	2.6
W+	GN49-0	8	76.5	15.0	34.4	2.2
W+	GN50-0	8	85.3	16.9	35.9	1.9
W+	GN65-0	8	81.5	37.3	38.3	3.0
W+	GN67-0	8	53.4	26.3	38.1	5.7

WM: male infection status (W-: uninfected, W+: infected), TBC: time before copulation (minutes), CD: copulation duration (minutes), SE: standard error

Table 6
Mating behavior in G1 and G5: ANOVAs

a. G1 experiment							
Source	df	Time before copulation			Copulation duration		
		Mean square	F	Pr>F	Mean square	F	Pr>F
WM	1	77.1199	0.05	0.8211	0.4370	0.01	0.9323
WF	1	103.0349	0.07	0.7939	444.3765	7.39	0.0084
LM(WM)	2	109.5601	0.07	0.9295	175.3110	2.91	0.0614
LF(WF)	2	4379.3054	2.93	0.0608	158.9480	2.64	0.0789
WM*WF	1	927.7630	0.62	0.4340	19.0701	0.32	0.5753
LM(WM)*LF(WF)	8	1645.0606	1.10	0.3756	81.1108	1.35	0.2364
Error	64	1496.5285			60.1431		

b. G5 experiment							
Source	df	Time before copulation			Copulation duration		
		Mean square	F	Pr>F	Mean square	F	Pr>F
WM	1	914.1791	0.29	0.5928	223.3507	2.55	0.1157
WF	1	14377.1940	4.55	0.0370	98.6940	1.12	0.2930
LM(WM)	6	6986.1415	2.21	0.0541	49.3286	0.56	0.7586
LF(WF)	6	3049.7289	0.96	0.4570	49.9115	0.57	0.7534
WM*WF	1	0.4776	0.00	0.9902	156.9030	1.79	0.1861
LM(WM)*LF(WF)	48	2650.7061	0.84	0.7358	102.6080	1.17	0.2797
Error	61	3162.5574			87.7295		

WM = male infection status (infected/uninfected), WF = female infection status (infected/uninfected), LM = line male, LF = line female. LM and Lf are nested within WM and WF, respectively.

Sex-ratio

In generation 5, we tested the effect of female infection status on sex ratio using females from four infected and four uninfected lines (GN40-W, GN42-W, GN43-W, GN45-W, GN50-0, GN52-0, GN65-0 and GN67-0), mated with infected and uninfected males. The results, presented in table 7, were analyzed by ANOVA after arcsine root transformation (table 8). Most importantly, the *Wolbachia*

female (WF) factor was not found significant, suggesting that *Wolbachia* does not push females to produce more daughters than sons. A significant interaction between male and female infection status was detected. Infected females tended to produce more males when mated with infected males rather than

Table 7

Sex ratio in G5: descriptive statistics

WM	WF	N	SR	SE
W-	W-	25	53.0%	1.3%
W-	W+	17	48.3%	1.7%
W+	W-	23	48.4%	1.4%
W+	W+	22	50.9%	1.1%

WM: male infection status (W-: uninfected, W+: infected), WF: female infection status (W-: uninfected, W+: infected), SR: sex-ratio (male / total number of adults), SE: standard error

uninfected males (male proportion 50.9% versus 48.3%), while the opposite was observed for uninfected females, who tended to produce fewer males when mated with infected males rather than uninfected males (male proportion 48.4% versus 53.0%). Overall, these effects were quantitatively very small.

Table 8
Sex ratio in G5: ANOVA

Source	df	Mean square	F	Pr > F
WM	1	0.0003	0.10	0.7526
WF	1	0.0018	0.52	0.4748
LM(WM)	6	0.0024	0.70	0.6504
LF(WF)	6	0.0054	1.57	0.1914
WM*WF	1	0.0293	8.54	0.0068
LM(WM)*LF(WF)	43	0.0045	1.32	0.2211
Error	28			

WM = male infection status (infected/uninfected)
WF = female infection status (infected/uninfected)
LM = line male, LF = line female LM and Lf are nested within WM and WF, respectively.

DISCUSSION

Parameter estimates

We were interested in determining whether the maintenance of *Wolbachia* in *Drosophila yakuba* represents a paradox based on current models. In the population under study, the observed infection frequency was 33.9% (95% confidence interval: 25.0%-42.8%), which is higher than a previous estimate performed at this same site (9.26%, $n = 54$, 95% confidence interval: 1.53%-16.99%) (Lachaise *et al.* 2000). Based on a smaller sample, infection frequency was estimated in a second site as 28.6% (95% confidence interval 4.9%-52.3%).

We investigated some of the parameters that are known to determine infection frequency at equilibrium: transmission efficiency, CI levels, effects on host fitness and sex-ratio. Transmission from wild caught infected females to their offspring was found perfect: no uninfected individual was detected among 208 tested. The same was true five generations later, where no uninfected individual was found among 111 tested.

In previous experiments based on lab-maintained lines, the *Wolbachia* variant from *D. yakuba* (which we will refer to as wAu_{yak}) was not thought to induce CI (S. Zabalou, A. Nirgianaki, S. Charlat, H. Merçot and K. Bourtzis, unpublished results). The absence of CI in

the field was thus expected, since CI expression is usually found higher in the lab than in nature (Turelli & Hoffmann 1995; Hoffmann *et al.* 1998). Infected G0 females did not show higher hatching rates than uninfected ones, but the sample was very small. In a bigger experiment with two infected and two uninfected lines, no CI was detected in generation 1. The same conclusion was derived from an experiment conducted five generations after collecting using four infected and four uninfected lines, although embryonic mortality was in average much higher, presumably due to five generations of inbreeding.

No effect of *Wolbachia* infection on female fertility was found: in crosses with uninfected males, infected females did not show higher hatching rates than uninfected ones. An effect of *Wolbachia* infection on fecundity was observed in G1, but this possibly results from the sample size. Consistent with this interpretation, the fecundity benefit was not detected in the G5 experiment, where additional lines had been included. Fecundity benefits have been observed in other dipteran species (Dobson *et al.* 2002). Additional experiments would be necessary to further investigate this issue in *D. yakuba*.

Although this trait is probably not an important component of female fitness, our CI-assay protocol also allowed us to investigate potential effects of female infection status on mating behavior, for which time before copulation (TBC) and copulation duration (CD) were taken as proxies. Although some effects were observed sporadically, no clear pattern emerged. In G1, infected females were found to mate longer but not earlier, while in G5 they mated earlier but not longer. If TBC and CD are indicators of male choice, these results can be interpreted as males preferring infected rather than uninfected females. However, the discrepancy between generations is confounding.

We examined sex-ratio effects in G5, and found no female biased progeny in offspring from infected females. Thus, sex-ratio effects do not seem to contribute here to *Wolbachia* maintenance. A significant (although quantitatively small), interaction between male and female infection status was observed, which we fail to interpret in adaptive terms.

***Wolbachia* maintenance (and its origin...)**

Infection dynamics models predict that in the absence of CI expression or sex ratio distortion, *Wolbachia* infections should be lost from natural populations unless beneficial to the host or perfectly transmitted from infected mothers to their offspring (reviewed in Hoffmann & Turelli 1997). Our observations fit with this prediction: here transmission was found to be perfect, and a possible (but doubtful) positive effect on host fecundity was observed. If, in

the lack of certainty, one neglects the fecundity effect, the picture is very similar to that obtained earlier for *wAu* in *D. simulans* (Hoffmann *et al.* 1996). In these two cases, *wAu* seems to be maintained like a neutral trait.

It is notable that intermediate infection frequencies have been observed in the two sites with reasonable sample size. Even the sample from Libreville, including only three individuals, was polymorphic. This observation prompts the question of the origin of uninfected flies, for which we see two hypothesis: these must derive either from cytoplasmic lineages that have never been infected or, if transmission efficiency is in fact less than perfect, from originally infected lineages.

Under the “never infected” hypothesis, two evolutionary scenarios can be proposed. First, *wAu_{yak}* might derive from recent horizontal transmission(s) of a non CI inducing *Wolbachia* behaving like a neutral trait. The observed pattern would then represent a transitory equilibrium between horizontal transfer and drift. This view would imply that horizontal transfers are sufficiently rare and/or recent for the only possible long term equilibrium (that is, fixation of the infection) not to have been reached. A second possibility is that *wAu_{yak}* has been once fixed in some, but not all, *D. yakuba* populations and that admixture followed. Such fixation could have occurred through recurrent horizontal transmission and drift, without CI expression, or much faster with the help of CI, which would then have been secondarily lost.

The “once infected” hypothesis, implies that maternal transmission is in fact less than perfect. The current situation might then be transitory, if infection leakage takes place at a higher rate than horizontal transmission, or stable, if the two process occur at similar rates. Here again, the possibility of an ancestral expression of CI followed by its secondary loss is not ruled out.

When looking at other *Drosophila* species, the success of *wAu* is patent: this bacterium infects the whole *Yakuba* complex (Lachaise *et al.* 2000) as well as *D. simulans* (Hoffmann *et al.* 1996). In *D. simulans*, where its effects have been investigated, *wAu* was not found to induce CI in populations from Australia (Hoffmann *et al.* 1996; Reynolds & Hoffmann 2002), Madagascar (James & Ballard 2000) or west-Africa (Charlat *et al.* 2003). Notably however, some intriguing results have been obtained using a population from Florida (Ballard *et al.* 1996). In this population, the *Wolbachia* infection was found to induce significant CI. Later sequencing results performed on the same lines suggested that *wAu* was responsible for this phenotype. It is thus not excluded that *wAu* might induce CI in some populations. The success

of this *Wolbachia* might reflect a high capability of horizontal transmission, or an impressive volatility of CI expression, rapidly lost in some populations after invasion.

The present work confirms that non-CI inducing *Wolbachia* can be maintained in natural populations, probably in a stable manner. Now that *Wolbachia* screenings are based on PCR rather than phenotypic effect, similar cases of infections without any apparent consequences will probably prove to be common; probably not as common as they really are, since such infections, as sex ratio distorters (Jiggins *et al.* 2001), can persist at low frequencies and thus remain out of sight.

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Discussion

Discussion

Par une combinaison d'approches théoriques et expérimentales, nous avons cherché dans ce mémoire à mieux cerner l'évolution de l'IC. De nos analyses théoriques, fondées sur l'hypothèse que les fonctions *mod* et *resc* peuvent varier indépendamment, il ressort que les types de compatibilité ne sont pas stabilisés par la sélection, contrairement à des suggestions antérieures (Turelli 1994). Les premiers résultats de la simulation suggèrent que pour des taux de mutation raisonnablement faibles, une évolution progressive des types de compatibilité est plus probable qu'une évolution brutale. Les résultats expérimentaux vont dans le sens de cette conclusion. Il apparaît en effet qu'une incompatibilité incomplète peut être observée entre variants étroitement apparentés. Enfin, l'étude du phénotype [*mod*-] chez *Drosophila simulans* et *Drosophila yakuba* suggère que la perte du phénotype d'IC n'empêche pas le maintien de l'infection. Dans cette discussion, nous examinons les conséquences de l'évolution des types de compatibilité sur les populations hôtes, avant de proposer des compléments et des améliorations possibles pour les approches développées dans ce mémoire.

1. Conséquences évolutives sur l'hôte

1.1. *Wolbachia* suicides et extinctions

L'IC peut être lourde de conséquences sur la valeur sélective moyenne des populations. Tout d'abord, au cours du processus d'invasion, de nombreux croisements sont incompatibles, en particulier lorsque les fréquences d'infections sont intermédiaires. On a d'ailleurs proposé de mettre à profit ce phénomène pour la lutte biologique (Dobson *et al.* 2002).

Notre modèle d'évolution des types de compatibilité suggère des effets encore plus marqués sur la valeur sélective moyenne. En effet, les résultats des simulations montrent que les populations présentent souvent un polymorphisme neutre de la fonction *mod*. A l'extrême, la dérive de variants suicides de type *mod_{BrescA}* au sein d'une population de type

modAresCA peut en théorie mener à l'extinction des populations. Enfin, le maintien par sélection balancée de variants de type *modBresCA* et *modAresCB* réduit également la valeur sélective moyenne. Les conséquences de ces phénomènes sur la démographie des populations hôtes restent à explorer. On peut cependant suggérer que l'impact sera négligeable dans des espèces où une grande proportion des individus meurt avant l'âge de reproduction, en particulier si l'effectif de la population adulte est principalement contrôlé par la compétition pour des ressources limitées.

1.2. Evolution des types compatibilité et balayages sélectifs

En envahissant une nouvelle population hôte, *Wolbachia* emporte avec elle le contenu d'un cytoplasme unique, réduisant ainsi de manière drastique la diversité mitochondriale. L'invasion des populations californiennes de *Drosophila simulans* par le variant *wRi* a permis de suivre ce phénomène en temps réel, et de démontrer que même si des individus non infectés persistent à l'équilibre, du fait d'une transmission maternelle imparfaite, le génome mitochondrial associé à *Wolbachia* atteint rapidement la fixation (Turelli *et al.* 1992; Ballard *et al.* 1996). La raison en est que les cytoplasmes non infectés présents à l'équilibre proviennent tous, à plus ou moins longue échéance, de lignages ayant secondairement perdu l'infection par transmission maternelle imparfaite.

Ce phénomène permet d'expliquer la faible diversité mitochondriale associée à des infections récentes. En revanche, si l'infection et le balayage sélectif qui l'accompagne sont suffisamment anciens, la diversité mitochondriale doit revenir à son niveau d'origine, en accord avec un modèle d'évolution neutre. Chez *Drosophila simulans*, la diversité mitochondriale associée aux différentes infections (la diversité intra-haplotype) est extrêmement réduite, bien que la diversité inter-haplotypes soit importante (Solignac & Monnerot 1986; Satta & Takahata 1990; Ballard 2000a). Notre modèle d'évolution des types de compatibilité suggère que ce patron ne reflète pas nécessairement des événements d'infection récents. En effet, si comme nous le pensons, de nouveaux types de compatibilité peuvent être fixés de manière récurrente au sein de populations déjà infectées, la diversité mitochondriale se verra constamment maintenue à un niveau anormalement bas.

1.3. Incompatibilité bi-directionnelle et spéciation

Parmi les conséquences potentielles de l'IC sur l'évolution des hôtes, la spéciation figure en première place : *Wolbachia* peut faciliter la divergence des populations si ces dernières sont infectées par des bactéries incompatibles. Le potentiel de l'incompatibilité bi-directionnelle comme facteur de spéciation est sujet à débats (Coyne 1992; Hurst & Schilthuisen 1998; Werren 1998; Weeks *et al.* 2002). Résoudre le problème nécessiterait de répondre à ces deux questions : (i) l'incompatibilité bi-directionnelle représente-t-elle une barrière forte à l'homogénéisation des populations par migration et (ii) la présence de *Wolbachia* bi-

directionnellement incompatibles dans des populations différentes d'une même espèce est-elle une situation vraisemblable ?

Des études théoriques récentes portant sur la première question suggèrent que des niveaux d'incompatibilité bi-directionnelle même relativement faibles peuvent faciliter la divergence de populations d'hôtes soumises à des pressions de sélections différentes (Telschow *et al.* 2002). En d'autres termes, l'incompatibilité bi-directionnelle peut faciliter un processus de spéciation déjà existant, à savoir l'adaptation de populations partiellement isolées à des conditions locales. D'un point de vue expérimental, ce phénomène n'a pourtant pas été démontré. En effet, dans les deux espèces où cette question a été abordée (*Drosophila simulans* et *Protocalliphora siala*), aucune corrélation entre incompatibilité bi-directionnelle et différenciation génétique au niveau nucléaire n'a été observée (Ballard *et al.* 2002; Baudry *et al.* 2003).

Qu'en est-il de la vraisemblance d'une incompatibilité bi-directionnelle entre populations conspécifiques ? Deux processus peuvent mener à une telle situation : transferts horizontaux et divergence des types de compatibilités. La possibilité du premier phénomène est démontrée par la présence, chez *Drosophila simulans*, de *Wolbachia* très divergentes dans des populations distinctes. Mon travail suggère que le second processus doit également être envisagé. A l'heure actuelle, aucune étude ne démontre la possibilité d'une divergence des types de compatibilité au sein d'une espèce. Un cas cependant est particulièrement intrigant : celui du moustique *Culex pipiens*, où des bactéries bi-directionnellement incompatibles apparaissent identiques sur la base du gène *ftsZ* (Guillemaud *et al.* 1997).

2. Perspectives théoriques

Notre modèle de simulation peut être considéré comme une première étape dans la prise en compte des pressions évolutives non déterministes pour l'évolution de l'IC, mais nous sommes encore loin d'une représentation réaliste. Parmi les développements possibles, l'intégration des trois paramètres suivants nous paraît particulièrement souhaitable : la structuration des populations, le polymorphisme intra-individuel, et enfin l'évolution des facteurs hôtes.

Les travaux relatifs aux conséquences de la structuration des populations sur l'évolution de l'intensité de *mod*, ont été évoqués en introduction (Frank 1998). Ils suggèrent que les variants présentant une forte intensité de *mod*, parce qu'ils permettent d'atteindre localement des fréquences d'infection élevées, sont sélectionnés en population structurée. En l'absence d'une modélisation rigoureuse, les conséquences de la structuration sur l'évolution des types de compatibilité sont difficiles à prédire. A première vue, il n'est pas exclu que la structuration des populations diminue la probabilité de fixation d'un nouveau type de compatibilité. Notons en effet que le polymorphisme de la fonction *mod*, considéré dans notre modèle comme un passage obligé pour l'évolution des types de compatibilité,

réduit momentanément la fréquence d'infection. Les sous-populations polymorphes pour *mod* risquent de ce fait d'être "diluées" par les sous-populations non polymorphes, réduisant ainsi la probabilité de fixation d'un nouveau type de compatibilité.

L'hypothèse d'une absence de polymorphisme intra-individuel est sans aucun doute une entorse à la réalité. En effet, l'existence des multi-infections démontre que plusieurs cellules bactériennes sont transmises à chaque descendant par les femelles infectées. L'expérience décrite dans l'article N°10 (annexe 4) nous montre que chez *Drosophila sechellia*, la bi-infection par les *Wolbachia* *wSh* et *wSn* est conservée de manière stable au cours des générations, même en l'absence de toute sélection pour le maintien des deux variants. Ce résultat suggère que l'effectif efficace intra-individuel est important. En d'autres termes, l'apparition d'un clone bactérien mutant n'implique pas nécessairement la disparition du clone d'origine au sein de l'individu hôte. Quelles peuvent en être les conséquences sur l'évolution des types de compatibilités ? Nous pouvons, sans trop de risques, avancer que les variations de la fonction *mod* seront peu affectées : au sein d'un individu, le maintien de plusieurs fonctions *mod* n'est pas sélectionné, car les propriétés de *mod* ne sont exprimées que chez les mâles. De ce fait, la fixation, par dérive, d'un clone mutant au sein d'une lignée cytoplasmique, devrait se produire avec la même probabilité, quel que soit l'effectif efficace intra-individuel (rappelons en effet que le taux de substitution neutre est indépendant de la taille des populations) (Kimura 1983). En revanche, il est probable que le maintien, au sein d'un même cytoplasme, de plusieurs fonctions *resc* soit imposé par sélection, du fait que les populations sont souvent polymorphes pour la fonction *mod*. Là encore, une amélioration du modèle actuel semble nécessaire.

Qu'en est-il finalement de l'évolution des facteurs hôtes ? Comme évoqué en introduction, une forte intensité de *mod* est délétère pour les mâles infectés si la fréquence d'infection est inférieure à 1. De la même manière, il est clair que la présence de *Wolbachia* suicides (de type *mod_{BRESCA}*) est délétère pour les mâles infectés comme pour les femelles infectées, si les partenaires compatibles sont rares ou absents. Pour autant, le processus d'évolution des types de compatibilité ne devrait pas se trouver affecté par l'évolution des facteurs hôtes. On imagine en effet difficilement par quel biais les hôtes pourraient limiter l'apparition ou le maintien de tels mutants. Il est intéressant de noter en revanche que la présence de *Wolbachia* suicides maintient une sélection continue pour des facteurs hôtes diminuant l'intensité de *mod*, même dans les populations où l'infection est fixée. En effet, dans une population porteuses de *Wolbachia* suicides, les mâles infectés comme les femelles infectées voient leur succès reproducteur réduit si l'intensité de *mod* est élevée. Ainsi, la prise en considération de l'évolution des facteurs hôtes ne devrait pas fondamentalement affecter l'évolution des types de compatibilité, mais pourrait contribuer à expliquer la répression de l'IC par l'hôte.

3. Perspectives expérimentales

Des développements expérimentaux peuvent également être proposés. En premier lieu, la confrontation au sein d'un même hôte de bactéries étroitement apparentées pourrait être étendue à d'autres *Wolbachia*. Le cas du moustique *Culex pipiens* semble potentiellement intéressant : dans cette espèce, des bactéries proches semblent bi-directionnellement incompatibles (Guillemaud *et al.* 1997), mais des variations du génome de l'hôte peuvent compliquer l'interprétation des résultats (Rousset *et al.* 1991). L'injection de ces différentes bactéries au sein d'une lignée homogène de *Drosophila simulans* permettrait de s'affranchir de ce problème. Plus généralement, l'injection dans une espèce modèle de nombreuses bactéries inductrices d'IC permettrait de mieux cerner la diversité et l'évolution des types de compatibilité. Rappelons toutefois la difficulté du transfert des infections de *Rhagoletis cerasi* vers *Drosophila simulans* (article N°9, annexe 3) illustrant les limites de cette approche.

Le problème déjà mentionné du polymorphisme intra-individuel mériterait d'être étudié expérimentalement. Comme mentionné plus haut, la persistance de plusieurs clones au sein d'un même individu peut affecter l'évolution des types de compatibilité. L'importance de ce phénomène dépendra directement de l'effectif efficace intra-individuel, c'est-à-dire du nombre de bactéries effectivement transmises à la descendance : si ce nombre est faible, la sélection pourra difficilement maintenir un état polymorphe. Comment déterminer l'effectif efficace intra-individuel ? Chez *Drosophila melanogaster* et chez l'hyménoptère *Nasonia vitripennis*, le nombre de bactéries présentes dans un œuf mature est de l'ordre de 10^6 (Breeuwer & Werren 1990; Hadfield & Axton 1999). Ce nombre n'est cependant pas informatif pour estimer l'effectif efficace, car il excède probablement de loin le nombre moyen de bactéries colonisant une cellule germinale. Une toute autre approche, fondée sur la mesure des taux de ségrégations, pourrait en revanche être utilisée. Le principe en est le suivant. Considérons une femelle porteuse de deux types de *Wolbachia* (w_1 et w_2), en quantités k_1 et k_2 . En théorie, les distributions respectives des quantités k_1 et k_2 dans la descendance de cette femelle suivent des lois binomiales dont le paramètre n est exactement ce à quoi nous nous intéressons. Ainsi, pour estimer n , il suffirait de déterminer k_1 et/ou k_2 chez la mère et chez de nombreux descendants, ce qui est désormais possible, grâce à la PCR quantitative. Notons cependant qu'une telle expérience ne peut être réalisée à partir d'une bi-infection "classique", impliquant deux variants bactériens phylogénétiquement distants. En effet, si les variants w_1 et w_2 sont trop différents, leurs taux de transmission respectifs ainsi que leurs capacités à se diviser dans cet hôte particulier, risquent de différer également, induisant un biais difficilement mesurable dans notre expérience.

Enfin, la *Wolbachia* suicide (*mod_{BRESA}*), pierre angulaire du modèle d'évolution présenté ici, reste à découvrir. Il est intéressant de noter que les protocoles d'élevage des organismes hôtes en laboratoire induisent un biais en défaveur de ce type de variants. En effet, les espèces modèles utilisées pour l'étude de *Wolbachia*, et en particulier la drosophile,

sont maintenues en lignées isofemelles : des lignées indépendantes sont fondées à partir de femelles de la nature. Ce type de maintien, même s'il est très utile en ceci qu'il permet de conserver la diversité initiale, présente un inconvénient majeur pour la *Wolbachia* suicide. En effet, une telle bactérie voit ses chances de survie réduites par les croisements incestueux qui sont le lot de la F1. D'une manière plus optimiste, on peut voir en ce phénomène un crible de choix pour détecter la *Wolbachia* suicide à partir des populations naturelles.

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Annexes

Annexe 1.

Article N°7 (revue). *Wolbachia*-induced cytoplasmic incompatibility

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En bref...

L'IC est une des stratégies permettant à la bactérie endocellulaire Wolbachia d'envahir les populations hôtes. Ce phénomène, initialement découvert chez le moustique Culex pipiens, dans les années 50, a depuis été observé chez de nombreuses espèces d'insectes et autres arthropodes. Après une présentation générale de Wolbachia et de l'IC, nous décrivons ici les différentes étapes de "l'histoire d'une infection": invasion d'une population hôte, co-évolution, et finalement perte, ou maintien à long terme. Dans une dernière partie, les applications, potentielles ou éprouvées, de l'IC à la lutte biologique sont discutées.

WOLBACHIA-INDUCED CYTOPLASMIC INCOMPATIBILITY

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1. Introduction

1.1. TAXONOMY, DISTRIBUTION AND NOMENCLATURE

Wolbachia belong to the α subdivision of proteobacteria (O'Neill *et al.* 1992). As all other members of their family (Rickettsiaceae), they are obligatory endocellular symbionts (Weiss and Moulder, 1984). First observed in 1924 by Hertig and Wolbach in the mosquito *Culex pipiens*, and described in details by Hertig in 1936, they were since detected in various Arthropod groups (Insecta, Collembola, Crustacea, Arachnida) (Werren *et al.* 1995a; O'Neill *et al.* 1997a; Vandekerckhove *et al.* 1999) as well as filarial Nematodes (Sironi *et al.* 1995; Bandi *et al.* 1998). Systematic surveys of insect communities revealed that at least 15% of the species are infected, making *Wolbachia* one of the most abundant endocellular bacteria (Werren *et al.* 1995a). Recent studies based on highly sensitive detection methods suggest to extend this estimation to 76% (Jeyapakash and Hoy, 2000).

As inferred from molecular data, *Wolbachia* form a monophyletic group (Roux and Raoult, 1995), among which five clades (A, B, C, D and E) can be distinguished. A and B diverged ~60 MY ago and form a monophyletic group including most Arthropod-infecting *Wolbachia* (Werren *et al.* 1995b). The E group is now represented by a unique *Wolbachia* strain, infecting a single arthropod species (Hexapoda, Collembola) (Vandekerckhove *et al.* 1999). A, B and E form together a monophyletic group (Vandekerckhove *et al.* 1999), out of which fall C and D, represented by Nematode-infecting *Wolbachia* (Bandi *et al.* 1998). Several genes have been used for phylogenetic purpose, such as 16S rDNA (O'Neill *et al.* 1992; Rousset *et al.* 1992; Stouthamer *et al.* 1993) and different protein coding genes (Werren *et al.* 1995b; Zhou *et al.* 1998; Van Meer *et al.* 1999), which all confirmed the main groupings. Highly variable ones also allowed understanding phylogenetic relationships on a finer scale (Zhou *et al.* 1998; Van Meer *et al.* 1999).

In spite of such a diversity, *Wolbachia* are denominated under a unique species name (*Wolbachia pipientis*). During an international meeting held in June 2000 (reported by Cook and Rokas, 2000; Charlat and Merçot, 2000), a nomenclature system was proposed. It was suggested to maintain this unique species name, but to name separately strains that had been shown to differ by any trait, either DNA sequences or phenotypic characters. Names should then be written using a *w* (for *Wolbachia*) followed by two or three characters and a subscript, indicating the strain origin and host species. As an example *w*No_{D.sim} refers to a *Wolbachia* strain naturally infecting *Drosophila simulans* in populations from Noumea (New Caledonia) while *w*Cer1_{R.cer} refers to one of the two *Wolbachia* strains infecting the cherry fruit fly *Rhagoletis cerasi* (M. Riegler, pers. com.). We will follow such a rule in this review.

1.2. WOLBACHIA TRANSMISSION MODE: A PARADOX?

Owing to its high predictive value, transmission mode is an essential trait of endosymbiont biology (Ewald, 1987). It allows the classification of endosymbionts along a continuum, ranging from complete vertical transmission (dependent on host reproduction) to complete horizontal transmission (independent from host reproduction). These two extreme strategies impose very different constraints on the evolution of symbiont/host interactions. This being so, horizontally transmitted symbionts can be deleterious to their host, while vertically transmitted ones more often provide benefits. Although horizontal transfers can occur, *Wolbachia* are mainly vertically transmitted, through egg cytoplasm. Thus, mutualistic relationships are to be expected. Such a situation is indeed observed between *Wolbachia* and Nematodes, in pathogenic filaria (Hoerauf *et al.* 1999), a major cause of morbidity throughout the tropics. In this respect, researches on *Wolbachia* are offering serious opportunities for medical applications. Conversely, in most cases, *Wolbachia* in Arthropods do not strictly speaking benefit their host. A solution to this apparent paradox is given by considering the amazing effects of *Wolbachia* on their host reproduction: feminization, male killing, thelytokous parthenogenesis and Cytoplasmic Incompatibility (CI). The three first phenotypes have in common to increase the proportion of females in infected females' broods, and thus directly advantage *Wolbachia* infected cytoplasm (for a review, see Pintureau *et al.* in this volume). CI-inducing *Wolbachia*, the subject of this chapter, have slightly different and probably more perverse consequences.

We will first describe the CI phenomenon, its distribution as well as the current knowledge about the mechanisms involved. Next, we will investigate the evolutionary dynamics of the associations of CI-*Wolbachia* with their hosts, which undoubtedly condition the long-term fate of this symbiosis. We will then discuss the evolutionary consequences of CI. Finally, the potential of CI-*Wolbachia* as biological control agents, as well as last advances in *Wolbachia* genomics, will be considered.

2. *Wolbachia*-induced CI

2.1. GENERAL DESCRIPTION

In 1952, Ghelelovitch reported the occurrence of reproductive isolation between different mosquito populations. He showed that owing to a maternally inherited factor, males from a given strain failed to produce progeny when mated with females from other strains, while the reverse cross was compatible. Laven (1967) further showed that in some cases, incompatibility occurred in both directions of cross. *Wolbachia* was identified as the causative agent, by Yen and Barr in 1971, which allowed to describe CI as an embryonic mortality of various intensity, occurring when *Wolbachia*-infected males mate with uninfected females (unidirectional CI) or females infected by a different *Wolbachia* strain (bidirectional CI) (figure 1).

Figure 1.

	W1 ♀	W2 ♀	∅ ♀
W1 ♂	W1	+	+
W2 ♂	+	W2	+
∅ ♂	W1	W2	∅

Unidirectional incompatibility is illustrated in crosses between infected (W1 or W2) and uninfected (∅) individuals. Bidirectional incompatibility is illustrated in crosses between individuals infected by two different *Wolbachia* strains. Infection status of the descent is indicated in circles.

As a consequence of unidirectional CI, *Wolbachia* can, as a first approximation, spread through uninfected populations. Indeed, while mating with infected males is detrimental to uninfected females reproduction, infected females are compatible with both infected and uninfected males. Infected cytoplasm is thus indirectly selected for, in a positive frequency dependent manner: as infection frequency increases, uninfected cytoplasm is more and more disadvantaged. Thus, CI induction allows *Wolbachia* to spread and then remain within natural populations. Other factors than CI, that may affect invasion dynamics, will be considered in detail later on in this chapter.

Since the time of its initial discovery in mosquitoes, CI has been described in Arachnida (Breeuwer, 1997), some Crustacea (Legrand *et al.* 1985; Moret *et al.* 2001) as well as in numerous insect orders, making it the most frequent and widely distributed of *Wolbachia* induced phenotypes (O'Neill *et al.* 1997a). Phylogenetic analysis suggested that CI-*Wolbachia* do not form a monophyletic group with respect to the *Wolbachia* strains that cause other phenotypes (Werren *et al.* 1995b; Zhou *et al.* 1998). In fact, the distribution of CI within *Wolbachia* general phylogeny makes parsimonious to assume that it was an ancestral *Wolbachia* property.

2.2. MECHANISMS

The so-called mod/resc model provides a general framework for the investigation of CI (Werren, 1997a). It assumes the existence of two bacterial functions: (i) mod (modification), the poison, is expressed in the male germline before *Wolbachia* are shed from maturing sperm and (ii) resc (rescue), the antidote, is expressed in the egg. If sperm has been affected by mod, zygote development will fail unless the appropriate resc is expressed in the egg. Although their molecular nature is currently unknown, the mod and resc functions are now characterized through a number of properties, which we report below. These properties will have to be accounted for by any hypothesis regarding the molecular nature of mod and resc.

mod intensity is variable. The percentage of unhatched eggs observed in crosses between infected males and uninfected females, which we will refer to as CI level, shows quantitative variations, ranging from 0 to 100 % (Poinsot *et al.* 1998). Thus, the molecule(s) involved in the mod function must potentially show variation, in quantity and/or activity. In some cases, variations in CI levels are caused by *Wolbachia* inherent properties (Giordano *et al.* 1995, Rousset and de Stordeur, 1994; Poinsot and Merçot, 1999). Interestingly, variations due to host effects were also shown to exist. Indeed, injection experiments, allowing transferring *Wolbachia* strains between species, demonstrated that CI level is affected by host nuclear background. As an example, a “strong” strain naturally infecting *Drosophila simulans* expresses a low CI level when injected into *D. melanogaster* (Boyle *et al.* 1993). Conversely, a “weak” strain naturally infecting *D. melanogaster* expresses a high CI level when injected into *D. simulans* (Poinsot *et al.* 1998).

mod and resc interact in a specific manner, as shown by the occurrence of bidirectional incompatibility. Simply speaking, any *Wolbachia* strain is only compatible with itself, suggesting that the molecules involved can exist under various forms, allowing specific recognition. Proteins are, of course, the best candidates. It is notable that several mod/resc interactions can take place within a single embryo as suggested by crossing experiments involving doubly infected individuals (infected simultaneously by two *Wolbachia* strains). Patterns of compatibility are exactly the ones expected if each resc interacts only with its mod counterpart: doubly infected males are compatible with doubly infected females only (Rousset and Solignac, 1995; Merçot *et al.* 1995; Perrot-Minnot *et al.* 1996; Sinkins *et al.* 1995). Interestingly, mod/resc recognition can, in some cases, be partial. Indeed, partial compatibility between different *Wolbachia* was reported in *Drosophila* by Poinsot *et al.* (1998). Surprisingly, the bacterial strains involved were phylogenetically distant. Two alternative explanations can be proposed to account for this result: (i) either bidirectional incompatibility did not evolve yet, or (ii) compatibility was lost and subsequently restored by evolutionary convergence. More data on the evolutionary rate of compatibility types is required for choosing between these two alternatives.

mod and resc are probably separate functions. Depending on the presence or absence of the mod and resc functions, four different CI-*Wolbachia* types can theoretically exist: mod+/resc+, mod-/resc-, mod+/resc- and mod-/resc+. The mod+/resc+ type corresponds to most strains described so far: they induce CI and rescue their own CI phenotype. The mod-/resc- type was shown to exist in *D. simulans* (Hoffmann *et al.* 1996). It

corresponds to strains that are both unable to induce CI and rescue the CI phenotype of other mod+ strains. The mod+/resc- type is suicidal and has never been observed: it cannot theoretically be maintained in natural population as it counter-selects its own presence. On the contrary, the mod-/resc+ type, unable of inducing CI but capable of rescuing CI induced by other strains, was actually shown to exist (Bourtzis *et al.* 1998; Merçot and Poinso, 1998a; Poinso and Merçot, 1999). The existence of such a *Wolbachia* strain strongly suggests that mod and resc are separate functions: if not separate genes, at least different gene domains. Other interpretations can however be proposed. mod and resc could represent a single molecule, with mod requiring higher concentrations. Alternatively, the mod-/resc+ strain could have a sex specific gene expression pattern, with a unique function being expressed in the female, not in the male. Let us emphasize here that the resc- status of a *Wolbachia* strain cannot be definitively fixed by crossing experiments. Indeed, such bacteria may be able to rescue the mod function of other strains, still undiscovered.

mod intensity is linked to bacterial density. The possibility of a relationship between *Wolbachia* density and CI level was investigated. Let us first consider studies focusing on bacterial density in male testes. It was shown in *D. simulans* that as CI level decreases with male aging (Hoffmann *et al.* 1986), so does *Wolbachia* density in male testes (Binnington and Hoffmann, 1989), as well as the number of infected spermocysts (Bressac and Rousset, 1993). CI level was also shown to correlate positively with the number of infected spermocysts in *D. melanogaster* (Solignac *et al.* 1994), and *D. simulans* (Merçot *et al.* 1995). Thus it seems that when comparisons involve variations associated to a given *Wolbachia* strain within a given species, the relationship between CI level and density in testes is clear. Interestingly, experimental interspecific transfers showed that this relationship was also observed in comparisons involving different hosts species: when $wMel_{D.mel}$ is transferred from *D. melanogaster* to *D. simulans*, a shift from low to high CI level is accompanied by a shift from low to high number of infected spermocysts (Poinso *et al.* 1998). Do CI level and density in male testes still correlate when different *Wolbachia* strains are compared? In *D. simulans*, $wMel_{D.mel}$ and $wRi_{D.sim}$ infect the same frequency of spermocysts and induce similar CI levels (Poinso *et al.* 1998). However, discrepancies appear if other *Wolbachia* strains are considered (in a single host or different hosts): some strains harbor low CI levels and high densities, while others harbor strong CI levels and low densities (Rousset and de Stordeur, 1994; Bourtzis *et al.* 1998; see also Bourtzis *et al.* 1996). Thus, it appears that the relationship between CI level and density in male testes, although well demonstrated when comparisons involve a single *Wolbachia* strain (in a single host or different hosts), breaks down when different *Wolbachia* strains are compared. Are similar conclusions drawn from density measurement in eggs? Here again, a positive correlation is observed when comparisons involve a single *Wolbachia* strain within a single host (Boyle *et al.* 1993), but it breaks down as soon as different *Wolbachia* strains and/or different hosts are compared (Giordano *et al.* 1995; Hoffmann *et al.* 1996; Clancy and Hoffmann 1997). Poinso *et al.* (1998) included both types of measurements: while a strong correlation is observed between CI level and density in testes, density in eggs and CI level appeared to be independent. Such a result is not surprising: one would indeed expect mod intensity to be more intimately linked to density in testes than in eggs, as mod is expressed during spermatogenesis.

mod prevents condensation of paternal chromosomes after fertilization. Cytological observations revealed that in crosses between males and females of different infection status, fertilization takes place normally (Ryan and Saul, 1968; Yen, 1975; Kose and Karr, 1995). In *Drosophila*, pronucleus fusion also occurs but paternal chromosomes show abnormal behaviors, remaining undercondensed while maternal chromosomes undergo mitosis (Callaini *et al.* 1996, 1997; Lassy and Karr, 1996). In the hymenopteran *Nasonia vitripennis*, paternal chromosomes are entirely lost, inducing complete haploidy (Breeuwer and Werren, 1990), while in *Drosophila*, they segregate more or less randomly, giving rise to haploid or aneuploid cells. The precise consequences on zygote development vary between species. Especially, diploid and haplodiploid organisms must be distinguished. In diploids, death occurs more or less shortly after fertilization (Callaini *et al.* 1996, 1997; Lassy and Karr, 1996). A more diverse range of outcomes occurs in haplodiploid species, owing to the fact that in such organisms, arrhenotokous parthenogenesis (male development from unfertilized haploid eggs) is commonly observed in the absence of infection. In *Nasonia*, *Wolbachia* induced haploidy leads fertilized eggs from incompatible crosses to develop into males (Ryan and Saul, 1968; Breeuwer and Werren, 1990). Conversely, CI induces the death of all fertilized embryos in *Leptopilina heterotoma*, another hymenoptera (Vavre *et al.* 2000) suggesting that in this case, embryos are aneuploid. In the haplodiploid acarid *Tetranychus*, a proportion of fertilized eggs develop into females while the others die (Breeuwer, 1997), suggesting that part of the embryos are diploid (not affected by CI) while the others are aneuploid. Such patterns are of high interest with regard to the investigations of CI mechanisms. Indeed, they provide opportunities to observe variations in the property of the *mod* function and, potentially to understand the origins of these variations.

3. Dynamics of CI-*Wolbachia*/host associations

Here we discuss the evolutionary dynamics of the associations between CI-*Wolbachia* and their hosts. Starting from a description of the different events involved in the invasion of a new species, we then consider the evolution of CI, and other relevant factors, once infection is established. This evolution undoubtedly conditions the long-term fate of CI-*Wolbachia*/host associations, which we finally discuss.

3.1. HOW CI-WOLBACHIA INFECT SPECIES

3.1.1. Co-speciation or horizontal transfer?

Two underlying processes can be envisaged when considering the present distribution of *Wolbachia* among arthropods: co-speciation and Horizontal Transfers (HTs). As numerous sequence data were obtained for *Wolbachia* and their hosts, it became possible to investigate this issue through a phylogenetic approach (O'Neill *et al.* 1992; Werren *et al.* 1995b; Zhou *et al.* 1998). Host and symbiont phylogenies appeared to be often incongruent, suggesting that if co-speciation may occur, it must be limited and cannot explain, on its own, *Wolbachia* distribution. HTs between species must thus be invoked, although it remains difficult to estimate their frequency.

By which means can *Wolbachia* jump between species? Several recent studies have focused on this issue. Based on the idea that such transfers require between-species intimate relationships, possibilities of *Wolbachia* HTs between parasitoids and their host were considered. Phylogenetic data suggested that *Wolbachia* can skip from host species to parasitoids (Vavre *et al.* 1999). Conversely, the reverse transfer (from parasitoids to hosts) seems to be less straightforward, and in any case less frequent (which may be due to the fact that when hosts are not killed by parasitoids, these latter are encapsulated, preventing from any exchange). Let us emphasize that such results, based on a phylogenetic approach, are weakened by the recent discovery that *Wolbachia* strains can exchange DNA: Werren and Bartos (2001) showed the 5' and 3' ends of a *Wolbachia* gene to have clearly distinct evolutionary origins, while F. Jiggins (pers. com.) demonstrated a strong lack of congruency between *Wolbachia* phylogenies based on two different loci. The existence of *Wolbachia* recombination, although of high interest, weakens any phylogenetic trees inferred from DNA sequences. If reliable phylogenetic data is to be obtained, the use of several different genes, allowing to identify robust nodes, is thus highly recommended.

HTs from hosts to parasitoids were also experimentally demonstrated. Indeed, Heath *et al.* (1999) reported that *Wolbachia* was transferred from *D. simulans* to its parasitoid *Leptopilina boulardi*, at a frequency near 1%, when uninfected wasps oviposit into infected host larvae. Furthermore, the newly acquired *Wolbachia* infections were maintained over generations, demonstrating that the germline had been efficiently colonized. Intraspecific HTs were also demonstrated. In isopod crustaceans, transfers have been shown to occur by a simple hemolymph contact, a route likely to be used in natural populations (Rigaud and Juchault, 1995). Furthermore, Huigens *et al.* (2000) recently reported that in *Trichogramma* wasps, when infected and uninfected individuals infest the same egg, *Wolbachia* transfers from infected to uninfected individuals occur at frequencies higher than 30%. These results demonstrate that in some conditions *Wolbachia* have the ability to be horizontally transferred, either within, or between species. It remains to be determined if the conditions required are very limited and if *Wolbachia* infection can efficiently develop and colonize germ cells in any new host.

Injection experiments between different host species provide possibilities for investigating the latter question. When performed between closely related species, such transfers are usually successful (Boyle *et al.* 1993; Giordano *et al.* 1995; Clancy and Hoffmann, 1997; Poinot *et al.* 1998). The outcomes of injections between distantly related species are less straightforward. Injections from mosquitoes into *Drosophila* (Braig *et al.* 1994), and from Hymenoptera into *Drosophila* were successful, but in the latter case, the infection was lost after several generations (Van Meer and Stouthamer, 1999). Furthermore, two of us (S.C. and H.M., together with M. Riegler) recently undertook injections between two closely related Diptera families. Current results suggest that the infection is highly unstable, due to a very low maternal transmission efficiency. It appears that the potential of *Wolbachia* to colonize efficiently the germline of new hosts is variable and might, in some cases, be a limiting factor of HTs.

3.1.2. Spreading of CI-inducing *Wolbachia*

Since the discovery of CI in mosquitoes, experimental and theoretical studies focused

on its invasion dynamics in natural populations. Early models (Caspari and Watson, 1959) demonstrated the unusually high invasion abilities of CI-*Wolbachia*. As a better knowledge of this symbiont biology has been gained, new parameters have been included, providing more realistic views (Fine, 1978; Hoffmann *et al.* 1990). Here, we present an overview of CI-*Wolbachia* invasion dynamics, without getting into equations. For a more detailed review on this issue, see Hoffmann and Turelli (1997).

What happens after a new CI-*Wolbachia* strain has been efficiently transmitted, either by HT or migration, into an uninfected population? Let us consider here the simplest situation: a *Wolbachia* strain perfectly transmitted (that is, infected females produce 100% infected progeny) and not affecting the fitness of its bearer (apart from its CI effect). As mentioned above, uninfected females suffer a fertility disadvantage when mating with infected males (the higher the CI level, the higher this disadvantage). Infected females thus reproduce more efficiently than uninfected ones: *Wolbachia* induce a selection against uninfected cytoplasmic lines, which indirectly advantages infected ones. Eventually, *Wolbachia* infection is expected to be fixed, even if CI is not 100%. Nevertheless, in this latter case, invasion will be slower.

Should other factors than CI be taken into account? Theory suggests that two parameters could have determining effects on the evolution of CI-*Wolbachia* frequencies: fitness effects on females and maternal transmission efficiency. Caspari and Watson (1959) considered the effect of a fitness reduction suffered by infected females (noted here f , varying from 0 to 1) together with that of CI (mod intensity, noted here m , varying from 0 to 100%). The main conclusions were the following: (i) 0 and 1 (i.e. extinction and fixation) are the only stable equilibrium frequencies and (ii) $p = f/m$ is an unstable equilibrium frequency, representing a threshold point below which frequency goes to 0 and above which it goes to 1. Thus, if $f \geq m$, *Wolbachia* is always lost (if it is not already fixed), and if $m > f \geq 0$, *Wolbachia* gets fixed if it reaches p (which is possible through random events). An important feature of this model is that it does not predict the stable coexistence of infected and uninfected individuals, which can yet be observed in natural populations (Turelli and Hoffmann, 1995). Fine (1978) and Hoffmann *et al.* (1990) showed that considering the effect of imperfect maternal transmission (noted μ , the fraction of uninfected eggs produced by infected females, varying from 0 to 1) could explain such a polymorphism. The main conclusions are the following: (i) 0 is a stable equilibrium frequency, (ii) p_s is a stable equilibrium frequency determined by f , m and μ and (iii) p_u is an unstable equilibrium frequency determined by f , m and μ , representing a threshold point below which frequency goes to 0, and above which it goes to p_s . Thus, the introduction of imperfect maternal transmission affects the value of the threshold frequency predicted by Caspari and Watson, but more importantly, it allows the stable coexistence of infected individuals (at frequency p_s) and uninfected ones (at frequency $1-p_s$).

Few case studies are complete enough to allow the testing of such theoretical models. Cage population experiments confirmed *Wolbachia* invasion abilities (Nigro and Prout, 1990). What about invasion dynamics in the wild? The most complete analysis to date concerns *D. simulans* and the spread of the $wRi_{D.sim}$ infection in California (Turelli and Hoffmann, 1995). The authors provided field estimates of the three key parameters mentioned above: fitness cost, transmission efficiency, and CI level. Although females seemed to suffer a slight fecundity reduction in laboratory conditions, this effect was not

detected in the field ($f = 0$). Conversely, transmission efficiency was shown to be perfect in laboratory conditions, but it was estimated that infected wild females produce in average 4% of uninfected progeny. Finally, CI was estimated to be close to 100% using males from laboratory stocks, but only 55% if wild males were assessed (since CI level strongly decreases with male age (Hoffmann *et al.* 1986), it was proposed that such a reduction of CI in the field was mainly due to the fact that, in average, wild males are older than males commonly used in CI experiments). Remarkably, field parameter estimation allowed authors to predict the infection spread at a rate comparable to the one observed in the wild through a monitoring of several independent populations over 5 years. Furthermore, predicted equilibrium frequencies from most natural populations were accurately concordant with observed ones. From this study, it appears that CI-*Wolbachia* invasion dynamics and equilibrium frequencies are well predicted by current theory. Let us note however that a few locations from the above study showed anomalous frequencies with regard to predictions of the model, possibly representing a tension zone between highly infected and uninfected populations. Another discrepancy between theory and reality comes from *D. melanogaster* infection frequency data. In this species, parameter estimates from the wild lead to predicted equilibrium frequencies clearly different from those observed in natural populations (Solignac *et al.* 1994; Hoffmann *et al.* 1994; Hoffmann *et al.* 1998). Although low and undetectable fitness advantages were invoked, such results suggest that other important parameters (possibly new *Wolbachia* induced phenotypes) remain to be discovered. The existence of non-CI-inducing strains in *D. simulans* (Hoffmann *et al.* 1996; Merçot and Poinot, 1998a), considered in more details further in this chapter, suggests similar remarks.

Let us mention here that estimation of the three key parameters often suggest that *Wolbachia* negative effects on host fitness are rare and limited (reviewed in Hoffmann and Turelli, 1997; Poinot, 1997). Thus, CI level and transmission efficiency appear to be the main parameters.

3.2. WHAT HAPPENS AFTER SPREADING?

How do CI, transmission efficiency and fitness cost evolve once infection has reached its equilibrium frequency? The answer to this question conditions the fate of CI-*Wolbachia* host associations in the long term: it determines to what extent *Wolbachia* infections are stable over time and hence, what type of relationships between CI-*Wolbachia* and their hosts are expected to evolve. We first discuss the evolution of these three key parameters from the bacterial point of view, then from the host side. Finally, we explore the outcomes of the evolution of bacterial and host determinants in combination.

3.2.1. Evolution of bacterial determinants

CI levels. Since CI-*Wolbachia* invade host populations owing to CI induction, one is intuitively tempted to consider that high CI levels are selected for. However, Prout (1994) and Turelli (1994) showed that this is not to be the case when the competing variants are compatible with each other. In order to illustrate this conclusion, let us consider a *Wolbachia* strain $\text{mod}_a/\text{resc}_a$ infecting a panmictic host population. Consider

now a mutant $\text{mod}_{a^*}/\text{resc}_a$ harboring a stronger mod intensity ($\text{mod}_{a^*} > \text{mod}_a$) but compatible with the original strain (resc_a can rescue mod_{a^*}). Will $\text{mod}_{a^*}/\text{resc}_a$ variants invade the population? The answer is no: males infected by $\text{mod}_{a^*}/\text{resc}_a$ induce a higher embryonic mortality than $\text{mod}_a/\text{resc}_a$ males (when mating with uninfected females), but $\text{mod}_{a^*}/\text{resc}_a$ and $\text{mod}_a/\text{resc}_a$ females are equally compatible with all types of males. Since *Wolbachia* are maternally transmitted, $\text{mod}_{a^*}/\text{resc}_a$ and $\text{mod}_a/\text{resc}_a$ variants have the same fitness. In fact, the occurrence of mod_{a^*} will induce an overall increase of the infection frequency, but this benefit goes both to $\text{mod}_{a^*}/\text{resc}_a$ and $\text{mod}_a/\text{resc}_a$ variants. Thus, it appears that from the bacterial point of view, variations in mod intensity between compatible strains are selectively neutral and thus evolve under genetic drift only.

Transmission efficiency and fitness cost. Turelli (1994), generalizing Prout's model (1994), has shown that selection among bacterial variants acts to maximize the number of infected progeny produced by infected females, which is directly determined by transmission efficiency and fitness costs. Thus, from the bacterial side, long-term evolution is expected to lead to high transmission efficiency and low fitness costs.

3.2.2. Evolution of host's determinants

Here we describe the selective forces acting on host genome with regard to the evolution of the three key factors. Let us notice that mitochondrial genome is not considered here: "host genes" refer to nuclear genes only.

CI levels. What selective pressures act on host genes that affect CI levels? Turelli (1994) showed that a reduction of CI levels is selected for. To illustrate this conclusion, let us distinguish the four types of individuals present in a host population: infected females (IF), infected males (IM), uninfected females (UF) and uninfected males (UM). IF and UM do not suffer from CI but are not advantaged either by strong CI levels. IM and UF do suffer from CI, and are selected for reducing CI levels. In other terms, host genes decreasing the incompatibility between IM and UF are selected for. Two types of such genes can be envisaged: those reducing mod intensity, selected for in IM and host rescue genes, selected for in UF. Thus, low CI levels are expected to evolve. Let us note that this conclusion is drawn only if infection is not fixed. More generally, the dynamics of host genes reducing CI will depend on the infection equilibrium frequency (which depends itself on CI levels).

Transmission efficiency and fitness cost. Turelli (1994) showed that regarding these two parameters, similar selective pressures act on hosts and symbionts. Low fitness cost are selected for, while maternal transmission tends to be maximized (because uninfected offspring are not protected from CI). Thus from the host side, long-term evolution is expected to lead to low fitness cost and high transmission efficiency. An interesting exception to this rule was recently shown to occur in hymenopteran species where CI induces male development instead of death. Vavre (2000) showed that in such species, host are selected for a reduction of transmission efficiency, owing to the fact that nuclear genes are efficiently transmitted in incompatible crosses.

3.2.3. Combined evolution of bacterial and host determinants

What if selection on hosts and symbionts are considered together? Mod intensity seems to be neutral for *Wolbachia*, while a reduction of CI levels is expected from selection on

hosts. High transmission efficiency and low fitness costs are selected for from both sides (if the special case of haplodiploids is not considered). Thus, if these three key factors do not interfere, host/symbiont co-evolution is expected to lead to low CI levels, low fitness costs and high transmission efficiency.

3.2.4. Interference between the three key factors

Turelli (1994) suggested that CI levels, transmission efficiency and fitness cost might be linked through a unique feature: bacterial density. This would in theory lead to trade-off densities, since selection pressures act in different directions: selection on CI level and transmission efficiency favor an increase of density, while selection on fitness cost tends to minimize density. Such interference would explain the above-mentioned discrepancies between field and laboratory parameter estimates in *D. simulans* (lower CI level, lower transmission and lower cost in the field, possibly due to a lower density).

What arguments actually support such relationships between the three key factors and density? As previously stated, CI level and density were shown to be positively correlated, but this relationship may have limited applications. Furthermore, there is no *a priori* reason to assume that density in embryos determines mod intensity: the way is long from the embryo to its spermatozoa... Is there any evidence for a correlation between density in embryos and transmission efficiency? Such a link is suggested by logic. However, few studies focused on this issue. Indirect evidence come from Poinot *et al.* (2000). What about the relationship between infection cost and density? Here again, a link would not be surprising, but no empirical data support it.

The three key parameters, if linked to density, are probably not linked to the same aspect of this latter: CI level must be linked to density in males reproductive tissue, transmission efficiency may be facilitated by high densities in ovaries, while the infection cost is probably affected by overall density (not tissue specific). Thus, any assumption of simple relationship between the three key factors should be considered cautiously.

3.2.5. Empirical data

Theory predicts that *Wolbachia*/host coevolution will lead to low CI levels, low infection cost and high transmission efficiency. Are such tendencies observed in reality? Injection experiments provide insights into this question. By comparing the parameter estimates in both natural and naive host (that is naturally uninfected), it is possible to determine the outcomes of co-evolution. Thus, when $wRi_{D.sim}$, naturally infecting *D. simulans* is transferred into *D. serrata* (naturally uninfected), CI level is increased while transmission efficiency is decreased (Clancy and Hoffmann, 1997). However, while $wRi_{D.sim}$ induces a fecundity deficit in its natural host, no such cost was detected in the novel one. Thus, injection into a naive host partially support the predictions.

Interestingly, when $wRi_{D.sim}$ is transferred into *D. melanogaster*, a species naturally infected by another *Wolbachia* strain, a shift from high to low CI level is observed (Boyle *et al.* 1993). Conversely, when $wMel_{D.mel}$, naturally infecting *D. melanogaster*, is transferred into *D. simulans*, a shift from low to high CI levels is observed (Poinot *et al.* 1998). These results put together suggest that *D. melanogaster* reduces CI in a non-specific manner. Solignac *et al.* (1994) suggested that *D. melanogaster* had experienced

Wolbachia infection for a longer time than *D. simulans*, which would explain such controls of CI levels. However, recent investigations show that some infections in *D. simulans* might be ancient (Charlat *et al.* unpublished results), suggesting to consider this explanation cautiously.

3.3. WHAT MAINTAINS CI?

In the above section, we concluded that CI levels were neutral from the bacterial point of view, while a reduction was selected from the host side, possibly leading to the loss of the mod function. However, CI-inducing *Wolbachia* are widespread and CI levels are often very high (Hoffmann and Turelli, 1997). Accordingly, it is very likely that CI is selected for. Three kinds of explanation can be proposed, which will be detailed here. First, the ability of *Wolbachia* to invade new species and to be maintained depends on its ability to induce CI. Second, population structure may affect the evolution of mod intensity. Finally, bidirectional incompatibility, and the evolutionary process leading to it, might favor an increase of CI levels.

3.3.1. Invasion and maintenance abilities

We proposed elsewhere to compare arthropods to a metapopulation within which the current distribution of *Wolbachia* strains was the consequence of the dynamics of extinction (loss) relative to colonization (gains) (Charlat and Merçot, 2000). Such a conception provides arguments for the evolutionary maintenance of CI.

First, CI inducing strains are more efficient than non-CI ones in colonizing new species. Indeed, the higher the intensity of mod, the lower the threshold frequency (that must be reached through random events for *Wolbachia* to invade deterministically), and the faster the invasion process. Thus, *Wolbachia* potential to be horizontally transferred induces a selection for high CI levels.

The second argument invokes selection at the population level. Although, within populations, variants inducing high CI levels are not selected for, bacterial populations that induce CI are less likely to go extinct than populations that do not. Thus, through a process of group selection (Hurst and McVean, 1996) CI may be evolutionary maintained.

3.3.2. Host population structure

Considering the competition between compatible strains harboring different CI levels, Franck (1997) showed that if infection is not fixed, and if host population is structured, high CI levels might be selected for. Indeed, population structure means that bacterial clones are not evenly distributed. A clone that induce a higher CI level will increase the infection frequency, but this increase will be more important locally. Thus, through a process of kin selection, strains inducing high CI levels advantage themselves by aiding relative symbionts in neighboring females. Franck (1997) suggested that weak population structuration is sufficient to explain the maintenance of high CI levels. Let us notice that such an argument also stands for selection on host: if population is structured, hosts genes that increase CI levels benefit themselves by aiding relative genes in infected females.

3.3.3. *The importance of bidirectional incompatibility*

Different CI-*Wolbachia* variants can infect separate populations within a given species. If populations come into contact, a competition occurs between *Wolbachia* variants. If these are incompatible, two outcomes are possible. The populations may become definitively isolated (a possibility which will be considered in more detail in the section below on CI and speciation). If complete isolation does not occur, one of the two variants will go extinct (Rousset *et al.* 1991). In such a case, selection will favor the strongest variant (in terms of mod intensity, transmission efficiency, and fitness effects). As transmission efficiency and fitness effects are supposed to be optimized by “within population selection”, strains will most probably differ by mod intensity. The highest mod intensity will hence be selected for. Thus, bidirectional incompatibility may be an important factor explaining the maintenance of high CI levels.

Furthermore, our current theoretical work suggest that evolutionary processes leading to new incompatibility types might also favor strong mod intensity (Charlat *et al.* unpublished results). By contrast to previous studies on this issue, our work includes recent results suggesting that mod and resc are independent functions (Merçot and Poinso, 1998a; Poinso and Merçot, 1999). Such an assumption allows the evolution of new compatibility types under a wider range of conditions than previously thought (Turelli, 1994; Werren, 1997b). It also suggests that new incompatibility types will invade more easily if mod intensity is at the same time stronger. Such a process may be a further explanation of why CI still exists.

3.4. INFECTION LOSS OR EVOLUTIONARY STABILITY?

We described some evolutionary forces that act to decrease CI level, and others that act to maximize it. The outcome of such conflicts is not straightforward to predict, since it is likely to depend on host biological traits such as population structure. When selection maximizes CI, infection is probably maintained in the long term.

Empirical data demonstrate that, at least in some cases, CI might be lost. Indeed, in *D. simulans*, two *Wolbachia* strains have been described that do not induce any detectable phenotype and probably derive from CI-inducing strains since they are very closely related and infect the same species as the latter. Apparently, their frequency is low and stable in the wild (Hoffmann, *et al.* 1996; James and Ballard, 2000; Charlat *et al.* unpublished results). Models do not predict that such mod- strains can be maintained, unless they confer a fitness advantage, which was yet not detected (Hoffmann *et al.* 1996). These may be too small to be experimentally spotted. Alternatively, undetected mod+ strains, compatible with these mod- variants, may occur at low frequencies in natural populations. Whatever the causal factor, it remains that mod- strains are maintained, albeit at a low frequency, suggesting that CI loss does not necessarily mean evolutionary instability. Most notably, since non-CI inducing strains were almost undetectable before PCR became available, it is possible that their discovery will become a relatively common event in the coming years.

4. Evolutionary consequences of *Wolbachia*-induced CI

4.1. CONSEQUENCES ON HOST POPULATION BIOLOGY

4.1.1. CI lowers population mean fitness

During the invasion process, the population average fitness is reduced. Considering the simplest case (no cost, perfect transmission and 100% CI), the average fitness drops to a minimum of 0.5 when bacteria infect 50% of the population (i.e. on average, half of the eggs do not hatch because of CI). Let us note that the population mean fitness is also lowered if infection equilibrium frequencies is not 1, that is, as soon as transmission is not perfect. Furthermore, our current work on the evolution of bidirectional incompatibility suggests that polymorphic situations may be maintained by selection (Charlat *et al.* unpublished results). Such a polymorphism can actually induce a mean fitness reduction of 50%. Thus, it seems that in diverse situations, the population mean fitness, and thus its capacity to grow, is strongly affected by CI-*Wolbachia*. In this respect, these symbionts represent an important feature of host demography and may strongly affect the structure of species communities.

4.1.2. Mitochondria hitchhike along with CI-*Wolbachia*

It has been well demonstrated that as *Wolbachia* invade, so do associated mitochondria (Nigro and Prout, 1990; Ballard *et al.* 1996). As a consequence, *Wolbachia* spread induces a strong reduction of mitochondrial diversity. Interestingly, it was demonstrated that even in cases where *Wolbachia* equilibrium frequency is not 1, the mitochondrial haplotype associated to the infected cytoplasm gets fixed. This is due to the fact that when *Wolbachia* is at equilibrium frequency, uninfected individuals derive from infected ancestors (Turelli *et al.* 1992). Such *Wolbachia* effect should be considered very seriously when inferring population histories from mitochondrial data. As an example, the patterns of mitochondrial diversity induced by CI-*Wolbachia* may mistakenly be interpreted as founder events.

Let us note that occasional paternal transmission of *Wolbachia* or HTs within populations may break the association between *Wolbachia* and mitochondria. In *D. simulans*, possibilities of rare paternal transmission seem to exist in laboratory conditions (Hoffmann *et al.* 1990). Furthermore, intraspecific HTs were shown to occur at very high frequencies in some parasitoid species (Huigens *et al.* 2000). In any case, population genetic studies in *D. simulans* demonstrate a linkage between *Wolbachia* and mitochondrial haplotypes (Ballard *et al.* 1996).

4.1.3. CI may promote speciation

Because of its ability to induce partial or complete isolation between host populations, CI has been investigated as a potential promoter of speciation (Werren, 1997b). The most complete study to date with regard to this issue concern three parasitoid wasp species of the genus *Nasonia*: *N. giraulti*, *N. longicornis* and *N. vitripennis* (Werren, 1997b). The first two diverged ~250,000 years ago, while their common ancestor diverged from *N. vitripennis* ~800,000 YA (Campbell *et al.* 1993). All three species are doubly infected by *Wolbachia*. Infection was shown to induce complete reproductive isolation between one of the older species pairs: *N. giraulti* and *N. vitripennis*

(Breeuwer and Werren, 1990; Bordenstein *et al.* 1998). Following antibiotic curing, fertile F1 hybrids are produced but there is severe F2 hybrid breakdown (Breeuwer and Werren 1995). These data demonstrate that CI-*Wolbachia* is involved in reproductive isolation, but since other reproductive (pre- and post-mating) barriers exist between these species, it is not known if *Wolbachia* were the original cause of speciation. Bordenstein *et al.* (2001) analyzed reproductive barriers in other species pairs and have shown that *Wolbachia* is involved in reproductive isolation in all cases. Furthermore, in the younger pair (*N. giraulti* and *N. longicornis*), CI seems to be the only isolating barrier to gene flow (except for weak sexual isolation), suggesting that if these species came into contact, *Wolbachia* could play a causal role in speciation.

Other systems potentially provide relevant information. Bidirectional incompatibility was shown to occur in *Culex pipiens* (Guillemaud *et al.* 1997). However, the involvement of *Wolbachia* was not clearly demonstrated. Furthermore, there is no evidence that geographical races in this species are due to *Wolbachia* (Werren, 1997b). In *D. simulans*, different incompatible strains also occur, but no genetic structuration, at the nuclear level, was observed (Ballard, 2000), suggesting that if this is a case of incipient speciation, we are still at the very first steps. Let us notice that in this species, CI is not complete (Merçot and Poinot, 1998b), and that the infection is not fixed in natural populations (James and Ballard, 2000), thus allowing for significant gene flow. The potential involvement of unidirectional incompatibility in speciation should also be considered. Although it limits gene flow in only one direction of cross, Shoemaker *et al.* (1999) recently provided evidence that unidirectional incompatibility can be a component of reproductive barriers. Indeed, the isolation between *Drosophila recens* and *D. subquinaria* was shown to be mediated by behavioral components in one direction of cross, while unidirectional incompatibility was an important factor in the reverse cross.

Although the involvement of CI-*Wolbachia* in speciation events is strongly suggested, no complete and direct evidence is available, as it is often the case in speciation study. However, the widespread occurrence of CI-*Wolbachia* and the potential of uni- and bidirectional incompatibility to cause reproductive isolation strongly motivates further investigations.

4.2. MUTUALISTIC RELATIONSHIPS

CI-*Wolbachia* are selected for a reduction of fitness costs. Going further, benefits to host are also to be expected, if infection is stable enough for these to evolve. Mutualistic relationships were actually observed in Hymenoptera (Girin and Bouletreau, 1995; Dedeine *et al.*, 2001), and invoked (but not detected) to explain the maintenance of non-CI strains in *Drosophila*. A striking example of the consequences that mutualistic endosymbiosis can have on evolution is that of mitochondria. Their endosymbiotic origin is well documented, especially from phylogenetic data. Strikingly, mitochondria fall within the α -proteobacteria subdivision of Eubacteria (Yang *et al.* 1985; Gray *et al.* 1989), as does the Rickettsiaceae family, to which *Wolbachia* belongs. Such a relatedness makes it tempting to speculate on the long-term evolutionary fate of *Wolbachia*. Complete genome sequencing projects, presented as a conclusion of this chapter, will undoubtedly tell us more on this issue, as remarkably illustrated for other endocellular symbionts (Andersson *et al.* 1998; Shigenobu *et al.* 2000).

5. Applied biology of CI-inducing *Wolbachia*

Wolbachia has been suggested as a potential tool for the development of novel, environmentally friendly, biotechnological strategies for the control of arthropod species that are major agricultural pests or disease vectors to humans, plants, and livestock or for the improvement of beneficial species (Beard *et al.* 1993a; Bourtzis and O'Neill, 1998; Bourtzis and Braig, 1999). Below are the potential applications for CI-inducing strains of *Wolbachia*.

First, *Wolbachia*-induced CI might be used to suppress natural populations of arthropod pests in a way analogous to Sterile Insect Technique (S.I.T.). S.I.T. technology involves the mass production and release of irradiated sterile male insects and is the current strategy used for the control of certain insect agricultural pests. One of the limitations of the S.I.T. programs is the competitiveness of released males. Radiation doses commonly used to sterilize males introduce secondary deleterious effects that reduce the fitness of these males. CI provides an alternative method to produce non-irradiated "sterile" males and as such reduces the cost of a given S.I.T. program by increasing the competitiveness of released males and thereby reducing the numbers need to be released for effective control. CI has been used in the past to introduce sterility into wild populations of mosquitoes. Indeed, several trials, sponsored by the World Health Organization, were undertaken in the mid 1960s in Burma and India to eradicate the filariasis vector species *Culex pipiens* and *C. quinquefasciatus*. By mass rearing and then releasing males that were incompatible with the target population, it was possible to effectively sterilize wild females and in one field trial completely eradicate mosquitoes from a Burmese village (Laven, 1967). Also, in the 1970's an international collaborative project took place in Central Europe and used CI strategies to control the European cherry fruit fly, *Rhagoletis cerasi*. Several successful field trials were performed but for financial reasons this project was never completed (Blümel and Russ, 1989; Boller, 1989). In addition to these field experiments, a number of laboratory and warehouse experiments in the United States of America have successfully applied *Wolbachia*-induced CI as a means of genetic control of the stored product pest, the almond moth, *Cadra (Ephesia) cautella* (Brower, 1978; 1979; 1980; Kellen *et al.* 1981). However, in order to use CI as an effective method to produce "sterile" males, it has to be combined with an effective sexing system, since released females that also carry *Wolbachia* would be capable of successfully mating with released males (Laven, 1967). In the absence of such technology, CI could be used in conjunction with lower doses of radiation than are currently used and still achieve higher competitiveness of males and also sterilize the few females that escape conventional sexing systems. This strategy has been experimentally tested in the mosquito *Culex pipiens* (Curtis, 1976) and it has been shown that application of low radiation doses can generate sterile females and cytoplasmically incompatible males are equally competitive to non-irradiated males (Sharma *et al.* 1979; Arunachalam and Curtis, 1985; Shahid and Curtis, 1987).

Second, *Wolbachia*-induced CI might be used as a mechanism to spread desirable genotypes into wild arthropod populations. For example, current research projects aim to develop genetically modified arthropods that will not be capable to transmit pathogens to humans, plants and livestock (Curtis, 1994; Pettigrew and O'Neill, 1997;

Ashburner *et al.* 1998; O'Brochta and Atkinson, 1998). However, an important practical concern exists over the efficacy of a given transgene to spread where population replacement is required (Ashburner *et al.* 1998). *Wolbachia* infections can be used as a spreading means in order the genetically engineered arthropods to replace natural target populations. In an elegant study by Turelli and Hoffmann (1995), it was shown that the *Wolbachia*-infected *D. simulans* Riverside strain was spreading at a rate of approximately 100 km a year, replacing the uninfected population in the Central Valley of California. Similar spreading of *Wolbachia* infections have been reported in other species such as the small brown plant hopper *Laodelphax striatellus* and the moth *Cadra cautella* (Ahmed *et al.* 1984; Hoshizaki and Shimada, 1995; Hoshizaki, 1997). Bidirectional incompatibility and multiple infections provide further tools for repeated sweeps into target natural populations. Indeed, bidirectional incompatibility phenomena and double infections have been described in natural populations of several arthropod species (O'Neill and Karr, 1990; Rousset and Solignac, 1995; Werren *et al.* 1995b; Perrot-Minnot *et al.* 1996; Bordenstein and Werren, 1998; Merçot and Poinso, 1998b; Wenseleers *et al.* 1998; Zhou *et al.* 1998; Jeyaprakash and Hoy, 2000). In addition, double and triple infected strains have been artificially generated in the laboratory. These strains are stable, express high levels of CI and replace double, single and uninfected strains in experimental cage populations (Sinkins *et al.* 1995; Rousset *et al.* 1999). Moreover, the identification of the *Wolbachia* genes responsible for CI will allow the introduction of these genes into the host nuclear genome and the induction of CI without the presence of *Wolbachia*. Theoretical models suggest that nuclear-coded CI genes will spread their host replacing target naïve populations along with any other chromosomally linked gene(s) (Sinkins *et al.* 1997; Curtis and Sinkins, 1998).

Third, *Wolbachia* might be also used as an expression vector in para-transformation strategies. Para-transformation is the method that uses symbiotic bacteria as vehicles for the introduction and expression of genes of interest into a target arthropod species and has been suggested as an alternative approach for the genetic manipulation of arthropods (Beard *et al.* 1993a; Ashburner *et al.* 1998). The symbiotic bacteria of the assassin bug *Rhodnius prolixus* (actinomycetes *Rhodococcus rhodnii*) and of tse-tse flies (S-endosymbionts) have already been used as expression vehicles (Beard *et al.* 1992, 1993b; Durvasula *et al.* 1997; Cheng and Aksoy, 1999). Moreover, a para-transformation approach is currently being evaluated for field releases of *Rhodnius prolixus* aiming to reduce the prevalence of the causative agent of Chagas' disease, *Trypanosoma cruzi* (Durvasula *et al.* 1997). It has to be noted that both *Rhodococcus rhodnii* and S-endosymbionts can be cultured in a cell-free medium and their genetic transformation was easily achieved by using shuttle plasmid vectors (Beard *et al.* 1992, 1993b). As regards *Wolbachia*, which is an obligatory intracellular bacterium, both a cell-free culture and a genetic transformation system are still missing. The fact that these bacteria can now be maintained in different insect cell lines (O'Neill *et al.* 1997b; K.B. unpublished data) and the recent isolation and characterization of endogenous phages and insertion sequences (Masui *et al.* 1999) will certainly facilitate current efforts for the genetic engineering of *Wolbachia*. In addition, homologous recombination approaches were successfully used for the genetic manipulation of other intracellular bacteria such as *Rickettsia*, *Chlamydia* and *Coxiella* (Tam *et al.* 1994; Suhan *et al.* 1996; Rachek *et al.* 1998, 2000) and are currently being applied to

Wolbachia as well (K.B. unpublished data). In each case, the genetically manipulated *Wolbachia* need to be reintroduced into the target hosts and express the desired gene in a spatially and temporally correct manner and finally to replace their native counterparts. These goals can be easily achieved since *Wolbachia* have been detected in all major tissues and transferred by a variety of methods into different hosts where they induced CI (Boyle *et al.* 1993; Braig *et al.* 1994; Chang and Wade, 1994; Rousset and de Stordeur, 1994; Giordano *et al.* 1995; Rigaud and Juchault, 1995; Clancy and Hoffmann, 1997; Bouchon *et al.* 1998; Grenier *et al.* 1998; Poinot *et al.* 1998; Dobson *et al.* 1999).

Wolbachia-based applications may be of broad use since these bacteria are present in a wide range of arthropod species and can also be transferred into naïve hosts. Perhaps, the ability of these bacteria to establish new infections and persist into their hosts for long time may be related with their potential to “escape” the host’s innate immune system (Bourtzis *et al.* 2000). However, several important factors need to be considered since they may influence the strength of CI expression. These include male host age, repeated copulation (Bressac and Rousset, 1993; Karr *et al.* 1998), larval density and diapause (Sinkins *et al.* 1995; Perrot-Minnot *et al.* 1996; Clancy and Hoffmann 1998) and environmental factors such as temperature (Hoffmann *et al.* 1986, 1990; Snook *et al.* 2000), food quality and natural occurring antibiotics (Stevens and Wicklow, 1992).

6. *Wolbachia* genomics, proteomics and post-genomics studies

Molecular, biochemical, genetic and classical microbiological studies have been hampered in *Wolbachia* because of their fastidious unculturable nature. However, recent advances in genomics have allowed deciphering the biology of obligatory intracellular bacteria such as *Rickettsia* and *Buchnera* (Andersson *et al.* 1998; Shigenobu *et al.* 2000). A European *Wolbachia* Consortium has recently been established, consisting of eight laboratories from six countries, and co-ordinated by one of us (K.B.). The aim of this Consortium, funded by European Commission, is to identify *Wolbachia* and host genes involved in *Wolbachia*-arthropod symbiotic associations, including the *Wolbachia* genes responsible for the induction of CI, parthenogenesis and feminization, by using an integrated genomics, proteomics and post-genomics (microarrays and bioinformatics) approach. The Consortium also aims to develop a genetic transformation system for *Wolbachia* that will facilitate further functional studies and genetic manipulation of the bacterium for applied purposes. The genomics component of the project consists of the complete and annotated genome sequence of three *Wolbachia* strains, respectively responsible for the induction of CI (*wNo_{D.sim}* strain from *D. simulans*), parthenogenesis (*wUni_{M.uni}* strain from *Muscidifurax uniraptor*) and feminization (*wVul_{A.vul}* strain from *Armadillidium vulgare*). Currently, the genome of the *wNo_{D.sim}* strain is being sequenced. Genome analysis will be complemented by proteomics and microarrays of the host and *Wolbachia* comparing RNA and protein extracts from: a) infected versus uninfected host strains and b) inducing a reproductive phenotype versus non-inducing *Wolbachia* strains. The identification of the genes involved in host-*Wolbachia* interactions will be a major breakthrough in deciphering the biology of this unculturable bacterium, understanding *Wolbachia*-host symbiotic

associations and uncovering the evolution of intracellular symbiosis. In parallel with the European *Wolbachia* project, another initiative, funded by the National Institute of Health of USA and New England Biolabs in collaboration with the Yale University (Dr. Scott O'Neill's laboratory), is in progress aiming to sequence two *Wolbachia* strains at the "Institute for Genomic Research" (Rockville, USA). The first strain induces CI in *D. melanogaster* (wMel_{D.mel} strain) while the second is present in the filarial nematode *Brugia malayi*. Interestingly, phylogenetic analyses have suggested a mutualistic relationship between the bacteria and their nematode hosts that is also documented by antibiotic treatments (Bandi *et al.* 1998). Indeed, tetracycline treatments inhibit development in early stages and reduce worm fertility (Genchi *et al.* 1998; Langworthy *et al.* 2000). Recent studies also showed that an endotoxin or lipopolysaccharide (LPS) from *Wolbachia* is a major cause of inflammatory responses induced directly by the filarial nematode (Taylor and Hoerauf 1999; Taylor *et al.* 2000). Comparative genomics of *Wolbachia* is expected to identify potential drug targets for filariasis control. Also, comparing the genome of *Wolbachia* with that of *Rickettsia prowazekii* (Andersson *et al.* 1998) may result in the identification of factors that determine host specificity and virulence of these intracellular pathogens. It is also expected that comparing the genomes of several intracellular organisms such as *Wolbachia*, *Rickettsia*, *Buchnera* including mitochondria will help to unravel the molecular pathways for the establishment of intracellular symbiosis.

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8. References

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Annexe 2.

Article N°8. On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: confronting the models with the facts

Poinsot, D., Charlat, S. & Merçot, H. 2003.

BioEssays, sous presse

En bref..

La terminologie mod / resc permet de distinguer les versants mâles et femelles de l'IC : l'induction de la mortalité embryonnaire et le sauvetage des embryons. Cette terminologie est très générale et purement conceptuelle. Des modèles plus concrets ont été proposés dans la littérature, qui peuvent être classés en trois catégories : le modèle "ralentisseur", le modèle "titration-restitution" et le modèle "clef-serrure". Nous les confrontons ici aux différentes propriétés de l'IC (incompatibilité bi-directionnelle, multi-infections, phénotype [mod- resc+], etc.). Les modèles ralentisseurs et titration-restitution ne sont pas catégoriquement rejetés mais rencontrent des difficultés, rendant nécessaire un certain nombre d'hypothèses supplémentaires, telles que la fixation des produits bactériens sur une grande variété de cibles, ou l'extinction sexe-spécifique de leur expression. Au contraire, le modèle clef-serrure nous est apparu satisfaisant et parcimonieux.

On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: confronting the models with the facts

Denis Poinso¹* Sylvain Charlat,² and Hervé Merçot²

Summary

The endocellular bacterium *Wolbachia* manipulates the reproduction of its arthropod hosts for its own benefit by various means, the most widespread being cytoplasmic incompatibility (CI). To date, the molecular mechanism involved in CI has not been elucidated. We examine here three different CI models described in previous literature, namely, the “lock-and-key”, “titration–restitution” and “slow-motion” models. We confront them with the full range of CI patterns discovered so far, including the most complex ones such as multiple infections, asymmetrical and partial compatibility relationships and the existence of *Wolbachia* variants that can rescue the host from CI but not induce it. We conclude that the lock-and-key model is the most parsimonious of the models and fits the observations best. The two other models cannot be categorically invalidated, but they encounter some difficulties that make additional hypotheses necessary. *BioEssays* 25:1–7, 2003. © 2003 Wiley Periodicals, Inc.

Introduction

Cytoplasmic incompatibility (CI) is a reproductive incompatibility observed in many arthropod species, which is caused by the endocellular bacterium *Wolbachia* (α -proteobacteria: Rickettsiaceae) (reviewed in Refs. 1,2). In its simplest form, CI can be described as an embryonic mortality that occurs when uninfected females mate with *Wolbachia*-infected males. Infected females are fully fertile regardless of the infection status of the male. As a consequence, infected females have more offspring on average. This allows the maternally inherited bacterium to invade new host populations.

The means by which *Wolbachia* induce CI are currently unknown, however, there is a general consensus that

Wolbachia must somehow modify the sperm, since embryonic development aborts when sperm from an infected male fertilize an uninfected egg, due to the loss of improperly condensed paternal chromosomes (a notable exception is found in haplo-diploid arthropods species, where the loss of paternal chromosomes is not lethal but leads to haploid embryos that develop normally as males). This modification of the sperm must take place at an early stage of spermatogenesis because the bacteria are shed from maturing sperm and eliminated in cytoplasmic “waste-bags”.⁽³⁾ It is also known that such modified sperm will be fully functional if *Wolbachia* are present in the egg, which implies that some sort of “rescue” is performed by those *Wolbachia*. These ideas were formalized by Werren,⁽⁴⁾ through the “*mod resc*” (modification/rescue) model, which involves two functions: *mod* modifies sperm while *resc* takes place in the egg and restores paternal material functionality. This *mod resc* (or poison antidote) model is a useful general concept: there is no restriction with regard to the actual nature of the *mod* and *resc* functions. Three biochemical models have been proposed so far to translate *mod* and *resc* into more concrete factors: the “lock-and-key” model, the “titration–restitution” model and the “slow-motion” model. The aim of the present article is to test these different propositions by confronting them with several key observations from studies of CI.

The models

1. The “lock-and-key” hypothesis (Fig. 1)

Figure 1

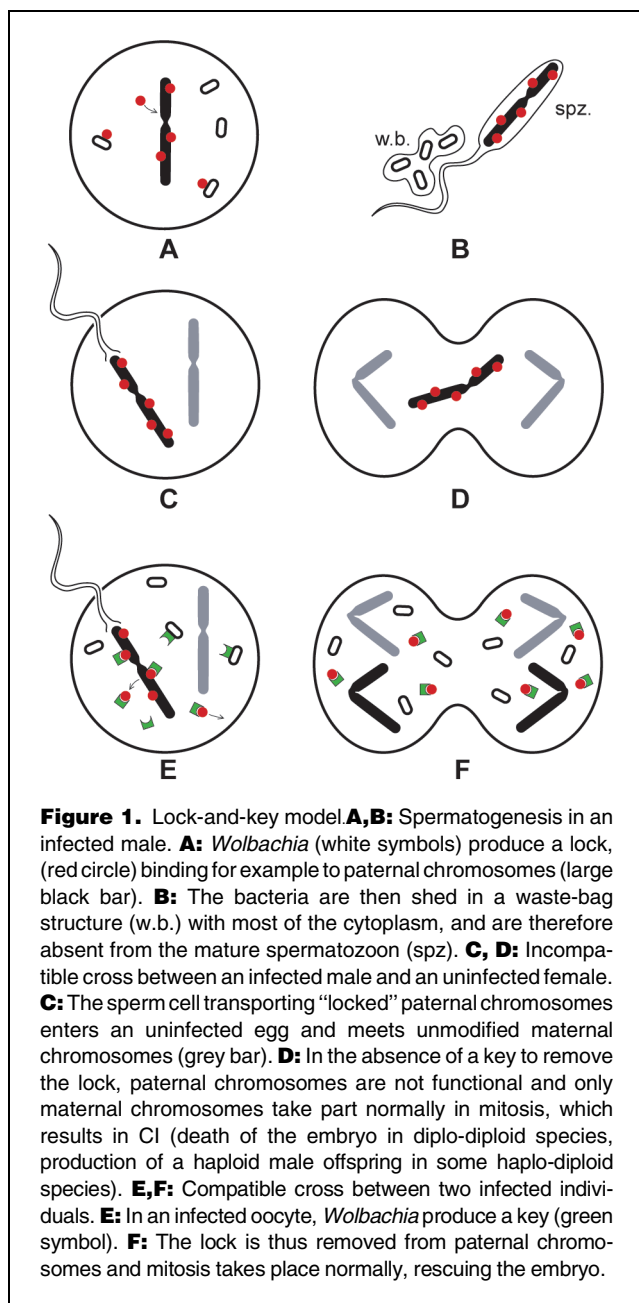
Following this model, the *mod* function is due to the production by the bacteria of a “lock” that binds to component of the paternal nucleus. Embryonic mortality occurs in crosses between infected males and uninfected females because the paternal material is “locked-in” and therefore unable to perform correctly. On the contrary, eggs infected by *Wolbachia* remain compatible with such modified sperm because the bacteria present in the egg produce a “key” that removes the lock (*resc* function). The two important features of this proposition are that (i) *mod* and *resc* do not result from the same molecular mechanism and are determined by different

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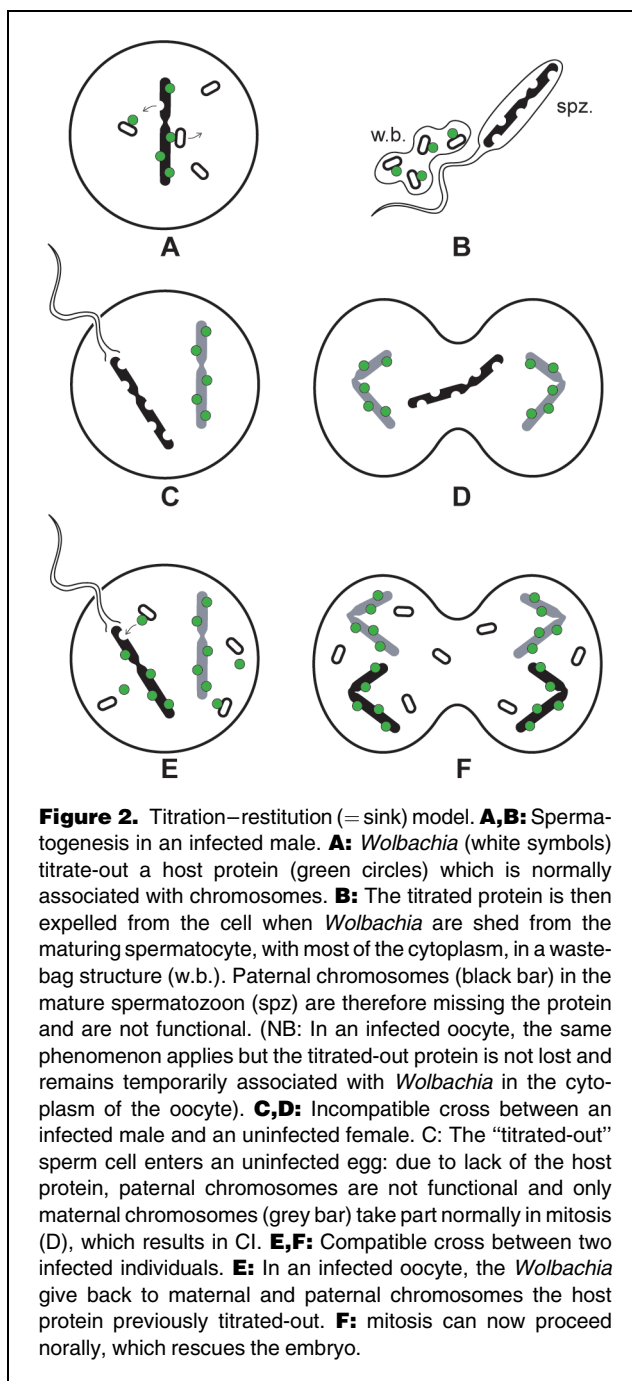


bacterial genes and (ii) *mod* penetrates the egg together with paternal chromosomes, allowing a direct physical interaction between *mod* and *resc* products. This model has been proposed in several papers,^(4–7) but molecular evidence for it is lacking so far.

2. The “sink” or “titration–restitution” hypothesis (Fig. 2)

Figure 2

Kose and Karr⁽⁸⁾ selected monoclonal antibodies raised against partially purified *Wolbachia* extracts. They first observ-



ed that, as expected, the anti-*Wolbachia* antibodies associated strongly with *Wolbachia* in infected *Drosophila simulans* eggs, and did not associate at all with condensed chromosomal DNA from the host. Yet, unexpectedly, the same antibodies produced a faint but reproducible signal in association with condensed chromosomes of the host if the egg was uninfected. Such a signal has also been observed on host DNA in *Drosophila simulans* spermatocytes (C. Lassy, H. Kose and T.L. Karr unpublished results quoted in Ref. 8) Furthermore,

anti-*Wolbachia* antibodies appeared to target histone-like host proteins.

Such observations led the authors to suggest that CI (that is, the *mod* function) might be due to *Wolbachia* removing some proteins normally associated with host chromosomes^(8,9) as previously suggested by Werren.⁽⁴⁾ Such titration would also occur in infected eggs prior to fertilization, as suggested by the absence of signal on host chromosomes in infected eggs. Presumably, the *Wolbachia* would give back the proteins to all chromosomes after fertilization (*resc* function). Under this view, *mod* and *resc* might be determined by the same gene(s) (the shift from titration to restitution after fertilization would then be triggered by host regulatory factors) or by different genes: one encoding a titrating factor, and the second encoding an inhibitor of titration, resulting in restitution.

3. The “slow-motion” hypothesis (Fig. 3)

Figure 3

Callaini et al.⁽¹⁰⁾ observed that, in incompatible *Drosophila* embryos, paternal chromosomes can condense and produce an anaphase-like aspect during the first mitosis, albeit *later* than maternal chromosomes do, suggesting that *mod* is merely delaying—and not completely blocking—the entry into mitosis. More recently,⁽¹¹⁾ Tram and Sullivan extended this observation to hymenopterans, and further showed that nuclear envelope breakdown, which marks the entry into mitosis, is also delayed for the paternal material.

These workers (see also Ref. 12) thus postulated that CI is due to *Wolbachia* altering the timing of the first mitosis and more specifically, that (i) *mod* is due to *Wolbachia* producing a factor that first binds to paternal chromosomes and then slows down their movements during the first embryonic mitosis, leading to unsynchronized paternal and maternal sets, and (ii) *resc* is caused by the similar modification of maternal chromosomes when *Wolbachia* are present in the egg, restoring a synchronous cycle between paternal and maternal complements.

The originality of this model is that the *resc* function is not caused by the removal of the slowing-down factor (otherwise we would be back to a subtype of the lock-and-key model) but to the production of the same factor in the egg. In other words, *mod* and *resc* result here from the same molecular mechanism and are determined by the same bacterial gene(s). Modified in the same fashion, the paternal and maternal set would be synchronized during the first mitosis. Although the delayed entry into mitosis of paternal material is now well supported, the “rescue” aspect of this model is still speculative: it remains to be demonstrated that maternal chromosomes movements are indeed slowed down when the egg is infected. In the present paper, when mentioning the “slow-motion” model, we will refer more particularly to the hypothesis that *mod* and *resc* indeed constitute a single slowing down factor.

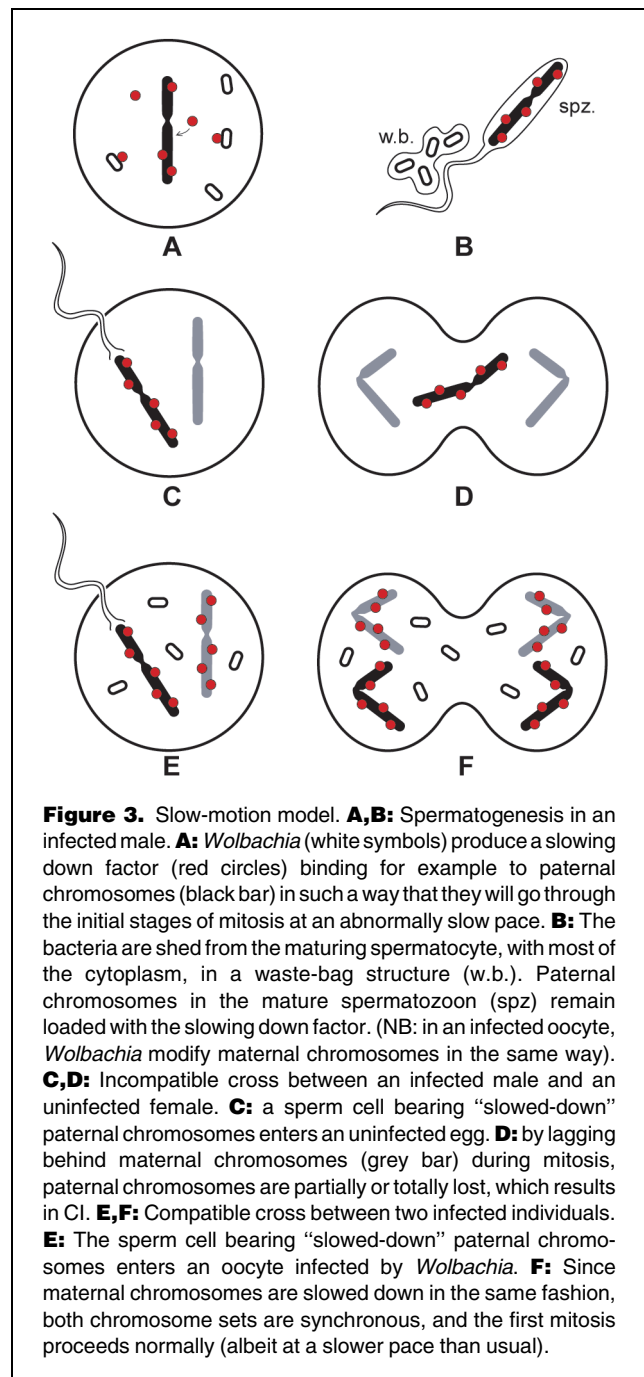


Figure 3. Slow-motion model. **A,B:** Spermatogenesis in an infected male. **A:** *Wolbachia* (white symbols) produce a slowing down factor (red circles) binding for example to paternal chromosomes (black bar) in such a way that they will go through the initial stages of mitosis at an abnormally slow pace. **B:** The bacteria are shed from the maturing spermatocyte, with most of the cytoplasm, in a waste-bag structure (w.b.). Paternal chromosomes in the mature spermatozoon (spz.) remain loaded with the slowing down factor. (NB: in an infected oocyte, *Wolbachia* modify maternal chromosomes in the same way). **C,D:** Incompatible cross between an infected male and an uninfected female. **C:** a sperm cell bearing “slowed-down” paternal chromosomes enters an uninfected egg. **D:** by lagging behind maternal chromosomes (grey bar) during mitosis, paternal chromosomes are partially or totally lost, which results in CI. **E,F:** Compatible cross between two infected individuals. **E:** The sperm cell bearing “slowed-down” paternal chromosomes enters an oocyte infected by *Wolbachia*. **F:** Since maternal chromosomes are slowed down in the same fashion, both chromosome sets are synchronous, and the first mitosis proceeds normally (albeit at a slower pace than usual).

Confronting the models with the facts

In this section, we present several important features of CI, each representing a test for the models described above. The basics of CI are as follows (i) when an infected male mates with an uninfected female, embryonic mortality occurs, but (ii) the very same male will be fully fertile if the female is infected by the same *Wolbachia*. Since the three CI models have been designed first to account for those two basic observations, they

can all explain them in a satisfactory manner. Accordingly we will not discuss those two features again. Yet, CI presents several other characteristics, which are not so easily explained by all models.

The resc function does not interfere with normal sperm cells

Eggs infected by one or several *Wolbachia* strains are fully compatible with sperm from uninfected males. No exception to this rule has been found so far.

1. Lock-and-key hypothesis. The “key” molecule present in the infected egg will not interfere with normal paternal material, since the key interacts only with its specific lock molecule.

2. Titration–restitution model. Implicitly, it is assumed that a normal paternal chromosomal set (carrying its usual load of associated proteins) will not be affected by *Wolbachia* making available more of these molecules upon its entry into the egg. We see no particular reason either to dismiss or to confirm this hypothesis.

3. Slow-motion hypothesis. Callaini et al.⁽¹⁰⁾ are aware that their model, without any additional hypothesis, would predict incompatibility between a normal sperm cell (paternal chromosomes with normal kinetics) and an infected egg (maternal chromosomes slowed-down by the bacterial factor). They postulate, therefore, that the amount of “slowing down factor” is sufficient in an infected egg to synchronize incoming normal chromosomes in step with the maternal set, when they write:⁽¹⁰⁾ “Presumably, the male chromatin recruits the *Wolbachia*-derived factor from the oocyte cytoplasm during replication of DNA. Maternal and paternal chromatin condensation are therefore coupled and the first mitotic division takes place successfully”. It remains to be established whether the first mitosis is systematically slower when the female is infected, as predicted by this model.

B. mod and resc interact in a specific manner

CI occurs when infected males mate with uninfected females. However, embryonic mortality is also observed when the two partners bear different *Wolbachia* strains.⁽¹³⁾ In such cases, CI occurs in both directions of cross, and is thus termed bi-directional (as opposed to uni-directional CI, occurring in crosses involving only one *Wolbachia* strain). Bi-directional CI demonstrates that *mod* and *resc* interact in a specific manner: a *Wolbachia* strain is only compatible with itself (some exceptions to this general rule are discussed below, see point 3).

1. Lock-and-key hypothesis. Allowance for bi-directional incompatibility is built into the basics of the lock-and-key

model. Indeed, one can envision the existence of a virtual infinity of possible lock/key combinations.

2. Titration–restitution hypothesis. A first hypothesis to explain bi-directional incompatibility between different *Wolbachia* variants would be that each variant titrates-out and restitutes a different DNA-binding host protein. Such an explanation might not allow for a very wide diversity of compatibility types. A second, more flexible, option would be that each *Wolbachia* has a specific “titration–restitution profile” among the proteins available on the host chromosomes. A small number of target molecules might then allow a larger number of different compatibility types.

3. Slow-motion hypothesis. This model would explain bi-directional incompatibility because paternal and maternal chromosomes affected by different slowing-down factors would be asynchronous. Two different hypotheses must be distinguished here. First, different slowing down factors might bind to the same sites on host chromosomes. Maternal and paternal factors would then compete to bind on paternal chromosomes. In some types of crosses, this would lead to maternal chromosomes being more delayed than paternal ones, which should result in the loss of maternal chromosomes. Yet, experiments using eye mutation markers in the hymenopteran genus *Nasonia* have shown that it is always the paternal set that is lost in both directions of cross.⁽⁵⁾ This hypothesis is thus unlikely, although this observation needs to be generalized to other *Wolbachia*/host associations. An alternative view is that the slowing down factors produced by different *Wolbachia* bind to different sites. Paternal chromosomes, already slowed down by the paternal factor, would be slowed down further by the factor of maternal origin upon its entry into the egg. Empirical observations would then fit the model's prediction: the loss of paternal chromosomes in all incompatible crosses. Thus, the slow-motion model requires that bi-directionally incompatible *Wolbachia* strains produce factors binding to different chromosomal sites, so that the effect of the slowing-down factor present in the egg cytoplasm can add to the effect of the factor produced in sperm.

C. Two Wolbachia variants can be partially and asymmetrically compatible

D. simulans females artificially transfected by the *Wolbachia* wMel, normally found in *D. melanogaster*, are partially capable of rescuing sperm of males infected with the *Wolbachia* wRi, naturally infecting *D. simulans* (i.e. only 25–30% of the embryos die) although males infected by wRi induce a nearly total embryo mortality (95–100%) when mated with uninfected females. In the reverse cross, wRi-infected females fully rescue sperm from males infected with wMel, although wMel-infected males induce total embryo mortality when mated with uninfected females.⁽¹⁴⁾ Thus, the *resc* function of wRi is fully

efficient against the *mod* function of *wRi*, which is trivial, but also against the *mod* function of *wMel*, which is quite unexpected, since the two variants are clearly distinct, based on two independent molecular markers.^(15,16) Likewise, one can conclude from partial compatibility in the reverse cross that the *resc* function of *wMel* is partially capable of rescuing embryos when faced with the *mod* function of *wRi*.

1. Lock-and-key hypothesis. To explain the above pattern, one could postulate that the locks of *wMel* and *wRi* are relatively similar. *wRi* would have a wider-spectrum key, allowing it to “open” both the *wRi* and *wMel* locks. On the other hand, *wMel* would have a more specific key, which would not be very good at opening the *wRi* lock, explaining the imperfect rescue in the other cross.

2. Titration–restitution hypothesis. The situation of *wRi* and *wMel* can be explained if the two variants remove and reconstitute the same host molecule, with *wRi* showing a higher affinity for it than *wMel*. In crosses between *wRi* males and *wMel* females, *Wolbachia* in the egg would not reconstitute enough host molecule to paternal chromosomes, resulting in partial embryonic mortality. On the contrary, in crosses between *wMel* males and *wRi* females, *Wolbachia* would reconstitute even more host molecule than necessary to paternal chromosomes, resulting in full rescue.

3. Slow-motion hypothesis. In this case, the *wRi/wMel* relationship can be explained if the slowing down factors produced by the two variants bind to the same sites on host chromosomes, with *wRi* showing a higher affinity for those sites than *wMel*. In crosses between *wRi* males and *wMel* females, *Wolbachia* in the egg would not be able to make maternal chromosomes as slow as paternal chromosomes resulting in partial embryonic mortality. On the contrary, in crosses between *wMel* males and *wRi* females, paternal chromosomes would be further slowed down by the maternal factor, resulting in full rescue.

D. Different mod functions do not exclude one another

Sperm from males infected simultaneously by two different CI-inducing *Wolbachia* will induce embryonic mortality if the eggs bear only one of the two *Wolbachia* variants.^(17–20) Moreover, cases of triple infections lead to similar conclusions: embryonic mortality occurs if females do not bear all the *Wolbachia* variants present in males.⁽²¹⁾ Therefore, a single sperm cell can bear the mark of two or three different *mod* functions simultaneously.

1. Lock-and-key hypothesis. Paternal nuclei bearing two different locks will remain impaired unless the *twocorresponding* keys are present in the egg.

2. Titration–restitution hypothesis. If each *Wolbachia* titrates-out and gives back a different (or a different spectrum of) DNA-binding protein(s), two different *Wolbachia* variants acting together in the same maturing sperm will lead to a new and unique pattern of titration for paternal DNA. Accordingly, this DNA will not be rescued in an egg bearing only one of the two *Wolbachia* variants.

3. Slow-motion hypothesis. If different slowing down factors act additively, that is, if they bind to different sites, paternal DNA affected by two *Wolbachia* will be more severely slowed-down than that modified by one of the two *Wolbachia* in the egg, leading to a failed mitosis.

E. Different resc functions do not exclude one another

Eggs infected simultaneously by two different and incompatible *Wolbachia* strains (say A and B) will not suffer from CI when fertilized by sperm cells from males infected by A, by B, or by A and B.^(17–20) This pattern holds true in the case of triple infections: females infected simultaneously by three *Wolbachia* are compatible with any male infected by one, two or three of these bacteria.⁽²¹⁾

1. Lock-and-key hypothesis. The *resc* function is due to the direct physical interaction between a key and its specific lock. There is, therefore, no reason why two different keys should exclude one another. If keys A and B are present simultaneously in the egg, any paternal nucleus locked by locks A, B or A and B will be rescued.

2. Titration–restitution hypothesis. If two or more *Wolbachia* differ in the (spectrum of) molecule(s) they titrate-out in spermatocytes and give back in the egg, *resc* functions are additive: in a bi-infected egg, each variant will give back two different sets of proteins and then restore compatibility with sperm missing partially (mono-infected male) or totally (bi-infected male) the molecules in question.

3. Slow-motion hypothesis. If the actions of different slowing down factors are additive, then an incoming paternal DNA bearing only factor A could be provided with factor B by the *Wolbachia* present in the egg, and be synchronized with maternal DNA affected by A and B.

F. mod and resc are functionally independent: the [mod- resc+] phenotype does exist

The *mod resc* notation⁽⁴⁾ allows CI *Wolbachia* to be classified in four theoretical phenotypic categories: (i) [*mod+* *resc+*], the “invasive” phenotype, where *Wolbachia* induces CI and rescues from it, (ii) [*mod–* *resc–*], the “helpless” phenotype, where *Wolbachia* is unable to induce CI nor to rescue from it, (iii) [*mod+* *resc–*], the “suicide” phenotype, where *Wolbachia*

is able to induce CI but unable to rescue its own effect and (iv) [*mod*− *resc*+], the “defensive” phenotype, where *Wolbachia* rescues CI from at least one type of *mod*+ variant, but is unable itself to induce CI. The [*mod*+ *resc*−] “suicide” phenotype has never been observed, but theory does not preclude its existence.^(22,23) On the contrary, the three other types have been found in the wild: [*mod*+ *resc*+],⁽²⁴⁾ [*mod*− *resc*−],⁽²⁵⁾ and [*mod*− *resc*+].^(7,26,27) The existence of this latter type demonstrates that *mod* and *resc* are functionally independent: *resc* can remain fully efficient while *mod* has been lost. How do the different models account for this finding?

1. Lock-and-key hypothesis. *mod* and *resc* are controlled by different genes. Thus, the emergence of *mod*− *resc*+ mutants can indeed be expected.

2. Titration–restitution hypothesis. One important aspect of this model is that the *resc* function is subordinated to a functional *mod*: obviously, *Wolbachia* can only give back host molecules that they are able to capture and store beforehand. Thus, *mod* must be functional prior to fertilization for *resc* to take place after fertilization. One possibility to circumvent this difficulty would be that in [*mod*− *resc*+] strains, *mod* (titration) is expressed in female hosts (allowing subsequent restitution) but not in males. Thus, the additional hypothesis of a sex-specific expression of *mod* is necessary.

3. Slow-motion hypothesis. This model postulates that *mod* and *resc* are determined by the same gene(s). The existence of the [*mod*− *resc*+] phenotype thus requires a similar additional hypothesis as above: the slowing down factor would be expressed in females, but not in males.

G. Different *mod resc* pairs have most probably evolved from a common ancestor

Although this point is not a demonstrated fact, both the number of different and bi-directionally incompatible variants and the existence of partially compatible variants make it the most parsimonious hypothesis. Let us consider an ancestral variant *mod*_A*resc*_A and how it could evolve into a new, bi-directionally incompatible variant *mod*_B*resc*_B.

1. Lock-and-key hypothesis. Since the lock-and-key hypothesis implies that *mod* and *resc* are controlled by different genetic determinants, the new *mod*_B*resc*_B could appear through an intermediate *mod*_B*resc*_A stage, following a process described in a recent theoretical paper.⁽²³⁾

2. Titration–restitution hypothesis. An intermediate *mod*_B*resc*_A type cannot occur here, because one can restore only what has been titrated. Therefore, any mutation from *mod*_A to *mod*_B also means that *resc*_A becomes *resc*_B. Such a new *mod*_B*resc*_B mutant would then face the very difficult task

of invading an incompatible *mod*_A*resc*_A population, where it would be strongly outnumbered and thus selected against.^(28,29) However, if we make the additional hypothesis that A and B types differ very slightly (weak bi-directional incompatibility), and if host populations are small, the *mod*_B*resc*_B type might get fixed by drift, just as any slightly deleterious mutation. The new variant would of course invade the population more easily if it was associated with positive effects on host physiology or if its rate of maternal transmission was higher than that of the previous variant.⁽³⁰⁾

3. Slow-motion hypothesis. Under this model, *mod* and *resc* are controlled by the same gene, so that the *mod*_B*resc*_A type cannot occur. The conditions for the emergence of new compatibility types from ancestral ones are thus as stringent as described above in the case of the titration–restitution model.

Conclusions

It follows from this comparative analysis of CI models that the titration–restitution and slow-motion models account for most observations, but require however the additional hypothesis that the [*mod*− *resc*+] phenotype is due to a sex-specific expression of bacterial genes. In addition, they would also seem to impose serious constraints on the evolution of new CI types. In contrast, the lock-and-key model accounts for all the facts known to date, and thus remains, in theory, the most parsimonious CI model currently available. Yet, it only requires a tiny but solid fact to bring down the nicest theoretical edifice, and given the accelerating pace of *Wolbachia* research, we might not have very long to wait for the solution of this half-century-old riddle.

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Annexe 3.

Article N°9 (manuscrit en préparation). *Wolbachia* transfer from a true fruit fly into the real fruit fly: investigating the outcomes of host / symbiont co-evolution.

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En bref...

*Chez *Rhagoletis cerasi*, la mouche du cerisier, deux variants bactériens ont été décrits: wCer1 et wCer2. Certaines populations présentent les deux variants (les individus sont dits bi-infectés), d'autres ne portent que wCer1 (les individus sont mono-infectés). En revanche, l'absence d'infection, ou la présence de wCer2 en mono-infection n'est jamais observée. Sur la base de croisements entre mâles de populations bi-infectées et femelles de populations mono-infectées par wCer1, il apparaît que wCer2 induit une forte IC. En revanche, les effets phénotypiques de wCer1 sont inconnus, et difficiles à étudier dans cette espèce au temps de génération démesuré. De plus, l'absence d'individus non infectés empêche la réalisation des croisements nécessaires. Nous décrivons ici les résultats d'une expérience d'injection cytoplasmique de *Rhagoletis cerasi* vers l'espèce modèle *Drosophila simulans*. Cette expérience a été réalisée dans deux buts immédiats: (i) séparer les deux variants bactériens, en vue de déterminer les effets phénotypiques de wCer1, (ii) tester l'hypothèse selon laquelle des associations *Wolbachia* / hôtes récentes (et à fortiori, les associations artificielles) sont caractérisées par une intensité de mod élevée, une transmission maternelle peu efficace, et un coût métabolique de l'infection. Malgré le succès de l'injection elle-même, le variant wCer1 s'est révélé incapable de se maintenir dans ce nouvel hôte. Au contraire, des lignées infectées par wCer2 ont pu être établies. En accord avec la prédiction, les taux de transmission observés sont extrêmement faible (environ 50%) et l'infection présente des effets négatifs sur la valeur sélective de l'hôte. En revanche, l'intensité de mod observée est extrêmement réduite (environ 40%). Sur la base de nos estimations, il apparaît clairement que ni wCer1 ni wCer2 ne pourraient être maintenu dans *Drosophila simulans* à l'état naturel. Ce résultat suggère que les transferts horizontaux entre espèces éloignées sont parfois impossibles, même si des vecteurs permettent de franchir efficacement la "barrière écologique".*

**WOLBACHIA TRANSFER FROM A TRUE FRUIT FLY INTO THE REAL FRUIT
FLY: INVESTIGATING THE OUTCOMES OF HOST/SYMBIONT CO-EVOLUTION**

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ABSTRACT

Wolbachia is an endosymbiont of diverse arthropod lineages, that can induce various alterations of hosts reproduction for its own benefice. Cytoplasmic incompatibly (CI) is the most common phenomenon. It results in embryonic lethality in crosses between males that bear *Wolbachia* and females that do not. In the cherry fruit fly *Rhagoletis cerasi*, *Wolbachia* seems to be responsible for previously reported patterns of incompatibility between populations. Here we report on the artificial transfer of two *Wolbachia* variants (*wCer1* and *wCer2*) from *R. cerasi* into *Drosophila simulans*, that was performed with two majors goals. First, separating *wCer1* & 2 in order to test their respective abilities to induce CI. Second, testing the theoretical prediction that recent *Wolbachia*/host associations should be characterized by high levels of CI, fitness costs to the new host and inefficient transmission from mothers to offspring. *wCer1* was unable to develop in the new host, resulting in its rapid loss after successful injection. *wCer2* was established in the new host. Transmission rates were low and the infection showed negative fitness effect, consistent with prediction, but CI levels were unexpectedly lower in the new host. Based on these parameter estimates, neither *wCer1* nor *wCer2* could be naturally maintained in *D. simulans*. The experiment thus suggests that natural *Wolbachia* transfer between species might be restricted by many factors, should the ecological barriers being bypassed.

Wolbachia is a maternally inherited α -proteobacteria and symbiont of arthropods (reviewed in O'Neill *et al.* 1997; Werren 1997; Bourtzis & O'Neil 1998; Stouthamer *et al.* 1999). This bacterium has an intracellular lifestyle and infections occur throughout host somatic and germ line tissues of insect species (Dobson *et al.* 1999). As a reproductive parasite it manipulates host reproduction and favours thereby its own dispersal in host populations. The most common *Wolbachia* effect described so far is cytoplasmic incompatibility (CI) (reviewed in Hoffmann & Turelli 1997; Charlat *et al.* 2002a). CI arises when infected males mate with uninfected females and results in embryonic lethality. Reciprocal crosses between infected females and uninfected males do not express CI. This pattern can be interpreted through a two functions model (Werren 1997): *Wolbachia* would somehow modify the sperm of infected males during spermatogenesis (modification, or *mod* function), leading to embryo death unless *Wolbachia* is present in the egg and restores viability (rescue, or *resc* function). The *mod* and *resc* functions seem to interact in a specific manner, because CI can be observed in crosses between males and females that are both infected, if the two partners bear different *Wolbachia* variants.

CI allows *Wolbachia* to invade host populations because it reduces the fitness of uninfected females relative to that of infected ones. Both theoretical and empirical studies (Caspari & Watson 1959; Fine 1978; Hoffmann *et al.* 1990; Turelli & Hoffmann 1995) have highlighted the key role of three parameters in the invasion dynamics: (i) CI level (the percentage of embryo killed by CI in incompatible crosses), (ii) the fitness effect of infection on female hosts (apart from CI) and (iii) the bacterial transmission efficiency from mothers to offspring. The above studies showed that the frequency of infected individuals presents a stable equilibrium depending on these three parameters, which is fixation only if maternal transmission is perfect and/or if CI level is 100%. The infection frequency reaches this stable equilibrium value only if it first passes a threshold frequency, which level also depends upon these three parameters.

CI is known for a variety of insect species, among which is the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). Early studies demonstrated high levels of incompatibility between populations (Boller & Bush 1974; Boller *et al.* 1976). The involvement of *Wolbachia* was shown more recently (Riegler & Stauffer 2002). Populations are either infected by a single *Wolbachia* variant, *wCer1*, or superinfected by two variants, *wCer1&2*, and incompatibility occurs between males from doubly infected populations and females from singly infected populations, suggesting the *wCer2* infection as the cause of CI (Riegler & Stauffer 2002). But the picture is not perfectly clear. First, although it is likely that

wCer1 once invaded the species through CI, the ability of this variant to induce CI cannot be tested, because populations lacking wCer1 have never been found. Second, the direct demonstration that wCer2 is responsible for CI has not yet been provided by a set of replicate crosses with individuals of known infection status. The establishment of standardised infected and uninfected laboratory lines is time consuming and not straightforward, given the long generation time and specialised biology of *R. cerasi*.

In this paper we report on the artificial transfer of *Wolbachia* from the true fruitfly *R. cerasi* into the real fruit fly, *Drosophila simulans* (Diptera Drosophilidae), an extensively studied *Wolbachia* host (reviewed in Merçot & Charlat 2003). This experiment was done with two major goals in mind. First, obtaining lines singly infected by wCer1 and wCer2, in order to test their ability to induce CI. Cytoplasmic injections have indeed proved to be an efficient technique for stimulating *Wolbachia* segregation (Charlat *et al.* 2002b). The second objective of this study was to test prediction regarding the consequences of *Wolbachia*/host coevolution on the three key parameters: maternal transmission efficiency, fitness effects and CI levels. Selection on host factors tends to increase the efficiency of maternal transmission and to decrease CI levels and fitness costs (Turelli 1994). Selection on bacterial factors tends to increase the efficiency of maternal transmission, to decrease fitness costs, while CI levels are neutral (Prout 1994; Turelli 1994). Coevolution is thus expected to lead to high transmission rates, low fitness costs and low levels of CI. Reciprocally, injection into a new host should lead to low transmission efficiency, negative fitness effects and high CI levels. The results presented are partially in agreement with these predictions. Indeed, a fitness cost to the host and low transmission efficiency are observed, as expected, but the level of CI is clearly reduced.

MATERIAL AND METHODS

***R. cerasi* and *D. simulans* lines**

Larvae of *R. cerasi* were collected from a wCer1&2 infected population on *Lonicera xylosteum* in Vienna, Austria, in 1999. After pupation, puparia were stored under the optimal conditions evaluated by Vallo *et al.* (1976). Emerging flies were kept in cages with water, adult diet and artificial egg laying devices according to Boller (1985).

D. simulans STC was used as a recipient for the *Wolbachia* from *R. cerasi*. STC is an inbred stock from the Seychelles archipelago, originally infected by two *Wolbachia*, *wHa* and *wNo*, cured from infection following a tetracycline treatment (Poinsot *et al.* 2000).

***Wolbachia* injection & line establishment**

The transfer of *wCer1* and *wCer2* into the *D. simulans* STC strain was performed by cytoplasmic injection (modified from Santamaria 1987). Using a micro-needle (Femtotips Eppendorf), cytoplasm was taken out from *R. cerasi* eggs and injected in the posterior part of recipient eggs. Donor eggs were obtained by dissection, directly from ovaries, allowing to get fresh and weakly differentiated embryos. Fresh receiver eggs were collected from the egg laying plates every hour. Recipient eggs were dechorionated manually prior to injection.

D. simulans females developing from injected eggs represent the generation 0 (G0). Each G0 female was crossed with one G0 male and was left for laying before its infection status was determined by PCR. The infection status of the offspring was determined by PCR on a mass extraction of 3 G1 females. In lines where infection was detected in G1, 10 G1 sisters were mated to their brothers, and left to lay separately before their infection status was determined.

During the experiment, all lines were maintained at 25°C at low larval competition in vials with axenic medium (David 1962). Rates of transmission from mothers to offspring came out to be low in transinfected lines, imposing stringent conditions for infection maintenance. Thus, at every generation, and for every transinfected line, six females were left to lay independently before their infection status was determined. Next generation was then started using offspring from infected females only.

Cytoplasmic Incompatibility tests

Individual crosses were done with 3 days old virgin males and 4-5 days old virgin females. Each cross was initiated by placing one male and one female in a vial with axenic medium. Copulation was monitored, allowing to discard those pairs where it lasted less than 15 minutes, to insure that sperm was actually transferred. The male was then removed and the female was supplied with an egg laying plate for 48 hours. Upon removal of the female, the eggs were placed at 25°C for 24 hours before egg hatch was measured by counting all eggs.

Laying plates with less than 20 eggs were discarded. All individuals from infected strains were checked by PCR for the presence of *Wolbachia*.

Maternal transmission rates

Maternal transmission was first roughly estimated as the proportion of infected female daughters from infected mothers during the line establishment, up to G10. The proportion of infected males was similarly assessed in G8, G9 and G10. If CI occurs, this infection rate is an overestimate of the actual transmission rate: CI will increase the proportion of infected adults, because uninfected eggs tend to abort. The actual maternal transmission rate of two lines was thus estimated after crossing infected females with uninfected males in G20.

Fitness effects measurements

Female fertility and fecundity were taken as parameters for the fitness effects of infections. These were investigated during CI assays experiments, and therefore using the same mating protocol. For fertility assays, uninfected males were crossed to infected and uninfected females and hatching rates were compared. For fecundity assays, infected and uninfected males were crossed with infected and uninfected females. Fecundity was estimated by the number of eggs laid per females in 48 hours.

PCR-RFLP and digestion

DNA was extracted from flies according to the STE method used by O'Neill *et al.* (1992). PCR primers used were 81F – 691R (Zhou *et al.* 1998) as well as *wCer1* and *wCer2* specific *wsp* primer pairs (Riegler & Stauffer 2002), *ftsZf1* – *ftsZr1* (Werren *et al.* 1995) and *16S* specific primer for *Wolbachia* (O'Neill *et al.* 1992). PCR reactions were done in reaction volumes of 12.5µl for the infection screening or in 50µl for post PCR procedures: 1µl or 4µl template DNA, 1x reaction buffer, 0.2mM dNTPs, 0.2µM forward and reverse primers, 0.5U or 2U of *Taq* DNA polymerase (Gibco) and sterile water was added to the final volume. PCR was run under conditions described by Zhou *et al.* (1998). *wsp* and *ftsZ* PCR products from *wCer1* infected *R. cerasi*, *wCer2* infected *D. simulans* and *wAu* of *D. simulans* were cycle sequenced using Big Dye (Perkin Elmer). Sequence products were loaded on an automatic

sequencer ABI 310. *wsp* PCR products of *wCer2* were digested with *Fnu4HI* (NEB) under the standard conditions recommended by the restriction enzyme provider.

Statistical analysis

CI and fertility data were analyzed with non parametrical tests (Wilcoxon). Fecundity data was analyzed by ANOVA.

RESULTS

Line establishment

1036 embryos of the uninfected *D. simulans* *STC* line were injected with cytoplasm of *wCer1&2* infected *R. cerasi*. From these, 82 developed into adult females, 51 of which were infected. The different infection types were *wCer1&2* (n = 31), *wCer2* (n = 12) and *wCer1* (n = 8). Thus, segregation between *wCer1* and *wCer2* already occurred after injection into generation 0 (G0). Transmission of *wCer1* and/or *wCer2* from G0 to G1 was found in 18 females. From these G0 females about 10 daughters were taken for line establishment. Only 3 out of 187 G1 females were superinfected with *wCer1&2*, 38 infected with *wCer2* and 8 with *wCer1*. *wCer1* was lost from all lines between G1 and G2, despite important efforts to detect rare infected G2 females. In G6, 6 isofemale lines remained infected by *wCer2*: RC20, RC21, RC33, RC45, RC50 and RC78. The six lines were from six different G0 females injected with *wCer1&2* cytoplasm. Uninfected lines RC20 \emptyset , RC21 \emptyset , RC33 \emptyset , RC45 \emptyset , RC50 \emptyset and RC78 \emptyset were founded using uninfected G1 females, sisters of the infected females used for the establishment of the infected lines.

Transmission rates

The infection rates in offspring from *wCer2* mothers were measured during the line establishment from generations 1 to 10, giving the following estimates: 54% in RC20 (n = 30, 95% confidence interval: 36.2%-71.8%), 61% in RC21 (n = 102, 95% CI: 51.5%-70.5%), 65% in RC33 (n = 50, 95% CI: 51.8%-78.2%), 80% in RC45 (n = 129, 95% CI: 73.1%-86.9%), 52% in RC50 (n = 43, 95% CI: 37%-66.9%), 86% in RC78 (n = 33, 95% CI: 74.2%-97.8%). These infection rates are overestimates of the maternal transmission rate as the

infection status of fathers was not checked. Indeed, the proportion of infected individuals could be greater in crossings between infected females and infected males than between infected females and uninfected males as CI selects for higher infection rates in the adult offspring.

The actual maternal transmission rates in RC21 and RC45 were estimated in G20 by crossing infected females with uninfected males. The transmission rates were 77% for RC21 ($n = 60$, 95% CI: 66.3%-87.6%) and 55% for RC45 ($n = 71$, 95% CI: 43.4%-66.6%).

CI assays

The expression of CI was tested by crossing uninfected females with infected and uninfected males. CI is observed if embryonic mortality is significantly higher when males are infected. This was investigated using four infected lines (RC21, RC45, RC33 and RC50) and their uninfected counterparts (RC21 \emptyset , RC45 \emptyset , RC33 \emptyset and RC50 \emptyset). As visible in Table 1, *wCer2* was found to induce CI in 8 experiments out of 10, although at a low level.

The ability of *wCer2* to rescue its own CI expression was tested by crossing infected males with infected and uninfected females. Rescue is observed if embryonic mortality is significantly lower when females are infected. This was investigated using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21 \emptyset and RC45 \emptyset). As visible in Table 2, a significant rescue was found in both experiments.

To test if this rescue was complete, infected females were crossed with infected and uninfected males. Rescue can be considered as complete if embryonic mortality is not significantly higher when males are infected. This was investigated using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21 \emptyset and RC45 \emptyset). As visible in Table 3, rescue was not found complete in the experiment involving the RC45 and RC45 \emptyset lines, while *P* was found just above the 5% threshold in the experiment involving RC21 and RC21 \emptyset . Thus, the data suggests that *wCer2* does not fully rescue its own CI. As discussed below, the imperfect transmission is likely to be the explanation.

Table 1.Does *wCer2* induce CI in *D. simulans*?

Gen	Male (Wolb)	Female (Wolb)	Nc	Ne	EM (%)	SE (%)	W	P
G8	RC21 (wCer2)	RC21∅ (∅)	15	1613	29.2	4.5		
G8	RC21∅ (∅)	RC21∅ (∅)	10	1093	11.7	5.9	2.607	< 0.01
G9	RC21 (wCer2)	RC21∅ (∅)	4	491	40.5	9.2		
G9	RC21∅ (∅)	RC21∅ (∅)	8	791	14.9	3.7	2.378	< 0.02
G10	RC21 (wCer2)	RC21∅ (∅)	9	1011	33.1	4.6		
G10	RC21∅ (∅)	RC21∅ (∅)	8	849	13.5	3.6	2.887	< 0.01
G8	RC33 (wCer2)	RC33∅ (∅)	5	584	14.7	4.5		
G8	RC33∅ (∅)	RC33∅ (∅)	6	593	13.9	4.3	0.183	<0.86
G10	RC33 (wCer2)	RC33∅ (∅)	14	1465	13.6	2.6		
G10	RC33∅ (∅)	RC33∅ (∅)	8	722	4.8	1.3	2.355	< 0.02
G8	RC45 (wCer2)	RC45∅ (∅)	19	1896	36.5	4.2		
G8	RC45∅ (∅)	RC45∅ (∅)	8	717	24.5	9.3	1.540	<0.13
G9	RC45 (wCer2)	RC45∅ (∅)	9	1077	64.6	9.5		
G9	RC45∅ (∅)	RC45∅ (∅)	8	920	11.9	8.3	2.983	< 0.01
G10	RC45 (wCer2)	RC45∅ (∅)	9	1061	44.8	9.9		
G10	RC45∅ (∅)	RC45∅ (∅)	8	819	5.8	1.3	3.464	< 0.001
G8	RC50 (wCer2)	RC50∅ (∅)	9	784	43.2	8.2		
G8	RC50∅ (∅)	RC50∅ (∅)	7	608	14.1	2.8	2.699	< 0.01
G10	RC50 (wCer2)	RC50∅ (∅)	4	289	33.8	8.9		
G10	RC50∅ (∅)	RC50∅ (∅)	5	528	6.8	1.3	2.449	< 0.02

Abbreviations: Gen (Generation following injection), Wolb (Wolbachia; ∅: uninfected), N crosses (number of crosses performed), N eggs (number of eggs counted), EM (mean embryonic mortality), SE (standard error), W (result of the Wilcoxon test), P (associated α probability). P is in bold when lower than 0.05. The Wilcoxon tests were performed by comparing each cross involving infected females with the corresponding control cross, where the female is not infected.

Table 2.Does *wCer2* rescue its own CI in *D. simulans*?

Male (Wolb)	Female (Wolb)	Nc	Ne	EM (%)	SE (%)	W	P
RC21 (wCer2)	RC21∅ (∅)	13	1502	35.4	3.9		
RC21 (wCer2)	RC21 (wCer2)	12	1249	22.8	3.0	2.393	< 0.02
RC45 (wCer2)	RC45∅ (∅)	18	2138	54.7	6.9		
RC45 (wCer2)	RC45 (wCer2)	20	1744	28.2	3.3	2.938	< 0.01

Same legend as Table 1. Data were pooled from two experiments (performed in G9 and G10), after testing for homogeneity.

Table 3.Does *wCer2* totally rescue its own CI in *D. simulans*?

Male (Wolb)	Female (Wolb)	Nc	Ne	EM (%)	SE (%)	W	P
RC21∅ (∅)	RC21 (wCer2)	13	1216	13.7	3.4		
RC21 (wCer2)	RC21 (wCer2)	12	1249	22.8	3.0	1.904	<0.06
RC45∅ (∅)	RC45 (wCer2)	15	1281	15.5	6.5		
RC45 (wCer2)	RC45 (wCer2)	20	1744	28.2	3.3	3.200	< 0.02

Same legend as Table 1. To increase sample size, data were pooled from two experiments (performed in G9 and G10), after testing for homogeneity. Crosses between infected males and infected females are the same as in Table 2.

Fitness effects

The effect of *wCer2* on female fertility can be tested by crossing uninfected males with infected and uninfected females. A positive or negative effect on fertility is detected if hatching rates differ in the two crosses. This was investigated using two infected lines (RC21 and RC45) and their uninfected counterparts (RC21Ø and RC45Ø). As visible in Table 4, *wCer2* was not found to affect female fertility.

Table 4.

Does *wCer2* affect female fertility in *D. simulans*?

Male (Wolb)	Female (Wolb)	Nc	Ne	Fertility (%)	SE (%)	W	P
RC21Ø (Ø)	RC21Ø (<i>wCer2</i>)	16	1640	85.8	2.4		
RC21Ø (Ø)	RC21 (<i>wCer2</i>)	13	1216	86.3	3.4	0.395	<0.7
RC45Ø (Ø)	RC45Ø (<i>wCer2</i>)	16	1739	91.2	4.0		
RC45Ø (Ø)	RC45 (<i>wCer2</i>)	15	1281	84.5	6.5	1.107	<0.27

Same legend as Table 1. To increase sample size, data were pooled from two experiments (performed in G9 and G10), after testing for homogeneity. Crosses between uninfected males and infected females are the same as in Table 3. Crosses between uninfected males and uninfected females are the same as in Table 1 (experiments D and E).

The effect of *wCer2* on female fecundity were tested by crossing infected and uninfected females with both infected and uninfected males (lines RC21 and RC45, RC21Ø and RC45Ø). The results, presented in Table 5, were analyzed by ANOVA (Table 6). In the experiment involving RC21 and RC21Ø, a surprising effect of male infection status was observed. Indeed, females appeared to lay significantly more eggs when mated with infected males. In this experiment, infected females were less fecund than uninfected ones, but this difference was not significant at the 0.05 threshold. In the experiment involving RC45 and RC45Ø, no effect of male infection was found. Again, infected females were less fecund than uninfected ones and here the difference was significant. The data thus suggest that *wCer2* reduces infected females fecundity.

Table 5.Does *wCer2* affect female fecundity in *D. simulans*? Descriptive statistics.

Male (Wolb)	Female (Wolb)	Nc	Ne	Fec	SE (%)
RC21Ø (Ø)	RC21Ø (Ø)	16	1640	102.5	4.7
RC21 (<i>wCer2</i>)	RC21Ø (Ø)	13	1502	115.5	5.9
RC21Ø (Ø)	RC21 (<i>wCer2</i>)	13	1216	93.54	7.0
RC21 (<i>wCer2</i>)	RC21 (<i>wCer2</i>)	12	1249	104.18	4.8
RC45Ø (<i>wCer2</i>)	RC45Ø (Ø)	16	1739	108.7	5.5
RC45 (<i>wCer2</i>)	RC45Ø (Ø)	18	2138	118.8	4.0
RC45Ø (<i>wCer2</i>)	RC45 (<i>wCer2</i>)	15	1281	85.4	8.1
RC45 (<i>wCer2</i>)	RC45 (<i>wCer2</i>)	20	1744	87.2	4.3

Same legend as Table 1. To increase sample size, data were pooled from two experiments (performed in G9 and G10), after testing for homogeneity. Abbreviations Fec (Fecundity)

Table 6Does *wCer2* affect female fecundity in *D. simulans*? ANOVAs**a. Lines RC21 and RC21Ø**

Source	df	Mean square	F	P
Male infection	1	1855.89	4.70	<0.04
Female infection	1	1390.95	3.52	<0.07
Male by Female	1	20.75	0.05	<0.82
Error	50	394.79		

b. Lines RC45 and RC45Ø

Source	df	Mean square	F	P
Male infection	1	602.32	1.27	<0.27
Female infection	1	12824.52	27.00	<10 ⁻⁴
Male by Female	1	292.81	0.62	0.44
Error	65	474.91		

Abbreviations: df (degree of freedom)

PCR-RFLP & sequencing

Sequenced *wsp* PCR products from single flies of RC21 and RC45 confirmed the presence of *wCer2* in these lines. *wCer2* and *wAu* differed in their *wsp* sequence by one mutation. This mutation site proved to be a restriction site and *wCer2* and *wAu* infections were differentiated by PCR-RFLP with *Fnu4H1*. *ftsZ* PCR products of *wCer2* infected *D. simulans* (GenBank accession number XXXXX), of *wAu* infected *D. simulans* Coffs Harbour (GenBank accession number XXXXX) and of *wCer1* infected *R. cerasi* (GenBank accession number XXXXX) were sequenced. *wCer2* and *wAu* shared the same *ftsZ* sequences, confirming

thereby their close genetic relationship. *wCer1* was more distantly related, and sequence divergences in *wsp* and *ftsZ* were similar.

DISCUSSION

Injection, segregation and infection loss

After injection from superinfected *R. cerasi* into *D. simulans*, *wCer1* and *wCer2* segregated in G0. In their original host, segregation of *wCer1* and *wCer2* was observed at a rate of less than 1% in field populations, whereby in all cases *wCer1* was the leaking variant (Riegler & Stauffer 2002). High segregation rates during injection most probably result from the low number of bacterial cells that are injected within a single recipient egg and actually survive.

Both *wCer1* and *wCer2* were still detectable by PCR in G1 following injection, suggesting that both variants reached the germ cells of G0 females. However, *wCer1* was lost from all lines between G1 and G2, suggesting that it was unable to develop properly in this new host, or to actively maintain itself in the germline. This loss was unfortunate, as it prevented us from determining the phenotypic effects of *wCer1*. But it was also an informative result. The incapacity of *wCer1* to develop in a new host might reflect a very tight and specific adaptation to the original host. This interpretation is consistent with the view that *wCer1* is in a more ancient infection in *R. cerasi* than is *wCer2*, as suggested by infection patterns in natural populations (Riegler & Stauffer 2002). On the contrary, *wCer2* was still present in G2. Although the efficiency of maternal transmission is low in *D. simulans*, imposing a stringent protocol for infection maintenance, we still possess, at the time of writing, the 6 lines deriving from 6 different G0 females.

CI levels, fitness effects and transmission efficiency

We found that *wCer2* can induce CI in *D. simulans*, although embryonic lethality is far from 100%. This confirms that *wCer2* is able to induce CI, and strengthens the view that it is responsible for the patterns of incompatibility observed between *R. cerasi* populations (Boller *et al.* 1976).

We observed that *wCer2* is able to rescue its own CI, but only partially so. This most probably results from maternal transmission being far from perfect: not all eggs are infected and thereby protected from CI. The transmission rates that would be necessary to explain the

imperfect rescue would be 57% for RC21 and 64% for RC45. Similar transmission rate values were observed for both lines at generation 20. Thus, it seems that *wCer2* is not strictly speaking self incompatible. Partial non-rescue is simply due to imperfect maternal transmission.

wCer2 does not affect female fertility, but seems to reduce female fecundity by at least 10%. Negative effects on host fitness have been reported previously in natural as well as artificial *Wolbachia*/host associations (Hoffmann *et al.* 1990; Clancy & Hoffmann 1997). Intriguingly, in one data set (involving lines RC21 and RC21 \emptyset), females were found to lay more when mated with infected males; a result which we fail to interpret in adaptive terms.

wCer1 was not transmitted after generation 1. *wCer2* had also a low transmission rate. This can be seen by the infection frequency observed during line maintenance, giving a mean value of 66% for the six trans-infected lines. Transmission efficiency *per se* was estimated at generation 20 in lines RC21 and RC45, giving a mean value 65.5%, which is much lower than any maternal transmission rate reported so far for natural *Wolbachia*/host associations.

Testing theory

Theory predicts that *Wolbachia*-host coevolution should lead to a decline of CI level and fitness costs, and to an increase of maternal transmission (Prout 1994; Turelli 1994). Inversely, strong CI, strong costs and low transmission rates are expected in new associations. Following Clancy & Hoffmann (1997), we tested this prediction by creating a new association and measuring parameters. As expected, fitness costs to the host and low transmission rates were observed. However, CI level was very low. We see two possible interpretations for this result.

First, *D. simulans* might actively repress the expression *wCer2*. This is plausible because *wCer2* is very closely related to *wAu*, a natural *Wolbachia* variant of *D. simulans*, that does not appear to induce CI in this host (Hoffmann *et al.* 1996; James & Ballard 2000; Reynolds & Hoffmann 2002; Charlat *et al.* 2003). Although *wAu* might have lost its ability to induce CI, regardless of the host background, a possibility remains that *D. simulans* actively and specifically represses its expression. This being so, *D. simulans* might recognise *wCer2* as *wAu*-like *Wolbachia* and thereby repress it.

Alternatively, the prediction that CI should be high in new associations might be incorrect. The expression of high CI might not be straightforward in any host background. Clancy and Hoffmann (1997) observed high levels of CI after transfer of *wRi* from

D. simulans into *Drosophila serata*. This could reflect the evolutionary closeness of *D. simulans* and *D. serata* rather than the ability of *Wolbachia* to express high CI in any background. In fact, high CI levels might not always be the sign of a recent *Wolbachia*/host association. Prout (1994) and Turelli (1994) demonstrated that within panmictic populations, bacterial variants inducing higher CI levels are not selected for, but Franck (1998) showed that if population is structured, bacterial variants inducing higher levels of CI are advantaged. Population structure might be sufficiently important for strong CI levels to be maintained in the long term.

The likelihood of horizontal transfers

From phylogenies of *Wolbachia* and their hosts, as well as direct observation, it is now clear that horizontal transfers between species can occur (Werren *et al.* 1995; Heath *et al.* 1999; Vavre *et al.* 1999; Huigens *et al.* 2000). *Wolbachia* in arthropods could be seen as a huge metapopulation with infected host species as habitats for various subpopulations (Charlat & Merçot 2000). Within host species, extinction and colonization might regularly occur through loss or gain of infection, and the current distribution of *Wolbachia* could represent a global and dynamic equilibrium between these two processes (Werren & Windsor 2000).

After the ideas of Combes (1995) it can be generalized that *Wolbachia* must cross three filters before it is established in a new host species: the ecological, the physiological and the population filter. The ecological filter is defined by the interaction between an existing and a potential new host species. It will condition the probability for *Wolbachia* of getting in contact with a new species, within an individual's body. The physiological filter is defined by the ability of *Wolbachia* to colonise the germline of an individual. Finally, the population filter conditions the ability of *Wolbachia* to invade and maintain itself in host populations, which depends on the values of the three main parameters: strength of CI, maternal transmission efficiency and fitness effects to the host (Hoffmann *et al.* 1990; Turelli & Hoffmann 1995).

Here, the ecological filter was bypassed as *Wolbachia* was intentionally injected into the new host. $wCer1$ and $wCer2$ were both established in the germ line. However, $wCer1$ was lost after the first generations, whereas $wCer2$ was maintained. The three parameters influencing *Wolbachia* invasion dynamics (CI level, transmission efficiency and fitness effects) were far from optimal. Based on formulas from Hoffmann *et al.*'s model (1990), and using the estimated parameter values, the only possible infection frequency at equilibrium for $wCer2$ is

0. In other words, should *wCer2* cross the ecological barriers by natural means, it would not be able to invade populations of *D. simulans*, nor would it be able to maintain itself starting from a high frequency. Our results thus suggest that horizontal transfers between evolutionarily distant species can be impossible. Within the *Wolbachia* metapopulation, sub-populations (i.e. *Wolbachia* variants) seem to be adapted to local habitats (species, or groups of closely related species). The population filter, the ability to invade host populations, might in fact be the most critical step, preventing *Wolbachia* from invading all arthropod species.

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Annexe 4.

Article N°10. *Wolbachia* segregation dynamics and levels of Cytoplasmic Incompatibility in *Drosophila sechellia*

Charlat, S., Bonnavion, P. & Merçot, H. 2003

Heredity, sous presse.

En bref...

Les deux variants bactériens présents chez Drosophila sechellia, wSh et wSn, sont respectivement proches des variants wHa et wNo, présents chez Drosophila simulans. D'autre part, les patrons d'infections dans les populations naturelles des deux espèces offrent un parallèle frappant: (i) chez D. sechellia, wSh peut être présent en mono-infection ou en bi-infection, alors que wSn n'est pas observé en mono-infection, (ii) chez D. simulans, wHa peut être présent en mono-infection ou en bi-infection, alors que wNo n'est pas (ou très rarement) observé en mono-infection. Des expériences antérieures ont montré que chez D. simulans, des lignées mono-infectées par wNo ou par wHa peuvent être obtenues par ségrégation et maintenues en laboratoire. Nous présentons ici une expérience similaire menée chez D. sechellia, montrant que des lignées mono-infectées par wSn ou par wSh peuvent être obtenues par ségrégation. Ces lignées nous permettent par ailleurs de tester les effets phénotypiques de wSh et wSn en mono-infection, et de les comparer aux effets des mêmes bactéries injectées chez D. simulans. Les résultats suggèrent que l'intensité de mod de wSh n'est pas influencée par le génome de l'hôte. Au contraire, l'intensité de mod exprimée par le variant wSn semble réprimée chez son hôte naturel.

Wolbachia segregation dynamics and levels of cytoplasmic incompatibility in *Drosophila sechellia*

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In *Drosophila sechellia*, the endocellular bacterium *Wolbachia* induces cytoplasmic incompatibility (CI): in crosses involving infected males, a partial or complete embryonic mortality occurs unless the female bears the same *Wolbachia* variant. *D. sechellia* is known to harbour two *Wolbachia* variants, namely *wSh* and *wSn*, closely related to *wHa* and *wNo*, respectively, two strains infecting the populations of *D. simulans* from the Seychelles archipelago and New Caledonia. Strikingly, the two species show similar infection patterns: in *D. sechellia*, *wSh* can be present on its own or in double infection with *wSn*, but individuals carrying *wSn* only do not occur; in *D. simulans*, *wHa* can be present on its own or in double infection with *wNo*, but individuals carrying *wNo* only do not occur, or occur at very low frequency.

Previous experiments on *D. simulans* showed that lines singly infected by *wNo* can be obtained by segregation, and stably maintained. Here we investigate this issue in *D. sechellia* through an 18 generation experiment, and show that *wSn* and *wSh* singly infected lines can arise by segregation. Using singly infected lines obtained in this experiment, we estimate the CI intensities of *wSh* and *wSn* in *D. sechellia*, and compare these to the CI intensities of the same *Wolbachia* injected into *D. simulans*. Our results do not suggest any consistent effect of the host species on the CI induced by *wSh*. On the contrary, it seems that *wSn* expression is repressed by host factors in *D. sechellia*. *Heredity* (2003) 00, 000–000. doi:10.1038/sj.hdy.6800211

Keywords: *Wolbachia*; cytoplasmic incompatibility; symbiosis; endocellular bacteria; genetic conflicts; *Drosophila*

Introduction

In *Drosophila sechellia*, as in many other Arthropod species, the endocellular bacterium *Wolbachia* induces an intriguing form of male sterility, known as cytoplasmic incompatibility (CI) (reviewed in Hoffmann and Turelli, 1997; Charlat *et al.*, 2002a). Males carrying the symbiont suffer a complete or partial loss of fertility when mated with uninfected females, but fertility is restored if the female harbours the same *Wolbachia* variant. Uninfected females thus suffer a fertility deficit relative to infected ones, the intensity of which depends on the frequency of infected males. The bacterium being transmitted by females only, through the egg cytoplasm, this highly deleterious effect on male hosts is not counter selected. On the contrary, infected females being fitter than uninfected ones, CI allows *Wolbachia* to invade uninfected host populations in a positive frequency-dependent manner and then maintain itself (Caspari and Watson, 1959; Turelli and Hoffmann, 1995). In fact, CI is only one of the various means by which *Wolbachia* manipulates its host's reproduction for its own benefit (O'Neill *et al.*, 1997; Stouthamer *et al.*, 1999). The molecular basis of CI is currently unknown, but there is some agreement that two bacterial functions at least are likely to be involved: *mod* (for modification) would be a sort of poison, affecting paternal chromosomes before *Wolbachia* is shed from maturing sperm and *resc* (for rescue) would

be a sort of antidote, saving the embryo from death when expressed in infected eggs (Werren, 1997; Poinot *et al.*, 2003).

D. sechellia is endemic from the Seychelles archipelago. It is known to carry two different *Wolbachia* strains: *wSh* and *wSn* (Rousset and Solignac, 1995; Bourtzis *et al.*, 1996). These two variants are distinguishable by several molecular markers (Rousset and Solignac, 1995; Werren *et al.*, 1995; Zhou *et al.*, 1998; Charlat *et al.*, 2002b). They belong to the two different main clades of Arthropods' *Wolbachia* (clades A and B), having diverged for about 60 million years. In laboratory strains, two different types of infected individuals can be found: those carrying *wSh* only (singly infected) and those carrying *wSh* and *wSn* (doubly infected). On the contrary individuals carrying *wSn* only are not observed (Rousset and Solignac, 1995).

D. simulans is very closely related to *D. sechellia* (Hey and Kliman, 1993; Kliman *et al.*, 2001). It is known to harbour several *Wolbachia* strains, among which are *wHa* and *wNo*, closely related to *wSh* and *wSn*, respectively (Rousset and Solignac, 1995; Werren *et al.*, 1995; Zhou *et al.*, 1998; Charlat *et al.*, 2002b). *wHa* and *wNo* infect the *D. simulans* populations from New Caledonia and the Seychelles archipelago, where the infection pattern strikingly parallels the situation of *D. sechellia*: some individuals harbour only *wHa*, while others carry both *wHa* and *wNo*, but individuals singly infected by *wNo* are absent or very rare (James *et al.*, 2002).

Experiments have shown that this pattern of infection in *D. simulans* is not because of the impossibility of *wNo* lines being obtained by segregation from double infection, and subsequently maintained (Poinot *et al.*, 2000). Here we address the same issue in *D. sechellia*, through

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an 18-generation experiment, based on a similar procedure, and show that *wSh* as well as *wSn* singly infected lines can be obtained by segregation and stably maintained. Using *D. sechellia* singly infected lines as well as *D. simulans* lines artificially infected by *wSh* and *wSn* (Charlat *et al*, 2002b), we estimate CI levels in these two species. Our results do not suggest any effect of the host species on the CI induced by *wSh*. By contrast, *wSn* expression seems to be repressed by host factors in *D. sechellia*.

Materials and methods

D. sechellia strains

S9 is a strain naturally infected by *wSh* (Bourtzis *et al*, 1996). *Dsech* is a strain infected by *wSh+wSn*, founded in the 1980s, originating from the Seychelles archipelago. *DsechTC* is an uninfected strain, obtained from *Dsech* by antibiotic treatment (Tetracycline).

Dse1 to *Dse14* are 14 lines used in the segregation experiment, each initiated using one single female from the *Dsech* strain. Among the singly infected lines obtained by segregation from these doubly infected lines (see below), three have been used in subsequent CI assays, which took place 30 generations after the end of the segregation experiment.

D. simulans strains

STC is an inbred uninfected strain obtained by antibiotic treatment (Tetracycline) from the *Seychelles* strain, naturally infected by *wHa+wNo*, derived from flies collected on Mahe island (Seychelles archipelago) in 1981.

ASh and *CSH* are isofemale lines, infected by *wSh*, obtained by cytoplasmic injection from *D. sechellia* into the *STC* strain (Charlat *et al*, 2002b). *ASn* and *BSn* are isofemale lines, infected by *wSn*, obtained by cytoplasmic injection from *D. sechellia* into the *STC* strain (Charlat *et al*, 2002b). CI assays involving *ASh*, *CSH*, *ASn* and *BSn* took place 40 generations after the injection into *D. simulans*.

Rearing conditions

In order to ensure optimal conditions for the maintenance of *Wolbachia* infections, host strains were maintained at 25°C, at low larval density, on axenic medium (David, 1962).

Segregation experiment

The present experiment followed a similar procedure to that of Poinot *et al* (2000). At generation G0, one female from each of the 14 doubly infected lines (*Dsech-1* to *Dsech-14*, infected by *wSh+wSn*) was crossed with two *DsechTC* males, before its infection status was checked by PCR. CI does not occur in such crosses, since males are not infected. Consequently, the hatching probabilities of the eggs laid by infected mothers do not depend on their infection status. From each of the 14 crosses, one G1 female was picked randomly and backcrossed with two *DsechTC* males, before its infection status was determined by PCR. A similar procedure was applied for 18 generations, allowing the total bacterial load as well as the proportion of *wSh* vs *wSn* to vary randomly.

Wolbachia detection and identification

The detection of *Wolbachia* and the distinction between the different *Wolbachia* variants were made by PCR. DNA was obtained according to O'Neill *et al* (1992) and the *wsp* gene was amplified according to Zhou *et al* (1998). Primer specificity allows distinguishing *wSh* (primer 178F and 691R) from *wSn* (primers 183F and 691R).

Measurement of embryonic mortality

Embryonic mortality was measured in *D. simulans* and *D. sechellia* using individual crossings between males aged 3–4 days and females aged 4–7 days. Mating was observed, and crosses where copulation lasted for less than 15 min were discarded. Inseminated females were individually placed at 25°C, on axenic medium, coloured with neutral red. *D. simulans* females were left to lay for 48 h and *D. sechellia* females for 96 h, owing to their lower fecundity. Laying boxes were left for an additional 24 h at 25°C so that all viable embryos could hatch, and finally placed at 4°C until egg counting. Embryonic mortality was then determined as the percentage of unhatched eggs. Laying boxes with less than 20 eggs were discarded. In *D. sechellia*, average egg number was 46.9, ranging from 20 to 96. In *D. simulans*, average egg number was 86, ranging from 31 to 142. For crosses showing 0% hatching, a fertility test was performed by crossing each parent with individuals harbouring a compatible infection. This procedure allows to distinguish between crosses where CI is 100% and crosses involving intrinsically sterile individuals. Crosses with one sterile parent were discarded. Finally, the infection status of parents was checked by PCR.

CI intensity

CI intensity (or CI level) is defined here as the percentage of embryos that do not hatch because of CI, in crosses between infected males and uninfected females. CI intensity experiments thus involve crosses between infected males and uninfected females (strain *DsechTC* in *D. sechellia*, strain *STC* in *D. simulans*). In each experiment, control crosses, involving uninfected males, were also performed, in order to determine the control cross mortality (CCM). This allows the calculation of a corrected CI (CI_{corr}), taking into account the embryonic mortality not caused by CI. If EM stands for the observed embryonic mortality, $CI_{corr} = (EM - CCM) / (1 - CCM)$ (Poinot *et al*, 1998).

Statistical analysis

All statistical tests were performed on SAS (1989, version 6.12) after root arcsine transformation.

Results

Segregation rates

The rate of segregation from doubly to singly infected cytoplasm was monitored through 18 generations, in conditions allowing the proportion of *wSh* and *wSn*, as well as the total bacterial load, to vary stochastically. The loss of *wSh* occurred in four lines out of 14 (*Dse5* at generation 4, *Dse6* and *Dse8* at generation 14, *Dse2* at generation 18). The loss of *wSn* occurred in only one line (*Dse14*, at generation 2). The complete loss of infection did not occur. At the end of the experiment, the

respective proportions of doubly infected, *wSh* and *wSn* lines were 64.3, 7.1 and 28.6%. In total, 230 crosses involved females still bearing *wSh*. Among these, *wSh* was lost in four crosses, making an overall loss rate of 1.7%. Similarly, *wSn* was lost in one cross out of 236 involving females still bearing this variant, making an overall loss rate of 0.42%. These two rates are not significantly different (Fisher's exact test: $P = 0.211$).

CI intensity in *D. sechellia* and *D. simulans*

The CI intensity of *wSh* was estimated in two *D. sechellia* lines: *S9* (naturally singly infected) and *Dse14-Sh* (obtained by segregation) and in two *D. simulans* lines: *ASh* and *CSh* (obtained by cytoplasmic injection (Charlat et al, 2002b)). The results are summarized in Table 1a. As expected, *wSh* was found to induce CI in all lines in both species: *t*-test comparisons with control crosses (Table 1c) are all significant, with $P < 0.001$ (not shown). The host effect was investigated by ANOVA (Table 2a). The *Line* factor was found significant ($P = 0.0028$). Indeed, important between-line variations occur in *D. simulans* ($CI_{\text{corr}} = 78.7$ and 50.0% for *ASh* and *CSh*, respectively), which are consistent with previous observations (Charlat et al, 2002b). Owing to this *Line* effect, the two species are not found to be significantly different, although the means are more than 20% apart ($CI_{\text{corr}} = 90\%$ in *D. sechellia* vs 67% in *D. simulans*). In other words, the between-species variation is not significantly higher than the between-line variation. Multiple comparisons, realised through the LSMEAN/TDIFF statement in SAS (1989), lead to similar conclusions: *S9* and *ASh*, although belonging to *D. sechellia* and *D. simulans*, respectively, do not differ significantly (not shown).

A similar experiment was carried out with *wSn*. CI intensity was estimated in two *D. sechellia* lines: *Dse5-Sn* and *Dse6-Sn* (obtained by segregation) and in two *D. simulans* lines: *ASn* and *BSn* (obtained by cytoplasmic injection (Charlat et al, 2002b)). The results are summarized in Table 1b. As expected, *wSn* was found to induce CI in all lines in both species: *t*-test comparisons with control crosses are all significant, with $P < 0.001$ (not

Table 2 CI intensity, ANOVAs

Source	df	Mean square	F	F denominator	Pr>F
(a) <i>wSh</i>					
Species	1	2.1308	4.10	Line	NS
Line	2	0.5211	6.40	Error	0.0028
Error	71	0.0814	—	—	—
(b) <i>wSn</i>					
Species	1	1.6577	25.15	Error	0.0001
Line	2	0.0524	0.80	Error	NS
Error	71	0.0659	—	—	—

Line is nested within *Species*.

shown). As shown in Table 2b, there does not seem to be any intraspecific variation in this case (*Line* factor nonsignificant). By contrast, the *Species* factor is found to be significant ($P = 0.0001$). Thus, *wSn* in *D. sechellia* induces a lower CI than in *D. simulans* (mean $CI_{\text{corr}} = 39\%$ vs 64%). Multiple comparisons lead to similar conclusions: within each species, the lines do not differ, but all interspecific comparisons are significant (not shown).

Discussion

Segregation rates

The rates of loss of *wSh* and *wSn* from initially doubly infected cytoplasm were followed through 18 generations, in conditions where the effect of CI (the selection of doubly infected embryos by doubly infected males) was relaxed. At every generation, infected females were backcrossed to uninfected males harbouring the same genomic background. The two types of singly infected cytoplasm were obtained and stably maintained. The transmission efficiencies of the two variants were not found to differ significantly. This experiment demonstrates that the absence of lines singly infected by *wSn* in

Table 1 CI intensity in *D. sechellia* and *D. simulans*: descriptive statistics

Host species	Male	Female	n crosses	n eggs	EM (%)	CI_{corr} (%)	SE (%)
(a) <i>wSh</i>							
<i>D. sechellia</i>	<i>Dse14-Sh</i> (<i>wSh</i>)	<i>DsechTC</i>	19	857	92.8	90.4	2.58
<i>D. sechellia</i>	<i>S9</i> (<i>wSh</i>)	<i>DsechTC</i>	17	713	90.4	87.3	3.89
<i>D. simulans</i>	<i>ASh</i> (<i>wSh</i>)	<i>STC</i>	23	1978	82.9	78.7	3.82
<i>D. simulans</i>	<i>CSh</i> (<i>wSh</i>)	<i>STC</i>	16	1230	59.5	50.0	7.67
(b) <i>wSn</i>							
<i>D. sechellia</i>	<i>Dse5-Sn</i> (<i>wSn</i>)	<i>DsechTC</i>	16	908	49.8	34.1	6.72
<i>D. sechellia</i>	<i>Dse6-Sn</i> (<i>wSn</i>)	<i>DsechTC</i>	23	1255	55.7	41.7	5.33
<i>D. simulans</i>	<i>ASn</i> (<i>wSn</i>)	<i>STC</i>	19	1831	70.4	63.0	3.77
<i>D. simulans</i>	<i>BSn</i> (<i>wSn</i>)	<i>STC</i>	17	1667	72.4	66.0	3.85
(c) Control crosses							
<i>D. sechellia</i>	<i>DsechTC</i> (uninfected)	<i>DsechTC</i>	28	1097	24.9	—	4.11
<i>D. simulans</i>	<i>STC</i> (uninfected)	<i>STC</i>	33	2589	19.5	—	2.37

EM: raw embryonic mortality, CI_{corr} : corrected CI, SE: Standard Error.

D. sechellia is not because of a perfect transmission of this variant, or to its lethality when present on its own. Interestingly, the complete loss of *Wolbachia* infection did not occur, suggesting that the total bacterial load did not decrease during the experiment. In other words, it seems that the emergence of singly infected cytoplasm was rather due here to stochastic variations in the proportions of the two variants than to a decrease of bacterial population size.

In a similar experiment realised in *D. simulans*, Poinsoit et al (2000) followed the segregation dynamics of *wHa* and *wNo* through 18 generations. Although the experiments were not performed together, a comparison can be made with the present results. The distribution of the various infection types at the end of the experiment with *D. simulans* can be considered: 33.3% doubly infected, 24.3% singly infected by *wHa*, 12.1% singly infected by *wNo* and 30.3% uninfected ($n=33$). These proportions contrast with those observed in *D. sechellia* (Fisher's exact test: $P=0.014$). In *D. simulans*, not only did singly infected lines of both types occur more frequently, but uninfected ones did as well. These results suggest that *wSh* and *wSn* in *D. sechellia* are more efficiently transmitted, than are *wHa* and *wNo* in *D. simulans*.

CI intensity in *D. sechellia*

The CI intensity of *wSh* and *wSn* was estimated in their natural host *D. sechellia*. Such estimations have been made previously for *wSh*, naturally present as a single infection (Rousset and Solignac, 1995; Bourtzis et al, 1996). In these two studies, the raw embryonic mortality in crosses between *wSh* males and uninfected females was, respectively, 71 and 70%. If one takes into account the control crossmortality (Poinsoit et al, 1998), the corrected CI levels are 62 and 66%, which is lower than what we observed (90.4% in *Dse14-Sh* and 87.3% in *S9*, the line used by Bourtzis et al, 1996). These differences are likely to be because of environmental factors, such as larval density, which are known to affect CI levels (Sinkins et al, 1995). Interestingly, we found that *wSh* expresses the same CI intensity in *Dse14-Sh* and *S9*, although the first line derives from the segregation experiment and the second is naturally singly infected. This is consistent with the view that natural singly infected lines derive from doubly infected ones through imperfect transmission.

Before our experiment, *D. sechellia* lines singly infected by *wSn* were not available. However, the CI intensity of *wSn* had been indirectly investigated by crossing doubly infected males with females bearing only *wSh* (Rousset and Solignac, 1995). In such crosses, the raw embryonic mortality was 35%. In this case, CI_{corr} is also 35% since the control crossmortality is 0%. This value is very close to the ones we observed in crosses involving males singly infected by *wSn* (34.1% in *Dse5-Sn* and 41.7% in *Dse6-Sn*), suggesting that the two variants in double infections do not affect each other (the intensity of one variant does not depend on the presence or absence of the other), consistent with several previous observations (Merçot et al, 1995; Rousset and Solignac, 1995; Rousset et al, 1999).

Based on our estimations of transmission efficiency and CI intensity, one can calculate the equilibrium frequencies expected under Hoffmann and Turelli's

model (1997). Assuming (i) that neither *wSh* nor *wSn* affect host fitness (apart from CI) and (ii) that the CI effects of the two strains are additive, the expected frequencies are the following: 99.17% doubly infected, 0.63% *wSh*, 0.19% *wSn* and 0.001% uninfected. Thus, *wSh* flies are expected to be three times more frequent than *wSn*, but both types of singly infected cytoplasm are expected to be rare. The very sparse data available on the frequencies of singly infected lines in the wild suggest that they might be more frequent (Rousset and Solignac, 1995). Two different factors might explain this discrepancy: our experimental conditions allow the expression of high CI levels and high transmission efficiency, both factors that increase the expected equilibrium frequencies of the double infection, and reduce the expected frequency of single infections. Gathering parameter estimates from the wild, together with additional data on the frequencies of the various infection types, might clarify this issue.

Evidence for a host effect

In order to investigate the possibility of host control on *wSh* and/or *wSn*, CI assays were realised in the foreign host *D. simulans*, using transinfected lines obtained by cytoplasmic injection (Charlat et al, 2002b).

Concerning *wSh*, the results do not suggest any variations between the two hosts. Within *D. simulans*, a significant difference was detected between lines. Previous results indicate that such differences do not remain stable over time, suggesting that they might be caused by uncontrolled environmental factors (Charlat et al, 2002b). Whatever the causal factor, this intraspecific variation in *D. simulans* lowers the statistical power of our comparison. Indeed, although the mean CI_{corr} in the two species is more than 20% apart, the difference is not significant. This highlights the importance of using more than one line to investigate such effects, in order to avoid erroneous conclusions.

On the contrary, *wSn* was shown to induce different CI levels in the two species. Consistent with previous observations, there does not seem to be any between-line variation for *wSn* within *D. simulans* (Charlat et al, 2002b). Thus, *wSn* expresses a higher CI in *D. simulans* than in its natural host (mean $CI_{corr}=39%$ in *D. sechellia* vs 64% in *D. simulans*). Overall, it appears that the differences between *D. simulans* and *D. sechellia* regarding the CI intensity of *wSn* and *wNo* are mainly because of host effects, and not to divergence between the two strains. Indeed, while the present results show that *wSn* induces a lower CI in *D. sechellia* than in *D. simulans*, previous experiments suggest that *wSn* and *wNo*, within *D. simulans*, induce similar levels of mortality (Charlat et al, 2002b). Under this view, one could predict that *wNo* and *wSn* should induce the same CI levels in *D. sechellia*. This remains to be investigated.

It is notable that the CI assays realised on *wSh* and *wSn* did not lead to identical conclusions regarding the host controls on CI levels: it appears that *wSn* only is repressed in *D. sechellia*. Indeed, if there might be a slight host effect for *wSh*, undetected in the present experiment, the tendency is rather that of a higher level of expression in *D. sechellia* than in *D. simulans*, suggesting that the host control observed here is *Wolbachia* strain-specific.

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Annexe 5.

Liste des publications.

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- Charlat, S., Calmet, C. & Merçot, H. 2001. On the *mod / resc* model, and the evolution of *Wolbachia* compatibility types. *Genetics*, **159**:1415-1422.
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Charlat, S., Calmet, C. & Merçot, H. Exploring the evolution of *Wolbachia* compatibility types: a simulation approach. **En préparation.**

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Riegler, M., Charlat, S., Stauffer, C. & Merçot, H. *Wolbachia* Transfer from a true fruit fly into the real fruit fly: investigating the outcomes of Host / symbiont co-evolution. **En preparation.**

Charlat, S., Riegler, M., Baures, I., Poinot, D., Stauffer, C. & Merçot, H. Incipient evolution of *Wolbachia* compatibility types. **En préparation.**

Charlat, S., Hurst, G.D.D. & Merçot, H. Evolutionary consequences of *Wolbachia* infections. **Soumis à *Trends in Genetics*.**