

Reproductive effects and geographical distributions of two *Wolbachia* strains infecting the Neotropical beetle, *Chelymorpha alternans* Boh. (Chrysomelidae, Cassidinae)

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Abstract

Wolbachia are maternally inherited endocellular bacteria known to alter insect host reproduction to facilitate their own transmission. Multiple *Wolbachia* infections are more common in tropical than temperate insects but few studies have investigated their dynamics in field populations. The beetle, *Chelymorpha alternans*, found throughout the Isthmus of Panama, is infected with two strains of *Wolbachia*, *wCalt1* (99.2% of beetles) and *wCalt2* (53%). Populations infected solely by the *wCalt1* strain were limited to western Pacific Panama, whereas populations outside this region were either polymorphic for single (*wCalt1*) and double infections (*wCalt1* + *wCalt2*) or consisted entirely of double infections. The *wCalt2* strain was not found as a single infection in the wild. Both strains caused cytoplasmic incompatibility (CI). The *wCalt1* strain caused weak CI (~20%) and the double infection induced moderate CI (~70–90%) in crosses with uninfected beetles. The *wCalt1* strain rescued about 75% of eggs fertilized by sperm from *wCalt2* males. Based on the relationships of beetle mtDNA and infection status, maternal transmission, and repeated population sampling we determined that the double infection invaded *C. alternans* populations about 100 000 years ago and that the *wCalt2* strain appears to be declining in some populations, possibly due to environmental factors. This may be the first study to demonstrate an association between widespread strain loss and environmental factors in the field.

Keywords: *Chelymorpha alternans*, cytoplasmic incompatibility, multiple infections, strain loss, *Wolbachia*

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Introduction

Wolbachia are a wide-spread group of endocellular bacteria (Rickettsiae) found in 15–76% of all insect species (Werren *et al.* 1995b; West *et al.* 1998; Jeyaprasaksh & Hoy 2000; Werren & Windsor 2000) that enhance their own transmission by manipulating host reproduction in various ways, including feminization of males, induction of thelytokous parthenogenesis, male-killing, and cytoplasmic incompatibility (CI) (O'Neill *et al.* 1997; Werren 1997; Stouthammer *et al.* 1999). Among insects, CI is a frequent effect of *Wolbachia*

and leads to the production of inviable eggs when uninfected females mate with infected males (Hoffmann *et al.* 1990; Reed & Werren 1995; Lassy & Karr 1996; Callaini *et al.* 1997). Infected females do not suffer losses through CI, thus the infection can spread due to the reproductive advantage the bacteria impart to infected females (see Hoffmann & Turelli 1997). Infections with two different strains are not uncommon in nature (Jeyaprasaksh & Hoy 2000; Werren & Windsor 2000) and in this case, double infected males are incompatible with single infected females bearing either strain (Rousset & Solignac 1995; Perrot-Minnot *et al.* 1996; Dobson *et al.* 2001). Double infections are thus expected to invade populations at the cost of single infections (Perrot-Minnot *et al.* 1996).

Few studies of the dynamics of multiple infections in natural populations have been carried out in detail and

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most concern *Drosophila* species. These studies show that complex compatibilities exist between multiple and single infections in *Drosophila*, i.e. CI effects of single strains may not be additive when strains occur as multiple infections (Charlat *et al.* 2002; James *et al.* 2002), strain segregation gives rise to single infections (Kondo *et al.* 1990; Solignac *et al.* 1994; Mer ot *et al.* 1995; Baudry *et al.* 2003), host factors may control strain density independent of the number of coinfecting strains (Ikeda *et al.* 2003; Mouton *et al.* 2003) and host genotype may affect the expression of CI (Rousset & Solignac 1995; Guillemaud *et al.* 1997; Charlat *et al.* 2003). Most studies of multiple infections include insects from temperate climates, though surveys for *Wolbachia* infections reveal that insects in the Neotropics are more likely to harbour multiple strains (34% of infected insects) than are insects in temperate zones (5–7% of infected insects; Werren & Windsor 2000). Here we present results of studies on the dynamics of two *Wolbachia* strains, *wCalt1* and *wCalt2*, in populations of the Neotropical tortoise beetle, *Chelymorpha alternans* Boh., in Panama and address possible environmental effects influencing the frequency of one strain.

C. alternans is found from sea level to approximately 1000 m elevation in disturbed but unburned habitats along forest, river and stream edges on various species of Convolvulaceae ('morning glory' family). Experimental studies indicate that *C. alternans* enters diapause under conditions simulating the dry season (Pullin & Knight 1992). Our observations indicate that as the dry season develops adults become increasingly scarce with immature stages largely absent. At the onset of the wet season, in late April of most years, adults reappear. In contrast, adults in nearly nonseasonal habitats near the Caribbean coast remain active and reproductive throughout the year (Windsor & Keller unpublished observations). Since elevated temperatures (Feder *et al.* 1999; Hurst *et al.* 2000; Snook *et al.* 2000) and host diapause (Perrot-Minnot *et al.* 1996) have been shown to affect the transmission of *Wolbachia*, it is possible that the distribution of some *Wolbachia* strains in tropical insects are affected by low heat tolerance or host adaptations to extreme environments. Because previous work showed that *C. alternans* was infected with at least two strains (Werren *et al.* 1995b) and this species is known to be distributed throughout Panama we investigated the distribution and reproductive effects of *Wolbachia* strains based on the following questions: (i) How many *Wolbachia* strains infect *C. alternans* and how are these strains distributed among host populations, i.e. is the double infection invading? (ii) Do *Wolbachia* in *C. alternans* cause CI and what are the effects of each strain? (iii) What is the maternal transmission rate of each strain and how does this affect bacterial invasion and persistence? (iv) How has the last *Wolbachia* sweep affected host mitochondrial diversity? and (v) Does climate affect the distribution of strains?

Materials and Methods

Beetle collection and maintenance

Beetles were sampled at 24 sites across Panama from December 1997 to November 2002, with some sites resampled up to four times. Adults and larvae were maintained in the laboratory (12 h light, 60% humidity, 26 °C) on fresh leaves of *Merremia umbellata* (Convolvulaceae). Leaves were soaked in a 2% solution of bleach (0.352 M NaClO) for 2 min, and rinsed three times in fresh water to remove the bleach. This treatment reduced fungal growth on beetles and their eggs. Non-extracted remains of adult beetles are stored as vouchers at 4 °C in 95% ethyl alcohol at the Smithsonian Tropical Research Institute (STRI) in the Republic of Panama. Beetles raised for CI studies were either first generation offspring of wild-caught females or were second or later generation antibiotic-treated (cured) stocks and were kept as virgins (2–5 weeks of age) until crosses were arranged. Beetles collected for the study of maternal transmission were allowed to lay 2–4 egg masses, then stored at –80 °C until tested for *Wolbachia*.

DNA template preparation

DNA was extracted from whole larvae or reproductive tissues of adults previously frozen at –80 °C. Insect tissue was ground in extraction buffer (5% Chelex 100 and 0.4% proteinase K in sterile deionized water) with a sterile pestle, vortexed for 10 s, then heated at 56 °C for 35 min then 95 °C for 10 min. After extraction the samples were again vortexed for 10 s, cooled to 4 °C and then spun in an Eppendorf centrifuge at 14K rpms for 2 min to sediment the Chelex and cellular debris. If a sample contained a lipid layer or was not clear then 10 µL of supernatant was removed, avoiding the lipid layer, then added to a tube containing 10 µL of sterile H₂O and respun at 14K rpms for 1 minute. This step usually eliminated problems of non-DNA contamination and interference in subsequent polymerase chain reactions (PCR). Extractions were used for both *Wolbachia* and *C. alternans* molecular studies and were held at 20 °C while in use, or at –80 °C for longer storage.

Strain identification

We cloned and sequenced portions of the *Wolbachia* 16S (483 bp) and *wsp* (560 bp) genes from one beetle from each of the Gamboa and Cana populations. The gene fragments were PCR amplified, ligated into a plasmid vector (pUC 19), transformed into *E. coli*, then plated onto 1.5% LB agar plates containing 0.095% ampicillin and XGal. Clones were isolated and each was resuspended in 50 µL of sterile water. One µL of each suspension was used in a 10 µL PCR reaction: 1 µL 10X buffer (ABI), 0.8 µL MgCl₂ (10 mM), 1 µL

nucleotide mixture (8 mM, equal parts each nucleotide), M13 forward and reverse primers (10 mM each), 5.65 μ L dH₂O, and 0.5 U of *Taq* polymerase (Amplitaq, ABI). Cycle sequence reactions were prepared using quarter reactions of the ABI Prism dRhodamine dye terminator kit and clones were sequenced on an ABI 377 automated sequencer. Sequences were aligned using SEQUENCHER version 3.1 (Gene Codes Corporation) and the sequences for each gene were compared to determine the number of strains present in each beetle.

We also directly sequenced partial fragments of *Wolbachia* 16S (952 bp), *ftsZ* (1003 bp), and *wsp* (560 bp) genes (O'Neill *et al.* 1992; Werren *et al.* 1995a; Zhou *et al.* 1998; MJ Research PTC – 200 thermal cycler) from one to two beetles from three populations, Gamboa (Panama province), Cana (Darien province) and Arenas (Veraguas province). Both forward and reverse DNA strands were sequenced as above.

To determine whether variants of each *Wolbachia* strain were present in *C. alternans* populations we sequenced a 490 bp portion of the *ftsZ* gene using *ftsZ* primers for one to two beetles from seven populations ($n = 12$ beetles, five beetle haplotypes) for the *wCalt1* strain and four populations ($n =$ four beetles, three beetle haplotypes) for the *wCalt2* strain. We also sequenced a 560 bp portion of the *wsp* gene for the *wCalt1* strain from one to three single infected beetles from five populations ($n = 11$ beetles, six beetle haplotypes).

Population sampling for *Wolbachia* strains

Strain-specific primers were created to enable us to track the distribution and temporal frequency changes of the two strains, *wCalt1* and *wCalt2*, in populations of *C. alternans*. These primers amplify a 490 bp region of the *ftsZ* gene: *wCalt1 ftsZ* F-5' CAAGCACTAGAAAAGTCGTTA, *wCalt1 ftsZ* R-5' AAGCCCTGGCATAACCATCAGA, *wCalt2 ftsZ* F-5' CAAGCGTTAGAGAAGTCATTG, *wCalt2 ftsZ* R-5' CAGTCTGGCATGATCATCAAA. PCR pro-

ocols followed Werren *et al.* (1995a). Separate PCR cocktails for each set of primers were prepared and run simultaneously for all populations sampled. A positive control containing both *Wolbachia* strains and a negative control containing no DNA were run with each PCR reaction. PCR products were visualized on 1% (w/v) TBE-agarose gels stained with ethidium bromide. Samples that tested negative for both strains were retested for extraction quality with insect-specific 28S rDNA primers (Werren 1995b) and were removed from the study if again no amplification products were produced. Because only the *wCalt1* strain was found as a single infection, it is to this strain that we refer when we mention the 'single infection'. A 'double infection' refers to the presence of both strains in the same beetle. A 'mixed infection' or 'polymorphic infection' refers to populations that have both double and single infected individuals.

Cytoplasmic incompatibility

Three experiments were performed to test the strength of CI within and between populations. First, reciprocal crosses were made between populations fixed for the double (*wCalt1* + *wCalt2*, Gamboa and Cana) and single (*wCalt1* only, Guarumal and Santa Fe) infections (see Table 1, Fig. 1 for locations). Next, reciprocal crosses were made between beetles originating from Gamboa stocks consisting of naturally double infected beetles (D), and single infected (S) and uninfected (U) beetles derived from double infected beetles by antibiotic treatment. Last, we performed reciprocal crosses between single and double infected beetles from Remedios, a population polymorphic for the infection types. We also crossed single and double infected Remedios lines with lines from Gamboa and Cana to determine whether the lack of CI among Remedios beetles was a property of males or females.

To cure Gamboa beetles of *Wolbachia* we injected 0.05–0.1 mL of sterile 0.9% rifampicin solution into 10 female and 10 male abdomens three times a week for two successive

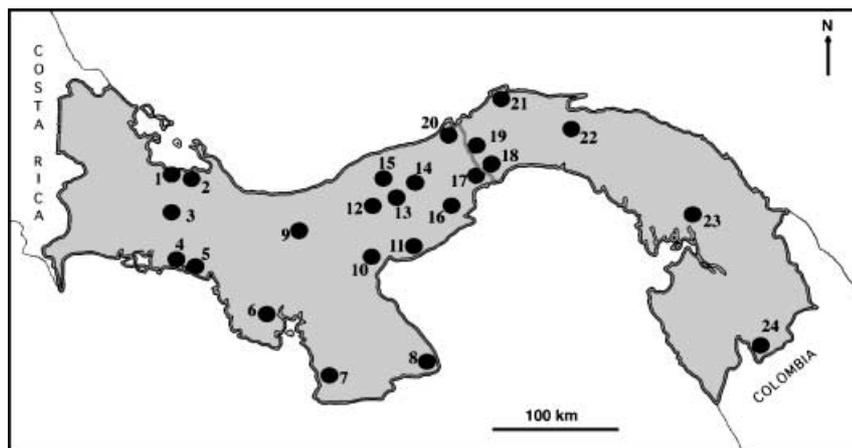


Fig. 1 *Chelymorchia alternans* populations sampled in Panama. The Panama Canal is depicted by the vertical grey line transecting the country.

Table 1 Collection information (site, province, coordinates, elevation in metres (m), dry season length and date of collection), infection frequencies (status: 2 – $w\text{Calt}1 + w\text{Calt}2$, 1 – $w\text{Calt}1$, 0 – uninfected); total number sampled (N) for each collection date; beetle haplotypes (H), and number of haplotypes sampled (n). Haplotypes from double infected beetles are in bold, italic font and haplotypes of single infected beetles are in regular font

Site#	Sites	Coordinates	(m)	# Dry Months	Date	Infection frequencies			N	H	n
						2	1	0			
1*†	Chiriqui Grande, Bocas del Toro	8°56" N; 82°09" W	20	3	August 1998	100%			4	2a	1
					June 2002	75%	25%				
					February 2003	100%	4		8		
2	Legani, Bocas del Toro	8°56" N; 82°03" W	50	na	February 2003	100%			1	2b	1
3	Los Planes, Chiriqui	8°35" N; 82°15" W	800	5	July 1999	87.5%	12.5%		8	1,7a,b	3
					November 2002	100%	9				
					June 1997	100%			4	6b,c,d	
4‡	Las Lajas, Chiriqui	8°10" N; 81°51" W	20	5	June 1999	86%	14%		14		5
					January 2002	50%	50%		20		
					July 1999	64%	36%		22	6b,b,d,e,e	
					December 1999	27%	73%		11		
					June 2001	24%	72%	4%	61		
					January 2002	28%	72%		22		
5‡	Remedios, Chiriqui	8°13.5" N; 81°50" W	30	5	November 2002	24%	76%		34		10
					December 1997		93%	7%	27	6e	
					July 2000		86%	14%	7		
					August 2001		100%		12		
					January 2002		100%		15		
					December 1997		100%		3	6f	
7†	Arenas, Veraguas	7°27" N; 80°52" W	20	5	October 1998				13		3
					July 2002		100%		18	1,6 g	
					May 1999		100%		14	5	
8	Pedasi, Los Santos	7°33" N; 80°2" W	30	11	July 2002				18	1,6 g	2
9	Santa Fe, Veraguas	8°32" N; 81°06" W	500	6	May 1999				14	5	2
10	Natá, Coclé	8°18" N; 80°31.5" W	50	na	January 2002				3	6 g	1
11	Anton, Coclé	8°22" N; 80°17" W	20	10	July 2000				10	6 g	2
12	Barrigon, Coclé	8°37" N; 80°36" W	100	8	October 1997				6	3a,3b,6a	3
					November 1998		89%	11%	9		
					July 2000		91%	9%	11		
13	La Pintada, Coclé	8°35" N; 80°26" W	100	na	July 2000	5%	95%		21	3a,8a,b	3
					May 2002		100%		6		
					May 2002	44%	56%		9	8a,a,c	
14	Toabre, Coclé	8°38" N; 80°20" W	100	na	May 2002				9	8a,a,c	4
15‡	Coclecito, Colon	8°49" N; 80°31" W	50	4	January 1998	70%	30%		10	1,8a	3
					July 2000	70%	30%		10		
					July 2002	82%	18%		17		
16†	Cerro Campana, Panama	8°41.2" N; 79°55.5" W	450	5	December 1999	100%			9	8a	1
					May 2000	100%			16		
					April 1999	100%			12	8a	
17†	Cerro Galera, Panama	8°56" N; 79°39.5" W	250	na	June 2002	100%			14		3
					July 1999	70%	30%		10	4b,8a	
					July 2001	44%	56%		9		
18	Curundu, Panama	8°59" N; 79°32" W	50	8	July 1999	70%	30%		9	4b,8a	2
					July 2001	44%	56%		9		
					May 1998	100%			8	4b,8a	
					December 1998	100%			10		
					May 1999	100%			14		
					December 1999	100%			10		
19*†	Gamboa, Panama	9°12" N; 79°42" W	60	4	April 2002	100%			17		2
					November 1998	89%	11%		9	8a	
					February 1998	100%			8	4b,8a	
20	Achiote, Colon	9°14" N; 80°02" W	20	3	January 2002	70%	30%		10		1
					July 1998	100%			3	4a,a,b	
					September 2000	100%			13		
21*‡	Portobelo, Colon	9°32" N; 79°40" W	10	4	December 2002	83%	17%		6		2
					January 1998	100%			8	4b,8a	
					July 1998	100%			10		
22‡	El Llano-Carti, Panama	9°18" N; 78°58" W	350	5	July 1998	100%			3	4a,a,b	3
					September 2000	100%			13		
					December 2002	83%	17%		6		
23	Santa Fe, Darien	8°28" N; 78°9" W	50	5	January 1998	50%	50%		10	4a,a	2
24*†	Cana, Darien	7°45.4" N; 77°41.6" W	500	5	November 1998	100%			15	4d	1
					April 2001	100%			10		
					September 2002	100%			31		

*populations sampled for *ftsZ* from *wcalt2*; †populations sampled for *ftsZ* from *wcalt1*; ‡populations sampled for *wsp* from *wcalt1*.

weeks. Ultimately, two unrelated females gave rise to our uninfected lines. One single infected line of the *wCalt1* strain was produced in the same manner. We did not encounter the second strain, *wCalt2* as a single infection in any partially cured beetles and therefore no crosses were made with beetles singly infected with this strain. Injected beetles were mated to each other, allowed to produce several egg masses, and then were frozen (-80°C) until tested for *Wolbachia* status. Three to five offspring from each brood were tested to verify the status of the infection in the brood. Uninfected lines were mated to each other to produce the second generation of uninfected beetles. These lines were then used in crosses after curing was again confirmed. The third and subsequent generations of single infected beetles were used for crossing studies.

Eight or more replicates for each reciprocal cross were made. While it was not possible to perform all crosses simultaneously, we initiated sets of crosses at the same time to investigate particular CI effects, i.e. within-site crosses for Remedios and Gamboa, and between-site crosses involving Gamboa beetles crossed with beetles from other populations. After producing at least 50 eggs, adults were frozen and later tested for the presence of the *Wolbachia* strains. Following larval hatching, we recorded the number of eggs in each egg mass and the number of emerged larvae. Hatch rates were calculated as the proportion of successfully hatching larvae averaged across females for each set of crosses. CI was calculated as the hatch rate from a particular cross divided by the average of all compatible crosses (95.6%). We tested the normality of both our original proportions and log and arc-sin transformed data using the Shapiro-Wilk *W* normality test (Sall & Lehman 1996). Because the hatch rates in most comparisons were not normally distributed we report results as medians and 10th and 90th quantiles and used nonparametric tests for comparing medians.

Since CI may be different under field and laboratory conditions we measured the hatch rates of eggs produced in the lab by wild-caught single and double infected females collected from a mixed population, Remedios. Data for females that appeared to be sperm-limited (hatch rates less than 20%) were removed prior to the analyses.

Infection rates of offspring from field-collected beetles

Wolbachia infections depend on efficient maternal (vertical) transmission for maintenance in insect populations. If transmission is less than 100% then the bacteria may only persist if they also induce strong CI. Because the frequency of the double infection varied among populations we measured the rate of maternal transmission by female beetles collected from three populations that varied in their frequency of the double infection: Gamboa, fixed for the double infection, Guarumal, fixed for a single infection of

the *wCalt1* strain, and Remedios, a population with both single and double infected beetles.

Female beetles were collected from Gamboa in 2000, Guarumal in 2001 and Remedios in 2001–02 and maintained in the laboratory (variable light, $26\text{--}28^{\circ}\text{C}$, 75% humidity) in separate containers that were examined every other day for egg production. Egg masses were transferred to sterile Petri plates and placed in an incubator (13L:11D, $26\text{--}28^{\circ}\text{C}$, 70–75% humidity) until larvae emerged 10–11 days later. We tested 10 offspring from each female for the presence of each strain using strain-specific primers. While PCR of *Wolbachia* from Guarumal single infected, 1-day old larvae gave reliable results, i.e. results similar to population estimates, we found that reliable PCR amplification of *Wolbachia* from offspring of Gamboa double infected females could only be obtained from three-week old adults. We have not further explored the reason for differences in PCR amplification of larvae from the different sites. Since there were double infected beetles in the Remedios population, we also tested Remedios progeny for *Wolbachia* as three-week old adults. Egg hatch rates were calculated as in the CI studies.

Climate and elevation effects on Wolbachia distribution

Rainfall patterns (and temperatures) are markedly different between Atlantic and Pacific sites and could affect the distribution of *Wolbachia* in host populations where temperatures are extreme or where the host experiences extended diapause due to the lack of rain. For that reason we compared the length of the dry season to the frequency of the less common strain, *wCalt2*, to determine whether the distribution of this strain was affected by climate. A dry season month was considered as a month in which the average rainfall was less than 200 mm. The rainfall data used in our comparisons were collected between 1956–1983 by the Panamanian Institute of Hydrological and Electrical Resources (I.R.H.E) with at least 10 years of collection data averaged by month for each site. Many of the I.R.H.E. weather stations were at or near most of our collecting sites ($n = 18$) but we were unable to match five sites (2, 10, 13, 14, 17; see Table 1, Fig. 1) with weather stations.

As an additional test of temperature effects on strain distribution we examined the frequency of the *wCalt2* strain with change in elevation because mean annual temperature drops 0.5°C with a rise of 100 meters in elevation. Our sites varied from 0–1000 m, corresponding to a range of approximately 5°C .

Beetle mtDNA

We sequenced a 1277 bp portion of the mitochondrial cytochrome oxidase gene (CO1) from two to 10 beetles from each of 24 *C. alternans* populations ($n = 63$ beetles).

CO1 was amplified by PCR using two primer pairs (Simon *et al.* 1994) in separate reactions, C1-J-1718 and C1-N-2191 (494 bp) plus C1-J-2183 and TL2-N-3-14 (783 bp) each in a volume of 25 μ L: 0.5 μ L DNA sample, 2 μ L 10X buffer (Applied Biosystems Inc., CA, USA), 2 μ L $MgCl_2$ (25 μ M), 0.5 μ L nucleotide mix (4 mM each), 0.5 μ L each primer (20 mM), 0.10 U *Taq* DNA polymerase (Amplitaq, ABI) plus distilled, deionized water. PCR cycling conditions were: 95 °C for 1 min, 35 cycles of (95 °C for 30 s, 45 °C for 1 min, 68 °C for 2 min), then 68 °C for 10 min. Sequencing was performed as previously described.

To test whether CO1 of *C. alternans* has evolved under neutrality we calculated Tajima's D (Tajima 1989) and Fay and Wu's H (Fay & Wu 2000) statistics and ran 10 000 coalescent simulations for each statistic to create 95% confidence intervals. Tajima's D is used to determine whether there is an excess of rare haplotypes, as expected after a selective sweep, population bottleneck or other processes such as background selection, and is based on the difference between two estimates of nucleotide diversity, θ_π and θ_W , where θ_π is the average of pairwise nucleotide differences and θ_W is the number of segregating sites within a population (Watterson 1975). Fay and Wu's H statistic is used to detect declines in genetic diversity due to selective sweeps or demographic events while being relatively insensitive to background selection (Fay & Wu 2000). H is calculated as $\theta_\pi - \theta_H$, where θ_H is an estimate of nucleotide diversity based on the frequency of derived variants. Diversity estimates and Tajima's D statistic were calculated using the program DNASP (Rozas & Rozas 1995) while the Fay and Wu H statistic was estimated using a program provided by J. Fay on the website (www.genetics.wustl.edu/jflab/htest.html). Two specimens of *Chelymorpha vittata* Champ., a close relative of *C. alternans* (Keller, Windsor and Werren unpublished data), were sequenced to provide an outgroup to determine the number of derived variants needed for the Fay and Wu H statistic. Because only two *C. vittata* specimens were sequenced and their sequences were identical, the number of *C. alternans* derived variants may be inflated due to an underestimate of polymorphisms in *C. vittata*.

An unrooted haplotype network was constructed with the program TCS ALPHA, version 1.01 (Clement *et al.* 2000) that uses statistical parsimony to infer haplotype relationships by the method of Templeton *et al.* (1992). Three ambiguities in the haplotype network were resolved by assuming the haplotypes were more likely to be related to haplotypes from the same population than to haplotypes from other populations (Crandall & Templeton 1993).

To determine whether host haplotype was correlated with host infection status, as is expected during and shortly after a *Wolbachia* sweep, we performed a contingency test comparing haplotype by infection status. Because sample sizes were small for individual haplotypes we combined

haplotypes into groups based on the inferred haplotype network: [1], [3a,b], [4a,b,c], [6a,b,c,d,e,f,g] and [8a,b,c].

To establish whether the mtDNA diversity of *C. alternans* is reduced compared to uninfected relatives we tested seven *Chelymorpha* species (*C. vittata*, *C. gressoria* Boh., *C. sp. nov.*, *C. testaceomarginata* Boh., *C. praetextata* Boh., *C. cinctipennis* Boh., and *C. cribraria* Fabr.) and three closely related *Stolas* species (*S. aenovittata* Champ., *S. pictilis* Boh., and *S. n. sp.*) for *Wolbachia* using *Wolbachia* general 16S primers but found no uninfected relatives to use for comparison.

Results

Strain Identification and strain variants

We recognized two strains, *wCalt1* and *wCalt2*, based on cloned products of the *Wolbachia* *wsp* gene from beetles of two populations (Gamboa and Cana) and by direct sequencing of the *wsp* and *ftsZ* *Wolbachia* gene fragments from beetles of three populations (Gamboa, Cana and Arenas). Cloning produced two *wsp* sequences [15 clones – *wCalt1* (2 clones), *wCalt2* (13 clones)] and one 16S sequence (23 clones). Direct sequencing produced identical *wsp* and 16S sequences to the cloned sequences of each gene, respectively, and two *ftsZ* sequences. Total sequence divergence between the strains was 11% for *wsp* (560 bp), 4% for *ftsZ*, not including indels (1003 bp), and 0% for 16S (948 bp). Sequences for each strain are deposited in GenBank, accession numbers AY566419–AY566426.

Both strains were found as single infections, thus verifying the independence of each strain. One population, Arenas, consisted of beetles infected with only the *wCalt1* strain and so we were able to identify the *wsp*, *ftsZ* and 16S sequences associated with this strain. Although the *wCalt2* strain was never found as a single infection in the field, we discovered one male infected with only the *wCalt2* strain from the Gamboa lab stocks and sequenced the three *Wolbachia* genes from this sample. We confirmed that the second set of *wsp* and *ftsZ* sequences was present in this beetle. The 16S sequence was the same as that associated with the *wCalt1* strain.

Comparisons of sequences for each strain from beetles of many populations and diverse haplotypes revealed no genetic variation for either strain [4 *wCalt2* *ftsZ* sequences (3 beetle haplotypes), 12 *wCalt1* *ftsZ* sequences (five beetle haplotypes), 11 *wCalt1* *wsp* sequences (six beetle haplotypes); Table 1].

Population infection frequencies and temporal changes

Nearly all (747/753) beetles sampled from 24 populations were infected with at least one *Wolbachia* strain, *wCalt1* (Table 1, Fig. 1). This strain was found as a single infection in eight populations and as a coinfection with *wCalt2* in 16

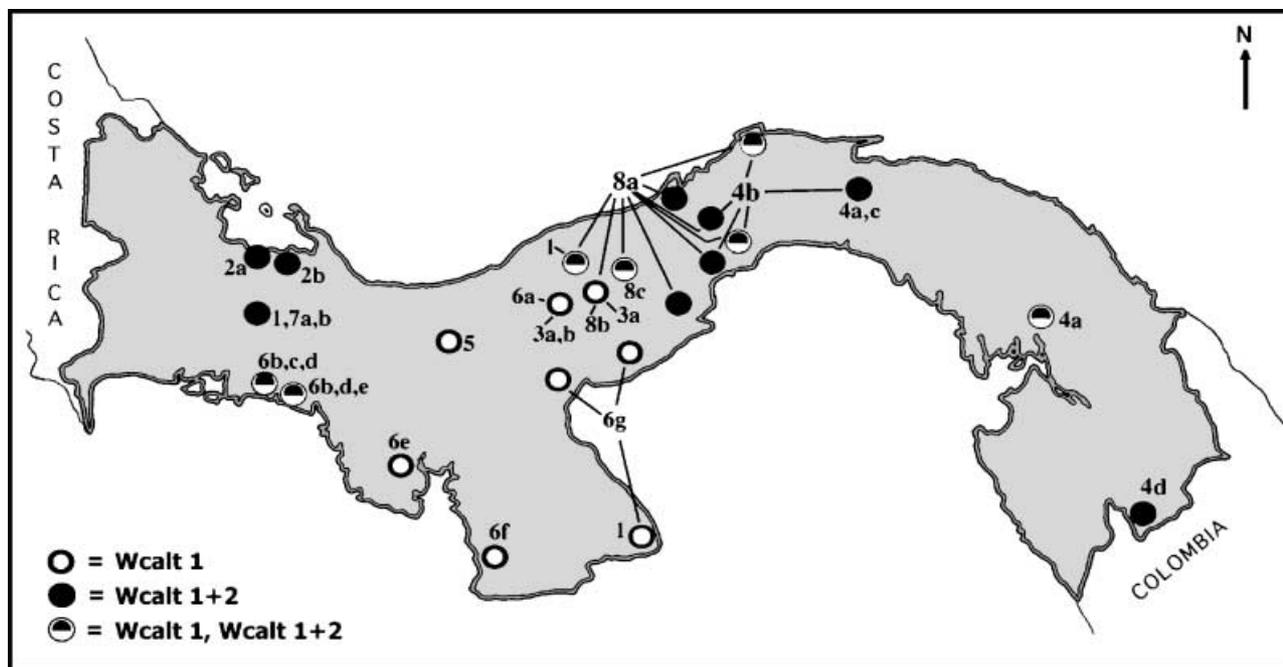


Fig. 2 *Wolbachia* infection status and *C. alternans* haplotype distributions in Panama populations. Open circles represent single infected populations, filled circles are double infected populations and half-filled circles are populations with both infection types. Haplotype numbers correspond to those in Table 1 and Fig. 6.

populations. The *wCalt2* strain was never detected as a single infection in field populations.

We noticed a distinct geographical pattern to single and double infections (Fig. 2). Populations in western Pacific Panama, including the Azuero and Sona peninsulas (populations 6–13) were almost entirely single infected (177 beetles; 96.6% single infected, 2.8% uninfected, 0.06% double infected). Outside of this region, seven populations (4, 5, 14, 15, 18, 21, 23) polymorphic for the infections (311 beetles; 47.9% double infected, 51.8% single infected and 0.3% uninfected) and eight populations with predominantly double infections ($N = 265$ beetles, 98% double infected, 2% single infected) occurred throughout Panama (populations 1, 3, 16, 17, 19, 20, 22, 24).

To determine the temporal dynamics of *Wolbachia* we resampled 11 populations over four years. We found that significant decreases in the frequency of the double infection occurred from 1999–2002 in two populations, Las Lajas (population 4, d.f. = 1, $G = 4.70$, $P < 0.05$) and Remedios (5, d.f. = 1, $G = 3.98$, $P < 0.05$). From July 1999 to December 1999, the frequency of double infected adults in the Remedios population fell from 70% ($n = 21$) to 27% ($n = 11$). Subsequent to this decline the frequencies of the infections in the Remedios population did not change significantly between sampling periods (December 1999–November 2002, $G = 0.124$, d.f. = 4, n.s., Table 1). Non-significant declines of the double infection occurred in Portobelo (21) (d.f. = 1, $G = 3.67$, $P > 0.05$) and Curundu (18) (d.f. = 1, $G = 1.19$, $P >$

0.10). Overall, the number of double infected individuals sampled at all sites declined significantly (i.e. a decline in the frequency of the *wCalt2* strain) in collections made during 2000–02 ($G = 9.44$, $N = 437$ beetles, d.f. = 1, $P < 0.01$).

Maternal transmission of *Wolbachia* by field-collected beetles

Host maternal transmission is one of the key elements, besides CI, affecting the maintenance of *Wolbachia* infections. Ideally, maternal transmission should be measured by mating infected females to uninfected males to avoid the loss of uninfected ova to the effects of CI, which would result in an inflation of the maternal transmission rate. A weakness in our design is that we used wild-collected females that were mated in the field, most likely to infected males, to measure vertical transmission. Transmission rates may be over estimated by a few percent, that is, females from these sites may have produced some uninfected ova that failed to develop due to CI. However, the egg hatch rates of these females were similar to the hatch rates of infected females crossed to uninfected males in later CI studies so our estimates of maternal transmission may not be overly exaggerated.

We investigated maternal transmission in three populations with different infection states, double (Gamboa), single (Guarumal) and mixed (Remedios). In Gamboa, transmission of both *Wolbachia* strains was 100% (10 females, 100

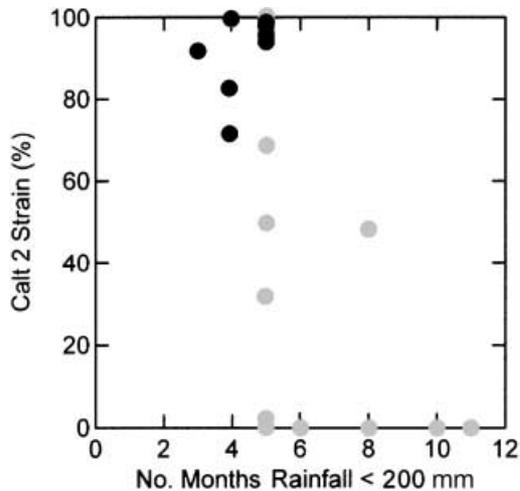


Fig. 3 Population frequency of the *wCalt2* strain plotted against the length of the dry season at each of 19 populations. Black points represent Pacific slope populations and grey points are Atlantic slope populations and points are staggered where they overlap at 0% and 100% values.

progeny) and in Guarumal transmission of the *wCalt1* strain was 99.2% with the remainder uninfected (eight females, 80 progeny). In Remedios, maternal transmission of the *wCalt1* strain by both double infected ($n = 12$, 115 offspring) and single infected ($n = 11$, 109 offspring) females was 98.7%. Transmission of the *wCalt2* strain by double infected females was lower, 83.1%.

Effects of climate and elevation on strain distribution

It is possible that climate influences the frequency of double and single infections, perhaps through effects on transmission. We therefore examined population infection frequencies and climate characteristics. The length of the dry season was negatively correlated with the distribution of the *wCalt2* strain (Fig. 3). Populations with five or more months of dry season had significantly lower frequencies of the *wCalt2* strain than those populations with shorter dry seasons (200 mm: $r_2 = 0.554$, $n = 19$, $P \leq 0.01$). Elevation, however, had no effect on the distribution of the *wCalt2* strain ($r_2 = 0.331$, $n = 22$, $P > 0.05$).

Cytoplasmic incompatibility – crossing studies

We tested the strength of CI within and between populations with four experimental designs: (i) crosses between naturally double (D) (Gamboa and Cana) and naturally single (S) infected populations (Guarumal and Santa Fe); (ii) crosses using GB lines that were naturally double infected, plus single and uninfected (U) lines derived from double infected lines by antibiotic treatment; (iii) crosses between naturally double and single infected beetles from

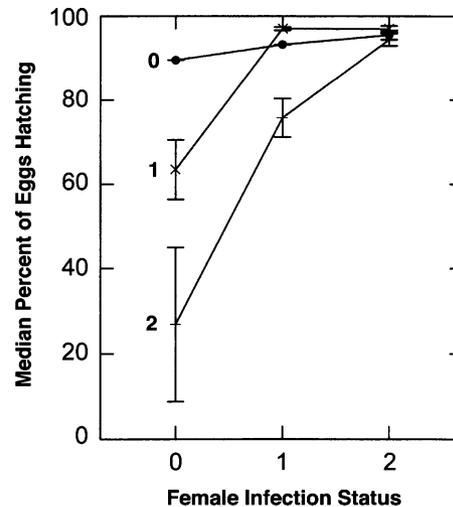


Fig. 4 Egg hatch rates for females with no *Wolbachia* (0), with the *wCalt1* strain (1), or with *wCalt1* + *wCalt2* strains (2) when crossed with males infected with 0, 1, or 2 strains. Male infection status corresponds to lines inside the figure.

the Remedios population; and (iv) crosses between Remedios beetles and double infected beetles from Gamboa and Cana.

We found that both infection types, single (*wCalt1*) and double (*wCalt1* + *wCalt2*), caused CI (Fig. 4, Table 2). Crosses between populations with double and single infections and crosses among Gamboa lines gave the same basic results. Compatible crosses produced 88.5–98.8% hatch rates, while the hatch rates of the incompatible crosses were significantly lower. For the D × S (male × female) cross, hatch rates were 62–78% (35–18% CI, MWU, $\chi^2 = 4.36$, d.f. = 1, $P = 0.037$), for the S × U cross, 56–74% (41–22% CI, MWU, $\chi^2 = 11.70$, d.f. = 1, $P = 0.001$) and for the D × U cross, 7–21% (93–78% CI) ($\chi^2 = 32.38$, d.f. = 1, $P = 0.001$). Therefore, consistent with other systems, double infected males are incompatible with single and uninfected females and single infected males are incompatible with uninfected females.

Individuals from the Remedios population gave different results. The median hatch rate for the Remedios D × S cross (89.5%) was within the range of compatible hatch rates from other populations (89.5%–97.7%, $N = 14$) but was significantly lower than the compatible Remedios S × S cross of 96.6% (MWU = 118, $\chi^2 = 4.012$, d.f. = 1, $P = 0.045$) indicating that the double infection in males of this population induces marginal CI (7.6%).

To determine whether the low CI of the Remedios D × S cross was a property of males or females we mated beetles from this population to beetles from Gamboa, a population where *Wolbachia* causes strong CI (Table 2). The cross of Gamboa D males to Remedios S females resulted in a moderately reduced hatch rate, 74.2%, that was significantly lower than the hatch rate of the Remedios D × S cross, 89.5%

Table 2 The median, n, and 90th and 10th quantiles for egg hatch rates (percent) from crosses, within and among sites, of virgin uninfected (0), single (1), and double infected (2) *C. alternans*. Gamboa 0 and 1 infection types were created by antibiotic-treatment of double infections

		Male Site							
		Remedios		Guarumal	Santa Fe	Gamboa		Cana	
Female Site	Infection	1	2	1	1	0	1	2	2
Remedios	1	96.9 [10] (100.0–51.4)	89.5 [16] (97.2–71.9)				95.6 [10] (98.6–74.3)	74.2 [12] (87.7–61.1)	13.1 [12] (77.4–0.00)
	2	96.8 [12] (100–66.8)	96.6 [11] (100–40.4)					97.6 [6] (100–61.2)	88.5 [11] (99.7–77.1)
Guarumal	1			97.2 [24] (100–80.5)				77.7 [20] (96.3–43.4)	
Santa Fe	1				97.7 [11] (100–42.4)			61.8 [11] (82.4–40.1)	
Gamboa	0	60.8 [11] (96.7–4.5)	53.0 [18] (79.1–7.3)			89.5 [17] (99.5–65.8)	73.7 [10] (93.4–36.8)	20.9 [24] (51.0–5.8)	6.8 [7] (81.8–0)
	1	98.0 [8] (100–94.2)	89.9 [8] (96.7–61.8)			93.2 [10] (98.3–66.4)	96.6 [10] (100–46.2)	73.1 [9] (91.1–45.8)	63.9 [8] (95.3–31.3)
	2			95.8 [21] (100–78.3)	96.2 [10] (100–74.2)	96.1 [23] (100–77.5)	98.8 [10] (100–57.6)	91.1 [40] (100–50.1)	95.8 [15] (97.3–64.2)
Cana	2					94.9 [12] (98.7–85.2)	95.7 [12] (99.7–45.6)	89.5 [12] (99.6–64.0)	

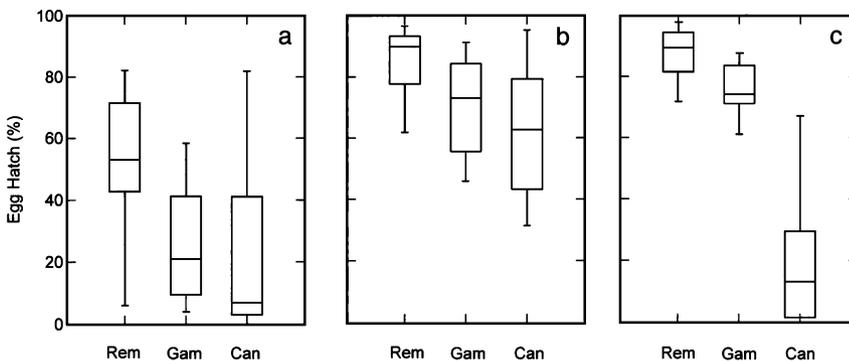


Fig. 5 The effects of double infected males from Remedios (Rem), Gamboa (Gam) and Cana (Can) populations (x-axis) on egg hatch rates when mated to (a) uninfected Gamboa females (b) single infected (*wCalt1*) Gamboa females, and (c) single infected (*wCalt1*) Remedios females.

(MWU = 116, $\chi^2 = 6.205$, d.f. = 1, $P = 0.013$), but not significantly different from the incompatible Gamboa D × S cross, 73.1% (MWU = 44, $\chi^2 = 0.096$, d.f. = 1, $P = 0.757$), indicating that the weak CI seen in Remedios was not due to sperm rescue by single infected females. We then crossed Remedios D males with Gamboa S females to compare the CI effect of Remedios males in this population. The resulting hatch rate, 89.9% (Table 2), was nearly identical to that of the Remedios D × S cross, 89.5%, and not significantly different from the compatible Gamboa S × S cross (MWU = 54, $\chi^2 = 1.244$, d.f. = 1, $P = 0.107$). As a further comparison of Remedios strains we crossed S and D Remedios males with Gamboa U females to determine whether the *wCalt2* strain in the double infection caused any greater incompatibility than the *wCalt1* strain alone. Though the hatch rate of the S × U cross, 60.8%, was somewhat higher than the D × U cross, 53.0%, and both were significantly lower than compatible crosses

(S × U: MWU = 27, $\chi^2 = 10.473$, d.f. = 1, $P = 0.001$; D × U: MWU = 17, $\chi^2 = 21.049$, d.f. = 1, $P = 0.001$), the difference between them was not significant (MWU = 115, $\chi^2 = 0.517$, d.f. = 1, $P = 0.472$) further indicating that the *wCalt2* strain in Remedios males may be responsible for little or no CI.

Cytoplasmic incompatibility — population comparisons

The level of CI induced by double infected males varied across populations (Fig. 5). Uninfected females from Gamboa stocks crossed with D males from three populations, Remedios, Gamboa and Cana, showed an increasing trend in the CI effect (i.e. decreasing egg hatch rate), respectively. Remedios males caused the least amount of CI (53%), compared to Gamboa (20.9%) and Cana males (6.8%) (V-W-test $\chi^2 = 14.93$, d.f. = 2, $P < 0.0006$, Tukey-Kramer HSD;

Table 2, Fig. 5a). A similar trend was seen when the D males were mated to single infected females from either the Gamboa (Fig. 5b) or Remedios (Fig. 5c) populations. Cana males induced the greatest amount of CI followed by Gamboa and Remedios males, respectively. Cana males had significantly reduced egg hatch compared to the other males (V-W-test $\chi^2 = 23.09$, d.f. = 2, $P < 0.0001$, T-K HSD) when crossed to Remedios S females, but the differences among males mated to Gamboa S females were not significant (V-W-test $\chi^2 = 3.44$, d.f. = 2, $P = 0.1789$).

Cytoplasmic incompatibility – field hatch rates

To investigate possible levels of CI in field populations of Remedios we examined the hatch rates of eggs produced by field-collected S and D females. We found that the hatch rates of these single (median 88.3%, range 33.2–98.5%, $N = 31$) and double (94.7%, 59.1–98.8%, $N = 16$) infected females did not differ significantly (MWU = 356, $\chi^2 = 0.770$, d.f. = 1, $P = 0.380$). This indicates that the double infection in Remedios causes weak to undetectable levels of CI in the field, however, larger samples sizes may reveal the weak CI effect uncovered in the laboratory experiments.

Mitochondrial DNA diversity and Wolbachia selective sweep

The mitochondrial haplotypes found in *C. alternans*, their inferred phylogenetic relationships and *Wolbachia* infection statuses are presented in Fig. 6. A total of 22 haplotypes with 23 segregating sites (18 synonymous and five non-synonymous mutations, 16 parsimony informative) were found among 63 infected beetles collected from 24 locations in Panama. Haplotypes were deposited in GenBank, accession numbers AY563955–AY563976. Genetic diversity estimates for all samples ($N = 63$), and subsets of double ($N = 36$) and single ($N = 27$) infected beetles are given in Table 3. Estimates of genetic diversity were similar between the double and single infected haplotypes due to the large number of haplotypes shared between them. We found no correlation between host haplotype and associated infection type ($G = 8.39$, d.f. = 4, $P > 0.05$). The inferred haplotype

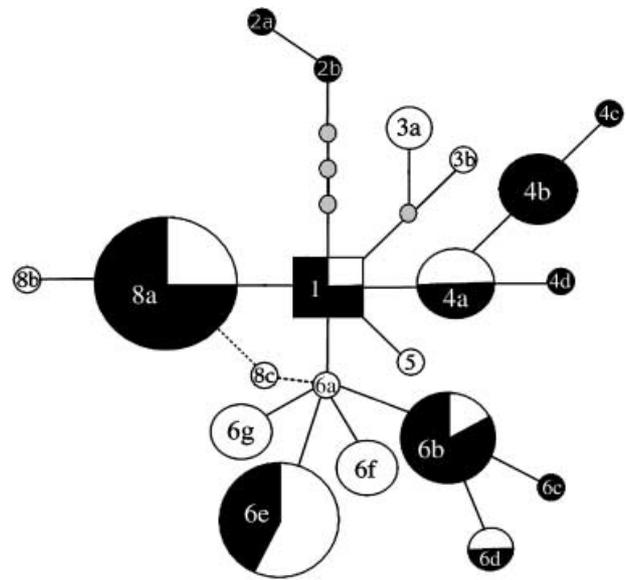


Fig. 6 Statistical parsimony network of *C. alternans* haplotypes. The square box represents the common ancestral haplotype and circles represent derived haplotypes. Circle size relates to sample size, one to 12 samples per haplotype, and shading corresponds to the proportion of double- (black) or single- (white) infected beetles with those haplotypes. Grey-filled circles represent unsampled haplotypes. The dashed line stands for an alternate haplotype connection. Each line between nodes represents one nucleotide substitution.

network (Fig. 6) shows that haplotypes of most single infected beetles are the same as or are derived from haplotypes of double infected beetles. This is consistent with the production of single *wCalt1* infections from double infected lineages.

To determine whether sequences were evolving in a non-neutral fashion, indicative of a recent selective sweep or demographic event such as a range expansion or population bottleneck, we performed Tajima’s D and Fay and Wu’s H-tests on (1) all sequences, and (2) separately for sequences from single and double infected beetles. CO1 appears to be evolving neutrally as all D and H-values were nonsignificant (Table 3). These tests provide no evi-

Table 3 Mitochondrial CO1 haplotype and nucleotide diversity estimates from single and double infected beetles. N – number of samples, π – nucleotide diversity, SD – standard deviation, θ – neutral expectation of π , D – Tajima’s D statistic, H – Fay and Wu’s H statistic

	N	Number of haplotypes	Haplotype diversity	Number of variable sites (S)	π	SD (π)	θ (S)	SD (θ)	D	H	% Pairwise genetic distance
All Sequences	63	22	0.929	23	0.0023	0.0001	0.0036	0.0012	-1.157	0.740	0.712
Single infections	27	15	0.937	12	0.0019	0.0001	0.0024	0.0010	-0.626	0.873	0.658
Double infections	36	13	0.908	17	0.0025	0.0001	0.0034	0.0013	-0.900	0.820	0.715

dence for a recent sweep (or bottleneck), indicating that the current infections must have been established long ago. The power of these tests to reject the null hypothesis, that of neutral evolution, depends on large samples sizes, i.e. > 50 , and a specific window of time since the demographic or selective event occurred because the addition of new mutations obscures events that took place in the remote past (Simonsen *et al.* 1995). Though our sample size was large ($N = 63$), the last *Wolbachia* sweep of *C. alternans* populations may have occurred too long ago to be detected by these tests. Assuming that the beetle DNA is evolving in a neutral fashion, as suggested by the neutrality tests, we roughly estimate the time since the last sweep to be 100 000–125 000 years ago based on the pairwise mitochondrial sequence divergence rate of approximately 2.3% per million years for invertebrate mitochondria (Brower 1994) and the synonymous substitution rate of 5.7% per million years in *Drosophila* (Tamura 1992).

Other *Wolbachia* Effects

It was not feasible in this study to measure the lifetime (one year) fecundity of *C. alternans*, so instead we measured the time it took females to produce 50 eggs (two egg masses) once they were paired with males of the same infection status. Among Gamboa beetles (antibiotic-treated and infected) we found a marginally significant female effect due to differences between uninfected (19.2 ± 1.3 se days), and single infected (14.5 ± 1.4 se days) females (K-W = 6.45, $\chi^2 = 6.452$, $P = 0.040$, d.f. = 2; Tukey-Kramer *post hoc* comparison of means, HSD = 0.324). However, double infected females (16.3 ± 1.0 se days) did not differ significantly in this measure from either single infected or uninfected females (MWU = 271.4, $\chi^2 = 0.197$, d.f. = 1, $P = 0.657$). The interpretation of these results are complicated because the data for each group were collected at different times and the grandsires of the single infected and uninfected lines had been treated with antibiotics.

Discussion

Basic theories of the invasion process of CI-*Wolbachia* suggest that once the frequency of *Wolbachia* in a population passes a critical threshold, the infection will spread due to the relative reproductive advantage the bacteria gain from inducing CI. (Caspari & Watson 1959; Hoffmann *et al.* 1990; Turelli 1994). Factors important in the spread of *Wolbachia* include the strength of CI induced, the efficiency of maternal transmission, and the relative fecundity of infected females. This applies both to single infections in uninfected populations and double infections in single infected or uninfected populations (Hoffmann & Turelli 1997). Because mitochondria and *Wolbachia* are maternally transmitted and thus co-segregate, analyses of mitochondrial variation can be

important in revealing patterns of *Wolbachia* invasions. As a *Wolbachia* sweep proceeds, the mitochondrial haplotype associated with the initial infection hitchhikes with the invasion and thus becomes linked to the invading strain(s) (Turelli *et al.* 1992; Rousset & Solignac 1995). Once the invasion is complete, intraspecific mtDNA diversity is reduced to the haplotype associated with the last invasion. Given the tight association between *Wolbachia* and host mtDNA, the reduction of mitochondrial variation serves as a genetic footprint of the movement of *Wolbachia* through host populations (Turelli *et al.* 1992; Shoemaker *et al.* 1999). With time, however, the correlation between haplotype and infection type may degrade with the accumulation of mtDNA mutations and strain loss (Solignac *et al.* 1994; Turelli 1994; James *et al.* 2002) and may be complicated when more than one strain is involved.

In the present study we found that the strains infecting *Chelymormpha alternans*, *wCalt1* and *wCalt2*, comprised two infection types — either a double infection of both strains or a single infection of only the *wCalt1* strain; the *wCalt2* strain was never found as a single infection. There was a distinct geographical pattern to the distribution of the infection types. Populations in a large region of western Pacific Panama were exclusively single infected whereas populations outside this region were either completely double infected or were polymorphic for single and double infections. We formed two general scenarios to explain the distribution of infection types. The first suggests that there is an ongoing sweep of a double infection that has replaced a pre-existing single infection in all populations of the country, except western Pacific Panama. The second suggests that the double infection has already swept across Panama and strain sorting has left some populations with mixed and single infections. The interpretation of our findings assumes that intraspecific horizontal and paternal transmission of *Wolbachia* (Hoffman *et al.* 1990, 1998) and paternal transmission of mitochondria (Kondo *et al.* 1990) are negligible and have not contributed to the distribution of *Wolbachia* or mtDNA among conspecific beetle hosts and thus associations of beetle haplotypes and *Wolbachia* strains are solely due to vertical (maternal) transmission.

Our results support the second scenario, a long-standing infection of two strains with secondary loss of the *wCalt2* strain in some populations. We found that: (i) the frequency of the double infection across populations was either stable or decreased with time; (ii) the *wCalt2* strain showed reduced maternal transmission in some populations; (iii) the levels of nucleotide diversity were similar for both single and double infected beetles; (iv) single and double infected beetles shared the same or similar haplotypes; and (v) tests of neutrality for the evolution of the mitochondrial CO1 gene revealed no evidence for a recent *Wolbachia* sweep. Our findings thus indicate that the last sweep occurred as a double infection in the distant past.

If the double infection is not currently invading single infected populations how do we explain the occurrence of exclusively single infections in populations of the western Pacific region and frequent single/double infection polymorphisms in others? Single infected populations may have formed during the original invasion if strain sorting occurred as the double infection swept through beetle populations, or the loss of the *wCalt2* strain may have occurred following the invasion. Several lines of evidence suggest the latter. First, four years of population sampling revealed fluctuations in the frequency of the *wCalt2* strain in several populations. Some populations, mostly those on the Pacific slope, showed declines in the frequency of the *wCalt2* strain while others, on the Atlantic slope, registered some loss but then recovered. Next, studies of maternal transmission indicated that transfer of the *wCalt2* strain to offspring was incomplete for some double infected females from one population (Remedios) polymorphic for the infections. This population was one that experienced a decrease and then stability in the frequency of the *wCalt2* strain. Further, the distribution of infection types within the haplotype network indicates that most single infections were formed after the initial sweep of the double infection, although there are two single infected haplotype lineages (three and five) that could have formed by loss of the *wCalt2* strain during the initial sweep. Considering only vertical transmission of the strains, a double infected haplotype could only be produced through an unbroken chain of double infected ancestral haplotypes that emerged since the initial invasion of the double infection. Haplotypes that are currently polymorphic for infection types must have originated from double infected ancestors. That extant ancestral haplotypes are found in both double and single infected beetles means that the loss of the *wCalt2* strain occurred since these haplotypes diverged from their double infected ancestors. Either loss of the *wCalt2* strain, and not the *wCalt1* strain, occurred randomly as each new haplotype in double infected beetles emerged or else recent environmental changes have caused nearly simultaneous loss of the *wCalt2* strain across several extant haplotypes.

Processes that might lead to the elimination of the *wCalt2* strain include host resistance, strain competition, and environmental curing. We have not explored the first two possibilities but have some evidence that the *wCalt2* strain may be restricted in its distribution by effects of the dry season in part of the range of the host. At sites where the dry season extends beyond four months, we found a significant decline in the frequency of the *wCalt2* strain. The length and intensity of the dry season vary across the Isthmus and beetle activity is ultimately constrained by rainfall. The Atlantic slope is perennially moist, experiencing fluctuations in rainfall with few months receiving less than 200 mm of rain and beetles are active year-round. The

Pacific slope is seasonally dry and hot with some regions receiving little or no rain for five or more months. During the Pacific dry season, which generally lasts from January through April or longer, host plants wither, beetles become scarce and may go into diapause until rain and longer days return (Pullin & Knight 1992). All populations with exclusively single *Wolbachia* infections occurred in the seasonal western Pacific region, an area that experiences a longer and more intense dry season than the rest of the country, five months and more. Most of the polymorphic populations also occurred in this area and along the Pacific coast. We found that populations that experienced an average of five months of dry season varied in the frequency of the *wCalt2* strain (0–100%). Five months may be the limit of tolerance of dry season conditions for the *wCalt2* strain.

Some populations that averaged five months of dry season might experience an occasional extended dry season due to an infrequent event, such as El Niño. Because we had access only to data averaged over 10 or more years these infrequent anomalies were not apparent. However, such an event may be sufficient to eliminate the *wCalt2* strain from some or all beetle hosts in the area. The loss of the *wCalt2* strain may happen because either: (i) this strain generally occurs at lower densities in host tissues than the *wCalt1* strain, so that during an extended dry season all bacterial titres are reduced, yet the density of the *wCalt2* strain, but not the *wCalt1* strain, falls below a critical threshold and the strain is lost; or (ii) physiologically, this strain is maladapted to long periods of high temperatures or host diapause.

It is known that both diapause (Perrot-Minnot *et al.* 1996) and intense heat for short periods (Werren 1997; Feder *et al.* 1999; Stouthammer *et al.* 1999; Hurst *et al.* 2000; Snook *et al.* 2000) reduce *Wolbachia* densities and even cure some insects of their bacterial infections in laboratory environments, so it is possible that these factors may have similar effects in natural insect populations. Extensive deforestation in Panama (Ibañez 2002) has contributed to the increased dryness of Panama and may ultimately be causing the decline of the *wCalt2* strain in this area. Certainly more studies are needed to determine what factors, heat, low humidity or host diapause are responsible for the loss of the *wCalt2* strain.

Strain variation among populations

Since *Wolbachia* and mtDNA cosegregate, we sequenced genes from each strain to determine whether the strains had also accumulated mutations. No variation was found for either *ftsZ* or *wsp* sequences of either strain. Lack of variation within *Wolbachia* strains is expected when an infection is invading (Turelli *et al.* 1992). It is more difficult to explain the lack of variation in long-standing infections where the host shows post-sweep genetic variation

(Shoemaker *et al.* 2003). One might expect that since host mitochondria and *Wolbachia* share the same history that parallel variation in *Wolbachia* genes might be detected. However, mitochondrial mutation rates are substantially higher than those typically found in bacteria, therefore the bacterial genes sampled may not have acquired detectable mutations in the time since the most recent sweep.

Cytoplasmic incompatibility and field hatch rates

We found that the double infection of *C. alternans* induced moderate CI in laboratory crosses with single infected beetles. However, we found no evidence that the double infection is supplanting the single infection in mixed or single infected populations. We even found that the frequency of the double infection was declining in two populations.

As with maternal transmission, the expression of CI varied among populations. Hatch rates from compatible crosses, both within and between populations, were reasonably consistent and ranged from 88.5–98.0%. However, the hatch rates of incompatible crosses, and thus CI, varied among populations. Double infected males from an eastern population, Cana, caused the strongest CI when mated to either uninfected or single infected females, whereas double infected males from a western population, Remedios, caused almost no more CI than single infected males when crossed with uninfected females, and very little CI when crossed with single infected females. Gamboa males gave intermediate CI results. The Cana and Remedios populations are approximately 610 km apart and Gamboa is located almost exactly midway between them. We have only just begun to explore the reasons for these site differences of CI but have evidence that environmental factors associated with the length of the dry season may be affecting the success of the *wCalt2* strain in some regions. Other possible explanations for the differences among populations include: (i) the occurrence of undetected strain variants among populations that might differ in their abilities to modify and/or rescue sperm; (ii) differences in strain titres among and within populations which result in variable CI levels when crosses are made between populations; (iii) varying host rescue mechanisms among populations which may be particularly effective against local strains but not other variants.

Because Remedios is a population polymorphic for the infection types, we studied the strains here in some detail. We found that the *wCalt2* strain of the double infection in Remedios has reduced ability to induce CI. Although we have not measured *Wolbachia* density in these beetles, it is possible that the low CI of double infected males is due to a low density of the *wCalt2* strain in this population. Other findings such as incomplete maternal transmission and a decrease in the frequency of the *wCalt2* strain in the Remedios population plus the fact that this strain is never found

as a single infection are in keeping with this theory. If the density of the *wCalt2* strain is lower in general this could explain why strain sorting always leads to single infections of only the *wCalt1* strain. Variable *Wolbachia* densities can lead to strain segregation (Sinkins *et al.* 1995; Clancy & Hoffmann 1997) and reduced CI levels (Breeuwer & Werren 1993; Perrot-Minnot & Werren 1999; Noda *et al.* 2001). However, nuclear restorer genes may also act to circumvent *Wolbachia* modifications (Turelli 1994).

We found that uninfected females were able to partially rescue sperm from double infected males, however, the success of rescue depended on the male's population of origin: Remedios (53% hatch rate) > Gamboa (20.9%) > Cana (6.8%). The double infection was also rescued by the single infection in females from two different populations where again the strength of rescue depended on the male's population of origin (Remedios > Gamboa > Cana). From these results it seems that the strength of CI is dependent on male rather than female factors, suggesting that sperm modification varies across populations. Variability of sperm modification could be due to variable *Wolbachia* titres (either total or strain-specific) in sperm cysts (Veneti *et al.* 2003). Since we have indications that the frequency of the *wCalt2* strain in some populations is negatively affected by the length of the dry season, it is possible that these conditions also affect *wCalt2* titres in males of these populations. Males with decreased *wCalt2* titres would have reduced CI in matings with single infected females and thus the double infection would not be able to invade where the negative effects of the dry season are extreme. Further studies of the effects of climate on local strain densities are needed to understand these population differences.

Dynamics of Wolbachia in a population polymorphic for the infection

Changes observed in the frequency of the *wCalt2* strain in some populations may be due to imperfect maternal transmission and possibly to environmental curing. In the Remedios population, we measured an 11–14% per generation loss of the *wCalt2* strain due to strain sorting whereas we were unable to measure any loss of the *wCalt1* strain. Likewise, in the lab, CI induced by the double infection was low, 7.6%. Given these results we might expect the frequency of the double infection to decrease with time. Initially, over a six month period we detected a significant decline in the frequency of the *wCalt2* strain in Remedios from 64% to 27% where it then remained stable in samples taken over the next three years.

To determine whether our observations of double and single infection frequencies in Remedios followed predictions, we used a model developed by Hoffman & Turelli (1997; pp. 65–67), that incorporates parameters for double infections and imperfect maternal transmission. Using the

Table 4 Field and laboratory values for parameters of the Hofmann & Turelli (1997) model of infection frequency shifts over time in double infected populations. The values for maternal transmission, μ , were estimated from eggs hatched in the laboratory from field-collected females. Values for infection frequencies, p , were estimated from field collections. Laboratory crosses between infection types produced the hatch rates, H . Fraction of offspring that did not bear the same infection status as their mother, u . Infection states, AB – double infection, A – single infection, 0 – uninfected

Parameters	June 2001	January 2002
uAB	0.132	0.234
uAB,A	0.088	0.234
uAB,0	0.044	0
uA,0	0	0
pAB	0.30	0.29
pA	0.67	0.67
p0	0.03	0.04
HA,AB	0.927	0.927
H0,AB	0.530	0.530
H0A	0.608	0.608

June 2001 values from field (f) and laboratory (l) studies (Table 4) for the model parameters, μ – segregation rate (l), H – relative hatch rate for incompatible matings (l), p – frequency of infection (f), F – relative fecundity of infected classes (l), the model predicted that the *wCalt2* strain in the Remedios population would disappear (frequency less than 1%) in 56 generations, nine to 14 years (four to six generations per year), due to incomplete maternal transmission and low CI. At the same time, the single infection would go to 95% fixation. However, population data collected in Remedios after the initial decline of the *wCalt2* strain do not fit these predictions. Rather than decreasing to 11–15%, as the model predicts, the frequency of the *wCalt2* strain has remained relatively stable over the last three years (Table 1). One reason for this stability may be that *C. alternans* has overlapping generations which violates the model's assumption of discrete generations. Overlapping generations may slow the rate of decline of the double infection if double infected adults of each generation survive throughout the breeding season. However, inaccuracies in our point estimates of the parameter values could also explain the deviation between observed and predicted values.

Our studies of *Wolbachia* in a Neotropical beetle show that *C. alternans* has a long-standing infection of two *Wolbachia* strains, *wCalt1* and *wCalt2*. The fates and natural history of each strain are different. The *wCalt1* strain occurs in all populations, induces weak CI, and has almost complete transmission, whereas *wCalt2* occurs in two-thirds of populations only as a double infection with *wCalt1*, induces moderate to strong CI in conjunction with *wCalt1* as a double

infection, and is not completely transmitted in all populations. Environmental factors associated with dry season conditions appear to limit the distribution of the *wCalt2* strain and may be responsible for its decline in some Pacific populations. Further studies are planned to evaluate the role of environment, bacterial titre and host effects on the dynamics of double infections in this system.

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