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Article

Experimental evidence of *Wolbachia* introgressive acquisition between terrestrial isopod subspecies

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Abstract

Wolbachia are the most widespread endosymbiotic bacteria in animals. In many arthropod host species, they manipulate reproduction via several mechanisms that favor their maternal transmission to offspring. Among them, cytoplasmic incompatibility (CI) promotes the spread of the symbiont by specifically decreasing the fertility of crosses involving infected males and uninfected females, via embryo mortality. These differences in reproductive efficiency may select for the avoidance of incompatible mating, a process called reinforcement, and thus contribute to population divergence. In the terrestrial isopod *Porcellio dilatatus*, the *Wolbachia* wPet strain infecting the subspecies *P. d. petiti* induces unidirectional CI with uninfected individuals of the subspecies *P. d. dilatatus*. To study the consequences of CI on *P. d. dilatatus* and *P. d. petiti* hybridization, mitochondrial haplotypes and *Wolbachia* infection dynamics, we used population cages seeded with different proportions of the 2 subspecies in which we monitored these genetic parameters 5 and 7 years after the initial setup. Analysis of microsatellite markers allowed evaluating the degree of hybridization between individuals of the 2 subspecies. These markers revealed an increase in *P. d. dilatatus* nuclear genetic signature in all mixed cages, reflecting an asymmetry in hybridization. Hybridization led to the introgressive acquisition of *Wolbachia* and mitochondrial haplotype from *P. d. petiti* into nuclear genomes dominated by alleles of *P. d. dilatatus*. We discuss these results with regards to *Wolbachia* effects on their host (CI and putative fitness cost), and to a possible reinforcement that may have led to assortative mating, as possible factors contributing to the observed results.

Key words: cytoplasmic incompatibility, introgressive acquisition, terrestrial isopod, *Wolbachia*

Hybridization plays an important role in plant and animal evolution (Arnold and Hodges 1995). Barton and Hewitt's definition of hybridization as "reproduction between members of genetically distinct populations" (Barton and Hewitt 1985) aims to show that this phenomenon is common and widespread. This definition encompasses a large array of situations dependent on the genetic distances between parental lineages. When parents are genetically close, hybridization can be beneficial as it may reduce inbreeding and increase genetic variability, creating novel genomic combinations with hybrids potentially fitter than parental types (Seehausen 2004). Contrarily, when individuals are genetically distant, hybridization can lead to outbreeding depression, due to disjunctions of adapted gene complexes (Dowling et al. 1997) or deleterious interactions between alleles from different populations (Dobzhansky–Muller incompatibilities). These latter mechanisms decrease hybrid fitness and gene flow between both parental lineages and contribute to ongoing speciation. Hybrid unfitness can also favor prezygotic isolation by inducing natural selection for the avoidance of maladaptive hybridization, a process named reinforcement (Servedio and Noor 2003).

A particular case of genetic incompatibilities between hybridizing populations results from the complex interactions between host nuclear genes and genes from cytoplasmic endosymbionts (Jaenike et al. 2006; Brucker and Bordenstein 2012). The idea that endosymbionts can influence both the reproductive success and the diversification of their host first emerged in literature in the study of the endosymbiont *Wolbachia* that has been clearly identified as the source of incompatibilities between *Culex pipiens* mosquito lineages (Laven 1967). After 60 years of intensive research, *Wolbachia* represent the most widespread intracellular bacteria detected in ecdysozoans, including insects, crustaceans, nematodes, mites, scorpions, and spiders (Werren et al. 2008). These maternally inherited bacteria transmitted by the eggs can evolve to obligatory symbionts (Hosokawa et al. 2010; Hoffmann et al. 2011; Taylor et al. 2013) but are facultative in most host species. *Wolbachia* bacteria are known as sex manipulator as they cause 4 major effects: feminization, cytoplasmic incompatibility (CI), male killing, and thelytokous parthenogenesis (Werren et al. 2008). These manipulations enhance the maternal transmission of *Wolbachia* (Werren et al. 2008; Landmann 2019) and also influence the reproduction of its hosts and their evolution. CI is the most widespread *Wolbachia* manipulation. It induces the mortality of embryos between males carrying *Wolbachia* and uninfected females, which promotes the spread of the bacterial infection by decreasing the brood size of uninfected females. If 2 populations with different infection status co-exist in sympatric situations, the high hybrid mortality induced by CI could theoretically promote isolation between lineages, regardless of their genetic relatedness, and may ultimately favor assortative mating through reinforcement (Bordenstein et al. 2001; Telschow et al. 2005, 2007; Jaenike et al. 2006).

Another important consequence of *Wolbachia* infection on the evolution of its host pertains to the fact that both *Wolbachia* and the mitochondrion are vertically transmitted from mother to offspring in the same egg cytoplasm. Such co-transmission results in a global concordance between the mitochondrial genetic signature and the infection status of the individuals. Hence, if *Wolbachia* increases in frequency in a population during a selective sweep due to CI or another mechanism, the mitochondrion will "take the ride" with it. This phenomenon, named "genetic hitchhiking," is well illustrated in the mosquitoes *C. pipiens* in which all individuals are infected with *Wolbachia* (Dumas et al. 2013; Sicard et al. 2019). In this

species, for which different *Wolbachia* phylogenetic groups were characterized around the world, the mtDNA diversification perfectly mirrored the *Wolbachia* ones, demonstrating the strict maternal transmission of the bacterium (Atyame et al. 2011, 2014; Dumas et al. 2013). The mitochondrion can even follow *Wolbachia* across species barriers during events of hybridization (Turelli et al. 2018; Cooper et al. 2019).

By inducing CI, *Wolbachia* bacteria can contribute to the divergence between closely related parental lineages harboring different infection status (Jaenike et al. 2006; Brucker and Bordenstein 2012). However, when hybridization is possible between uninfected and infected individuals, hybrid zones can also be the source of introgressive acquisition of *Wolbachia* and of nuclear gene admixture. Hybrid zones are thus considered to be a major source of *Wolbachia* transfer between genetically differentiated hosts (Cooper et al. 2019). In this study, we addressed experimentally the question of the consequence of CI induced by *Wolbachia* on hybridization between 2 subspecies of the crustacean isopod *Porcellio dilatatus*, which can be sympatric, as observed in the south of France (Legrand et al. 1978; Sicard et al. 2014). Although the prevalence of *Wolbachia* in natural populations of the 2 species is still poorly known, to date, individuals of *P. dilatatus petiti* (lineage named "petiti") were always found to be infected with the *wPet* strain while individuals of *P. dilatatus dilatatus* (lineage named "dilatatus") were found to be either uninfected or infected with the *wDil* strain, depending on the sampled population (Sicard et al. 2014). Whereas *wDil* induced ~70% CI in crosses between infected "dilatatus" males and uninfected "dilatatus" females, *wPet* decreases the fertility of uninfected or *wDil*-infected "dilatatus" females fertilized by "petiti" males of >95%. Therefore, both *Wolbachia* strains found in the 2 *Porcellio* subspecies have been shown to be responsible for bidirectional CI of asymmetric intensity (Sicard et al. 2014). However, full fertility of uninfected "dilatatus" females can be restored by the infection of *Wolbachia*, demonstrating that reproductive isolation between the subspecies is essentially dictated by *Wolbachia*-induced CI (Legrand et al. 1985, 1986; Sicard et al. 2014). In this study, we experimentally investigated whether *Wolbachia*-induced CI in *Porcellio* subspecies would prevent hybridization between infected "petiti" and uninfected "dilatatus" or on the opposite promotes the spread of *Wolbachia* and the associated mitochondrial haplotype in the initially uninfected genetic nuclear background.

Materials and Methods

Porcellio dilatatus lineages

The individuals used in this study belong to 2 lineages from 2 *P. dilatatus* subspecies named "petiti" and "dilatatus." Strong specific features in both morphological traits (Legrand et al. 1974) and sex determination modes (heterogametic male XY for "dilatatus" and heterogametic female ZW for "petiti"; Legrand et al. 1980) allow to distinguish 2 different subspecies (Legrand et al. 1974, 1978). Afterwards, these divergences were corroborated by genetic data based on mitochondrial markers (i.e., rRNA and COI; Sicard et al. 2014) but a part of this genetic divergence could be due to the geographical distance between sampling points where the lineages were sampled. The lineage "petiti" infected with the CI *Wolbachia* strain *wPet* was originally sampled in 1971 on Saint-Honorat island (Alpes-Maritimes, France: 43.51°N, 7.05°E), whereas the lineage "dilatatus" has been created from *Wolbachia*-free individuals sampled in 1988 in Rom (Deux-Sèvres, France: 46.29°N, 0.29°E).

Individuals belonging to the 2 lineages were maintained separately in large boxes (60 × 40 × 18 cm) containing hundreds of animals under natural photoperiod, at 20°C and with food *ad libitum* (dried lime leaves and carrots). In 2008, we setup 5 types of population cages each containing 40 individuals (1:1 sex ratio) belonging to “*dilatatus*” and “*petiti*” lineages in different proportions (% “*dilatatus*” [%d]/% “*petiti*” [%p]): 6 replicates of population cages “100% ‘*dilatatus*’”: 100%d/0%p (“*dilatatus*” control), 6 replicates of population cages 80%d/20%p, 6 replicates of population cages 50%d/50%p, 6 replicates of population cages 20%d/80%p, and 6 replicates of population cages “100% ‘*petiti*’”: 0%d/100%p (“*petiti*” control). All cages were maintained for 7 years under controlled laboratory conditions representing around 7 terrestrial isopod generations.

DNA extraction

In both 2013 and 2015, 10 individuals were randomly collected in each population cage (i.e., 5 conditions × 6 replicates × 2 years for a total of 600 individuals). Among these samples, we successfully extracted the total genomic DNA of 596 individuals using standard phenol–chloroform extraction protocol (Kocher et al. 1989).

Mitochondrial haplotype and infection status

We aimed at monitoring the *Wolbachia* infection status of individuals and the associated mitochondrial haplotype. To assign the mitochondrial haplotype (“*dilatatus*” versus “*petiti*”) of each of the 596 analyzed individuals, we amplified by simplex PCR their 16S mitochondrial (16Sm) gene (16SF: 5'-CGCCTGTTTATCAAAAACAT-3'; 16SR: 5'-CTCCGGTTTGAAGTCAGATC-3'). PCR was conducted in the Bio-Rad MyCycler™ Thermal Cycler, in a final volume of 10 µL, with an annealing temperature of 57°C and a final concentration of 0.2 µM for each primer using the QIAGEN® (Venlo, The Netherlands) kit (named QIAGEN® Multiplex kit) according to the manufacturer's standard amplification protocol. PCR fragments were purified with 1 µL of Mix EXoAnP for 1 h at 37°C and digested with the *VspI* restriction enzyme, which allows the discrimination between the “*dilatatus*” 16Sm haplotype (1*VspI* restriction site) and the “*petiti*” 16Sm haplotype (no *VspI* restriction site) after agarose gel electrophoresis. Each individual was also tested for infection with *Wolbachia* by amplifying the bacterial gene *gatB* (Baldo et al. 2006). The *gatB* primers were used in combination with those of the P43 microsatellite molecular marker (Michaud et al. 2016), as an amplification control, in the same multiplex. PCR conditions were the same as described above. Afterwards, we statistically compared the frequencies of individuals carrying *Wolbachia* (and the 16Sm *petiti* haplotype) between studied years using Chi-square tests combined with a Yates correction as implemented in Graphpad InStat software. For these tests, we combined the data for cages with the same initial proportions of the 2 lineages and did not consider cages in which lineages were not mixed.

Microsatellite genotyping

We aimed to investigate nuclear genome admixture between “*dilatatus*” and “*petiti*” during the course of the experiments. To this end, we used 10 microsatellite molecular markers: P3, P13, P23, P25, P30, P36, P39, P40, P43, and P47 (Michaud et al. 2016). Three multiplexes of loci were designed: one grouping the P3, P25, and P47 loci, another the P30, P40, and P43 loci, and the third grouping loci P13, P23, P36, and P39. Then, 1 µL of each PCR product was added to 9 µL formamide and 0.5 µL ROX standard (Life

Technologies) and resolved by electrophoresis on an ABI PRISM R_3130 Genetic Analyzer. Genotypes were called by GeneMapper® software (Applied Biosystems) version 3.7, followed by visual verification.

Microsatellite genetic diversity

We used the software MICRO-CHECKER with 1,000 iterations (Van Oosterhout et al. 2004) to identify the presence of null alleles or scoring errors due to stuttering. Linkage disequilibria were assessed using exact tests (1,000 permutations) implemented in FSTAT version 2.9.3 <https://www.softpedia.com/get/Science-CAD/FSTAT.shtml> (Goudet et al. 2002). Departures from Hardy–Weinberg expectation were analyzed for each population cage using Fisher's exact tests (1,000 permutations) as implemented in GENEPOP on the web (Rousset 2008). For these tests, we adjusted the level of significance for multiple testing using a Bonferroni correction.

We estimated the genetic polymorphism over all microsatellite markers for each population cage. Thus, we estimated the genetic diversity with allelic richness (AR), expected heterozygosity (He), and Fis computed with FSTAT version 2.9.3 (1,000 permutations).

Microsatellite genetic structure

Genetic differentiation between population cages was estimated by F_{ST} computed with FSTAT version 2.9.3 (Weir and Cockerham 1984; Goudet et al. 2002). We adjusted the level of significance for multiple testing using a Bonferroni correction. At individual scale, we examined also the global genetic structure using Bayesian approach implemented in STRUCTURE version 2.3.4 (Pritchard et al. 2000). We conducted analyses with the admixture model and considering independent allele frequencies between populations. This method allows inferring the relative contributions of source populations (genetic clusters) to each individual's genome, the number K of genetic clusters being defined by the user. As recommended by Evanno et al. (2005), we replicated 20 independent runs of STRUCTURE for each values of K genetic cluster ranging from 1 to 10. Each run had a burn-in of 10 000 and a total number of 1,000,000 iterations. We derived the most likely number of genetic clusters (K) by applying the method described in Evanno et al. (2005) and implemented in STRUCTURE HARVESTER version 0.6.9 (Evanno et al. 2005; Earl and vonHoldt 2012). For each analyzed individual, we obtained the averages of the ancestry coefficients to each genetic cluster, for the most likely value of K . From these, we calculated the mean ancestry coefficients for each type of population cages.

We then aimed at assessing whether the mean ancestry coefficient to a given genetic cluster within population cages differed from expectations under the null hypothesis that the relative genetic contribution of the 2 lineages remained stable over time. For this, we estimated what should be the mean ancestry coefficient to genetic clusters in cages with a mixture of both lineages at the start of the experiment (2008), taking into account any bias favoring a particular genetic cluster by STRUCTURE. To do so, we used a linear regression function ($y = a + bx$) where y is the mean ancestry coefficient to the cluster associated to the lineage “*dilatatus*” (named as “*dilatatus*” genetic cluster) and x is the original proportion of individuals from this lineage in the cage, that is, the theoretical mean ancestry coefficient under our null hypothesis. However, x corresponds the mean theoretical ancestry coefficient only if the genetic signatures of the 2 lineages have remained stable during the experiment. This can only be certified in control (nonmixed) population cages. x and y were thus taken from the 2 types of

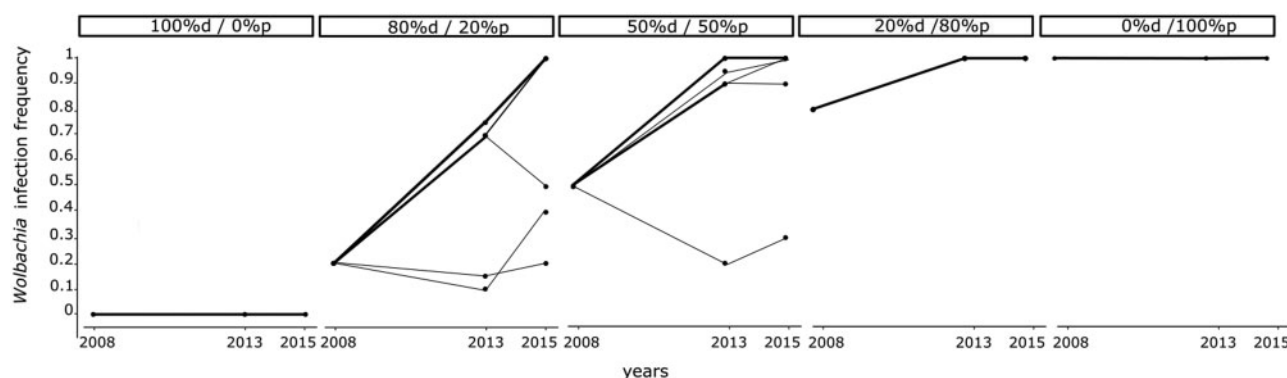


Figure 1. Evolution of *Wolbachia* infection frequency across time, including all replicates of the different population cages, classified by type of population cages (100% d/0% p, 80% d/20% p, 50% d/50% p, 20% d/80% p, and 0% d/100% p). A thick line illustrates an overlay of data for some replicates of different population cages.

control population cages in 2013 and 2015, such that x is either 0 or 1. Based on this 2-point regression, we estimated y for $x = 0.2, 0.5$, and 0.8 , corresponding to cages with a mixture of both lineages. We compared these estimated values (assuming our null hypothesis) to the mean observed ancestry coefficients for each type of population cage using Mann–Whitney tests implemented in Graphpad InStat software.

Hybridization analysis

We investigated hybridization between the 2 lineages by inferring the microsatellite genotypes to parent and hybrid category using the method implemented in the program NEWHYBRIDS (Anderson and Thompson 2002). To evaluate the reliability of the method on our dataset, we generated “artificial” hybrids of known ancestry for assignment. To this end, we first randomly selected 50 individuals from the control population cages (i.e., 100% d/0% p and 0% d/100% p) and their genotypes were implemented as “parental genotypes” in the software HYBRIDLAB version 1.0 (Nielsen et al. 2006) to simulate random mating and provide *in silico* genotypes for artificial hybrids. Thus, we artificially generated 2 parental categories (i.e., “*dilatatus*” [$n = 2,200$] and “*petiti*” [$n = 2,200$]) and 4 hybrid categories (i.e., F1 hybrids [$n = 1,100$]; F2 hybrids [$n = 1,100$]; and backcrosses: F1 X “*dilatatus*” [$n = 1,100$] and F1 X “*petiti*” [$n = 1,100$]).

We combined these 8,800 artificially generated individuals with the 596 individuals from population cages and used NEWHYBRIDS software to assign each “individual” to one of the 6 pre-defined categories: pure subspecies “*dilatatus*” or “*petiti*” or hybrids (i.e., F1 hybrids; F2 hybrids; F1 X “*dilatatus*” and F1 X “*petiti*”). NEWHYBRIDS was run with Jeffrey’s prior, a burn-in of 10,000 followed by 1,000,000 Markov chain Monte Carlo iterations. Allelic frequencies from control population cages (i.e., 100% d/0% p and 0% d/100% p) served as priors. We tested our approach by estimating both its efficiency and accuracy. The efficiency represented the proportion of artificially generated individuals inferred to the right categories (i.e., number of artificially generated individuals correctly identified/total number of artificially generated individuals in the category) (Vähä and Primmer 2006). The accuracy represents the proportion of artificially generated individuals that truly belongs to the category where they have been inferred (i.e., number of artificially generated individuals correctly identified on the total of individuals inferred in the category).

Results

Growing *Wolbachia* infection frequency and mitochondrial hitchhiking

Results from 16S_m amplification, combined with the enzymatic digestion, were in accordance with expected results in control population cages (i.e., 100% d/0% p and 0% d/100% p) and perfectly matched the *Wolbachia* infection status. Indeed, infected individuals always had “*petiti*” mtDNA corroborating an exclusive maternal co-transmission of *Wolbachia* and mitochondrion.

During the studied period of 7 years, *Wolbachia* showed a global growing infection frequency in population cages in which “*dilatatus*” and “*petiti*” were mixed together (Figure 1). From 2008 to 2013, we observed a global significant increase in infection frequency of *Wolbachia* in population cages where the bacterium was initially present regardless the initial proportions ($\chi^2_1 = 11.56$, $P < 0.001$). In 2015, *Wolbachia* reached fixation in 3 out of 6 population cages with initial infection frequency at 20% and in 4 population cages among 6 with initial infection frequency at 50% (Figure 1 and Supplementary Material 1). No significant change in infection frequency was observed between 2013 and 2015 in population cages 80% d/20% p ($\chi^2_1 = 3.49$, $P = 0.06$) and in population cages 50% d/50% p ($\chi^2_1 = 0.25$, $P = 0.62$; Figure 1).

Microsatellite analysis and hybridization detection

We detected no linkage disequilibrium between microsatellite makers (P -value adjusted after Bonferroni correction: 0.001). However, several markers showed null alleles that were most probably linked to homozygosity excess in line with departures from Hardy–Weinberg found in several loci in the different population cages (Table 1). The homozygosity excess might be explained by founder effect and maintenance of the lines in laboratory conditions for several decades before our experiments. Indeed, the low number of founder individuals, at the origin of the “*dilatatus*” and “*petiti*” lineages, may have led to a strong genetic bottleneck combined with an increasing effect of the genetic drift. It is important to note that each locus showed at least 1 diagnostic allele in one of the 2 lineages.

Hybridization rate

Individuals showing admixed ancestry coefficients were detected in all mixed population cages (Figure 2). These admixed individuals certainly resulted from mating between individuals belonging to the 2 initial lineages, demonstrating that hybridization occurred. As

Table 1. Genetic indices for population cages estimated from 10 microsatellite markers: the AR, He, and F_{is} values are indicated

Year	2013					2015					All
d/p proportion	100%d/ 0%p	80%d/ 20%p	50%d/ 50%p	20%d/ 80%p	0%d/ 100%p	100%d/ 0%p	80%d/ 20%p	50%d/ 50%p	20%d/ 80%p	0%d/ 100%p	
P3											
Null alleles	No	No	No	No	No	Yes	No	No	Yes	Yes	—
AR	4.95	4.99	6.18	6.96	6.33	4.00	5.44	6.29	6.39	4.44	6.42
He	0.75	0.76	0.78	0.78	0.74	0.71	0.74	0.72	0.75	0.62	0.78
F _{is}	0.03	0.06	0.09	0.01	−0.05	0.22	0.16	0.05	0.47	0.53	0.20
P47											
Null alleles	No	No	No	No	No	No	No	No	No	Yes	—
AR	2.00	2.00	2.43	3.58	3.83	2.00	2.00	2.00	2.43	3.11	2.88
HE	0.18	0.41	0.38	0.55	0.61	0.21	0.33	0.26	0.49	0.53	0.44
F _{is}	−0.10	0.02	0.13	0.13	0.06	0.04	−0.14	0.09	−0.05	0.24	0.16
P25											
Null alleles	No	Yes	No	No	No	No	Yes	No	No	No	—
AR	2.85	3.00	3.00	3.79	2.96	2.00	3.99	3.46	2.84	2.70	3.69
He	0.40	0.50	0.61	0.56	0.45	0.37	0.50	0.54	0.53	0.31	0.60
F _{is}	0.02	0.24	0.07	0.13	−0.05	−0.12	0.31	0.17	−0.06	0.10	0.28
P30											
Null alleles	No	No	No	Yes	Yes	No	No	No	Yes	No	—
AR	2.00	3.87	7.82	6.61	8.22	2.00	3.43	4.81	6.05	7.17	6.88
HE	0.36	0.51	0.68	0.71	0.82	0.35	0.47	0.62	0.72	0.74	0.67
F _{is}	0.03	0.13	0.14	0.34	0.19	0.12	0.01	0.06	0.26	0.10	0.24
P43											
Null alleles	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	No	—
AR	2.92	2.82	2.00	4.00	3.97	1.00	1.90	1.43	1.00	1.00	3.51
He	0.28	0.18	0.41	0.57	0.59	0.00	0.07	0.02	0.00	0.00	0.31
F _{is}	0.75	0.71	1.00	0.36	0.41	NA	1.00	0.00	NA	NA	0.73
P40											
Null alleles	No	No	Yes	No	No	No	No	No	No	No	—
AR	3.32	4.47	6.10	6.55	6.67	2.00	4.31	5.90	5.91	4.79	6.14
He	0.19	0.34	0.63	0.73	0.67	0.18	0.30	0.62	0.67	0.62	0.63
F _{is}	0.13	0.00	0.24	0.10	−0.16	0.08	0.11	−0.03	−0.14	−0.02	0.22
P39											
Null alleles	No	No	No	No	No	No	Yes	No	No	No	—
AR	1.00	2.97	3.83	3.82	3.97	1.00	3.81	3.97	3.68	4.00	3.85
He	0.00	0.23	0.42	0.62	0.65	0.00	0.30	0.48	0.51	0.66	0.52
F _{is}	NA	0.05	−0.14	0.06	0.07	NA	0.33	0.04	0.08	−0.02	0.28
P23											
Null alleles	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	—
AR	2.00	3.69	4.75	5.36	5.65	1.99	3.79	4.64	5.31	2.00	5.17
He	0.13	0.34	0.39	0.69	0.70	0.13	0.33	0.50	0.66	0.04	0.55
F _{is}	−0.07	0.30	0.73	0.72	0.81	−0.06	0.35	0.25	0.59	0.00	0.64
P13											
Null alleles	No	Yes	No	No	No	No	No	No	No	Yes	—
AR	1.00	3.81	4.80	4.90	3.89	1.00	2.82	4.28	4.39	4.81	4.74
He	0.00	0.32	0.52	0.71	0.53	0.00	0.23	0.42	0.66	0.62	0.55
F _{is}	NA	0.35	0.18	−0.15	−0.05	NA	−0.11	0.17	0.04	0.19	0.33
P36											
Null alleles	No	No	No	No	Yes	No	No	No	No	No	—
AR	1.96	2.99	2.91	2.69	3.00	1.99	2.98	2.90	2.68	2.00	2.94
He	0.09	0.28	0.47	0.51	0.59	0.13	0.32	0.41	0.50	0.29	0.50
F _{is}	−0.04	−0.13	−0.10	0.01	0.41	−0.07	0.00	0.11	−0.01	−0.20	0.30
All											
AR	2.45	3.51	4.55	5.06	5.05	1.89	3.50	4.09	4.22	3.78	4.81
He	0.25	0.40	0.54	0.66	0.64	0.22	0.36	0.46	0.55	0.46	0.56
F _{is}	0.10	0.14	0.21	0.17	0.17	0.08	0.15	0.09	0.15	0.13	0.31

The F_{is} values significantly different from 0 (*P*-value threshold after Bonferroni correction *P* = 0.0005) are in bold italic. NA = Not Available.

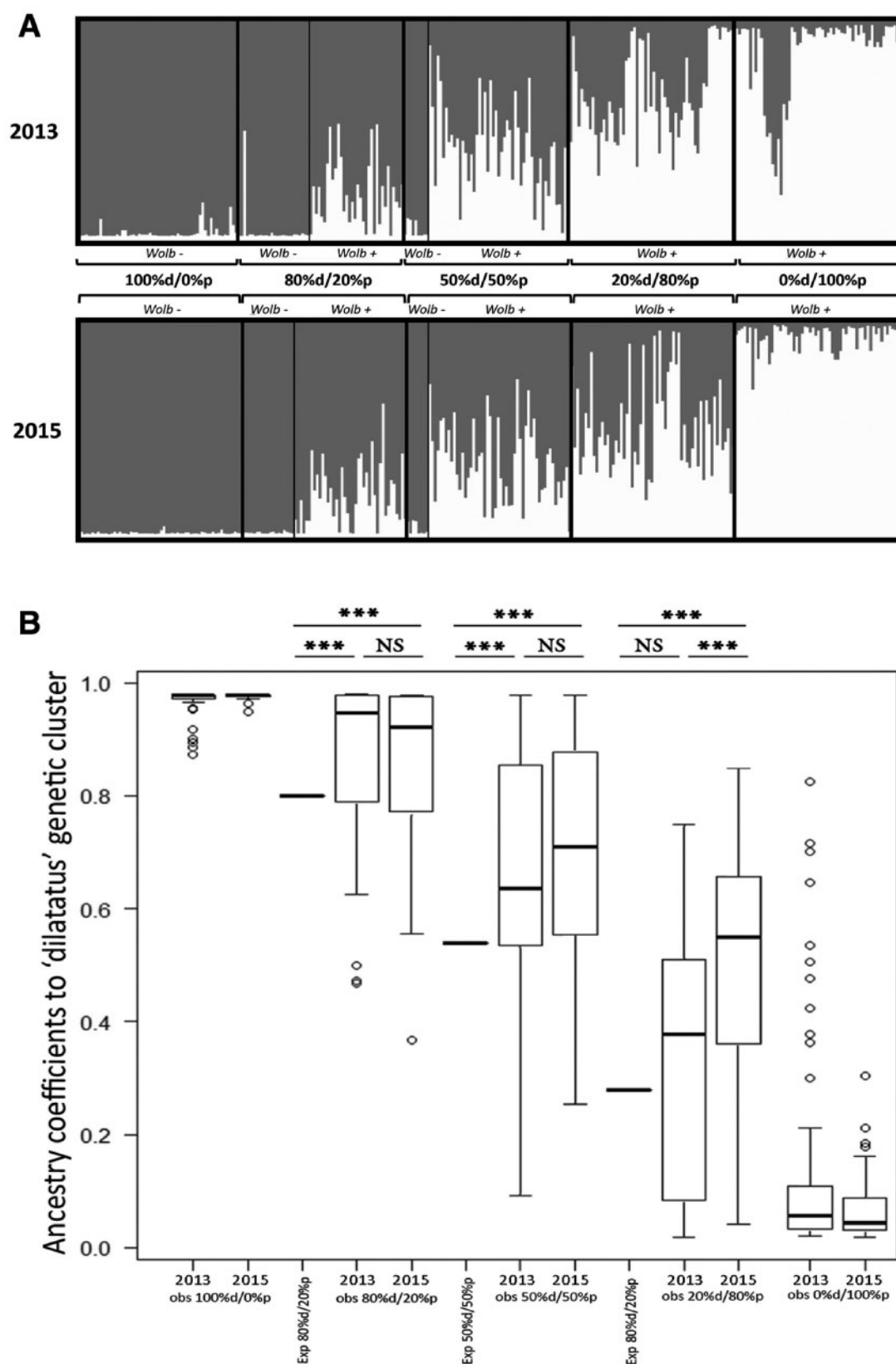


Figure 2. (A) Bar plot from STRUCTURE based on microsatellite data and showing clustering of investigated individuals assuming $K=2$. A vertical bar represents each individual. Each color corresponds to the coefficient of ancestry to the inferred genetic clusters (gray= genetic cluster associated to "*dilatatus*" and white= genetic cluster associated to "*petiti*"); black lines separate the individuals from different types of population cages. (B) Ancestry coefficients to "*dilatatus*" genetic cluster according to conditions (i.e., population cages and studied year as well as expected rates). NS: non-significant, *** $P < 0.0001$.

Table 2. Inferences to different parent and hybrid categories by NEWHYBRIDS, using microsatellite genotypes from individual sampled from different population cages (upper part). The lower part shows assignments of artificial generated individuals, which were performed to assess the performance of the method.

	New Hybrids categories							
Individuals from experimental population cages	<i>dilatatus</i>	<i>petiti</i>	F1	F2	F1x <i>dilatatus</i>	F1x <i>petiti</i>	Hybrid number	Hybrid rate (%)
2013								
100% <i>dilatatus</i>	55	0	0	0	2	0	2	4
80%d/20%p	36	1	0	11	11	0	22	37
50%d/50%p	14	1	0	32	12	0	44	75
20%d/80%p	0	19	0	39	2	0	41	68
100% <i>petiti</i>	1	46	0	10	2	0	12	20
Total 2013	50	21	0	82	25	0	107	60
2015								
100% <i>dilatatus</i>	59	0	0	0	1	0	1	2
80%d/20%p	32	0	0	12	15	0	27	46
50%d/50%p	13	1	0	28	18	0	46	77
20%d/80%p	0	9	0	41	9	0	50	85
100% <i>petiti</i>	0	54	0	5	0	0	5	8
Total 2015	45	10	0	81	42	0	123	69
Artificially generated individuals							Efficiency	
<i>dilatatus</i> (n = 2,200)	2,198	0	0	0	2	0	100%	
<i>petiti</i> (n = 2,200)	0	2,060	1	4	0	135	94%	
F1 (n = 1,100)	0	0	933	25	129	13	85%	
F2 (n = 1,100)	4	18	117	605	174	182	55%	
F1x <i>dilatatus</i> (n = 1,100)	59	0	90	24	927	0	84%	
F1x <i>petiti</i> (n = 1,100)	0	235	80	129	3	653	59%	
Total inferred	2,261	2,313	1,221	787	1,235	983	—	—
Accuracy	97%	89%	76%	77%	75%	66%	—	—

expected, NEWHYBRIDS inferred individuals to different hybrid or parental categories in all population cages, considering an individual as assigned to a category when its posterior probability of belonging to that category was >0.80 . NEWHYBRIDS inferred individuals to F2 hybrids or backcrossed (F1 X “*dilatatus*” hybrid) categories in all cages except in control population ones. However, no individual was inferred to F1 hybrids (consistent with the fact that the first sampling was carried out 5 years after the population cages were setup) nor to F1 X “*petiti*” hybrid categories (Table 2). These inferences are robust according to the estimations of both the efficiency and accuracy of our approach (Table 2). The proportions of hybrids remained stable between 2013 and 2015 ($\chi^2_2 = 0.33$, $P = 0.85$; Table 2). However, differences were observed between the types of population cages, with the 80%d/20%p cages presenting lower proportion of hybrids rate compared with 50%d/50%p and 20%d/80%p ones (Table 2).

Increase of the “*dilatatus*” nuclear genetic signature

F_{ST} values revealed significant genetic differentiation in all pairs of population cages (mean $F_{ST} = 0.191$; Table 3). Results from both STRUCTURE and STRUCTURE HARVESTER programs inferred the highest ΔK for 2 genetic clusters ($K = 2$) (Evanno et al. 2005) corresponding to the genetic signatures of the 2 subspecies (Figure 2A and Supplementary Material 2). Indeed, control “*dilatatus*” population cage (i.e., 100%d/0%p) revealed high ancestry coefficients to genetic cluster 1 associated with “*dilatatus*” genetic cluster (means ancestry coefficients: 0.96 and 0.97 in 2013 and 2015, respectively; Table S2 in Supplementary Material 2) although control “*petiti*” population cage (0%d/100%p) revealed high ancestry coefficients to genetic cluster associated to “*petiti*” genetic cluster (means ancestry

coefficients: 0.86 and 0.92 in 2013 and 2015, respectively, Table S2 in Supplementary Material 2). However, individuals from the control “*dilatatus*” cages revealed a more specific genetic signature, displaying significantly higher ancestry coefficients than individuals from “*petiti*” cages (in 2013: Mann–Whitney U-statistic = 628, $P < 0.0001$; in 2015: Mann–Whitney U-statistic = 311.5, $P < 0.0001$). In some 80%d/20%p and 50%d/50%p population cages, *Wolbachia* did not reach fixation. All uninfected individuals from these population cages were analyzed separately, showing that all but one individual (which could be considered as “an uninfected hybrid”) was pure “*dilatatus*” (see “*wolb-*” individuals in Figure 2A). Individuals infected with *Wolbachia* in mixed “*dilatatus*”/“*petiti*” population cages were all hybrids (see “*wolb+*” in Figure 2A).

As predicted by the null hypothesis, the ancestry coefficients inferred for “*dilatatus*” genetic cluster remained stable between 2013 and 2015 for population cages 100%d/0%p and 0%d/100%p (for 100%d/0%p: Mann–Whitney U-statistic = 1,500.5, $P = 0.25$; for 0%d/100%p: Mann–Whitney U-statistic = 1,600, $P = 0.30$) making possible the calculation of the average ancestry coefficients between 2013 and 2015. These average ancestry coefficients inferred for “*dilatatus*” genetic cluster for population cages 100%d/0%p and 0%d/100%p were 0.965 and 0.11, respectively. These coefficients led to the linear regression equation: $y = 0.855x + 0.11$ (0.855 being $[0.965 - 0.11]/1$). Based on this equation, the estimated mean ancestry coefficients to “*dilatatus*” genetic cluster of 2008 population cages 80%d/20%p, 50%d/50%p, and 20%d/80%p were 79%, 54% and 28%, respectively. The observed mean ancestry coefficients to “*dilatatus*” genetic cluster (for 2013 and 2015) are represented in Figure 2. According to the null hypothesis that the

Table 3. Mean pairwise F_{ST} values for each population cages ("dilatatus"/"petiti" %) comparison (below diagonal)

	100% d/0% p		80% d/20% p		50% d/50% p		20% d/80% p		0% d/100% p	
	2013	2015	2013	2015	2013	2015	2013	2015	2013	2015
100% d/0% p 2013	—									
100% d/0% p 2015	0.033	*								*
80% d/20% p 2013	0.171	—								*
80% d/20% p 2015	0.312	0.203		*						*
50% d/50% p 2013	0.419	0.307	0.117	—						*
50% d/50% p 2015	0.006	0.033	0.193	0.056						*
20% d/80% p 2013	0.040	0.005	0.212	0.329	0.437					*
20% d/80% p 2015	0.103	0.037	0.126	0.221	0.320	0.039				*
0% d/100% p 2013	0.236	0.124	0.095	0.130	0.215	0.104	0.030			*
0% d/100% p 2015	0.558	0.437	0.118	0.046	0.111	0.243	0.132	0.055	—	*
			0.344	0.143	0.089	0.572	0.448	0.334	0.182	—

All P -values were significant with a threshold of 0.001 after Bonferroni correction (*)

mean ancestry coefficient was constant during time, it appeared that, since 2008, the inferred averages of ancestry coefficients of "*dilatatus*" have significantly increased in all mixed cages even in those where *Wolbachia* have reached fixation (Figure 2B).

Discussion

In this study, we used individuals from 2 *P. dilatatus* laboratory lineages (the uninfected "*dilatatus*" lineage and the *wPet* *Wolbachia* infected lineage "*petiti*"), in different proportions to settle population cages and monitor *Wolbachia* infection, nuclear microsatellite and mitochondrial markers 5 and 7 years after their setting up. By this way, we showed that *Wolbachia*-induced CI in *Porcellio* subspecies did not prevent hybridization between infected "*petiti*" and uninfected "*dilatatus*" and led to the introgressive acquisition of *wPet* in genetic background dominated by "*dilatatus*" alleles.

The genetic differentiation between "*dilatatus*" and "*petiti*" lineages was assayed using nuclear microsatellite markers in order to evaluate the potential hybridization between them. Consistent with previous findings, based on a mitochondrial marker (Sicard et al. 2014), our results using nuclear microsatellite markers clearly revealed distinct genetic signatures between "*dilatatus*" and "*petiti*" lineages. However, this genetic divergence could be partially attributed to the geographic distance between sampling points as well as laboratory maintenance procedure since their sampling (~50 years). Besides, it was known that fertility was not affected when an uninfected male "*dilatatus*" was crossed with a "*petiti*" female (Legrand et al. 1978, 1980) while fertility of the reciprocal cross was strongly reduced due to *Wolbachia*-induced CI (Legrand et al. 1978; Sicard et al. 2014). With the help of specific microsatellite alleles, we were able to detect, in this study, hybrids belonging to the F2 and back-crossing categories, both after 5 and 7 years following the setting up of cages. These results demonstrate that F1 hybrids were fertile and do not provide evidence for outbreeding depression. Unidirectional CI and the initial proportions of "*petiti*" individuals compared with "*dilatatus*" ones in our population cages certainly explain the lower hybridization rate observed in 80% d/20% p (around 40%) population cages compared with 20% d/80% p ones (around 75%). Indeed, as "*petiti*" males cannot generate hybrid due to CI, only a very limited number of "*petiti*" females were able to generate hybrids in population cages with initial proportion of "*petiti*" at 20%. We observed, in ~7 generations, a global increase in *Wolbachia* frequency over time in our (non-control) population cages even though infection frequency remained stable or even decreased in 2 population cages (1 in 80% d/20% p and 1 in 50% d/50% p; see Figure 1), likely due to stochastic effects. Fixation of *Wolbachia wPet* was only reported in 3 80% d/20% p and 4 50% d/50% p population cages (Figure 1). In comparable cage experiments conducted with *Aedes aegypti* transinfected with *wAlbB* from *Aedes albopictus*, having a similar CI penetrance, only 7 generations were required for full fixation with a 20% initial release (Xi et al. 2005). The slower invasion of *Wolbachia* observed in our experiment is unlikely due to imperfect vertical transmission, as the absence of discordance between mitotype and *Wolbachia* indicates a perfect vertical transmission of both "*petiti*" mitochondrion and *Wolbachia wPet* during the 7 years of the experiment. However, several other nonexclusive hypotheses could explain such slowdown in *Wolbachia* invasion: (1) assortative mating (preferentially among uninfected individuals; Moreau et al. 2001); (2) differential male competitiveness in the access to the sexual partner (Beltran-Bech and Richard 2014); and/or (3) a cost of harboring *Wolbachia* in terms of survival and brood sizes (Braquart-

Varnier et al., 2008; Sicard et al., 2010). Concomitantly to the growing infection frequency of *Wolbachia* *w*Pet, which was exclusively found in “*petiti*” individuals when we started the experiment, we did not observe an increase of the nuclear genetic signature of “*petiti*” from microsatellites data, but on the contrary an increase of the “*dilatatus*” genetic signature (Figure 2; Supplementary Material 2). In principle, in cages that contained 50% of “*dilatatus*” and 50% of “*petiti*” at the beginning, there is no obvious reason why CI should favor the microsatellite alleles of a particular lineage. Indeed, although infected males (initially from the “*petiti*” lineage) should be less efficient at producing hybrids due to CI, the same is true for noninfected females from the “*dilatatus*” lineage. The better performance of “*dilatatus*” alleles can supposedly come from (1) better general performances of “*dilatatus*” in laboratory conditions, (2) better mating performance for the latter, and/or (3) a fitness cost of carrying *Wolbachia* that may reduce the fitness of hybrids coming from infected (“*petiti*”) mothers. In addition to difference of mating performance and *Wolbachia* infection, reinforcement mechanisms, leading to the evolution of assortative mating to circumvent CI deleterious consequences, might also be involved, as previously described in several insect species (Bordenstein et al. 2001; Jaenike et al. 2006). In the 80%*d*/20%*p* population cages, the highly prevalent “*dilatatus*” males might have preferentially mated with females from their own lineage (i.e., assortative mating). In this context, assortative mating would explain the lower hybridization rate observed in the 80%*d*/20%*p* population cages compared with 20%*d*/80%*p* ones. A modeling approach simulating various degrees of fitness cost induced by *Wolbachia* and various levels of mating preference/performance, combined with behavioral experiments on mate preferences and reproductive success, could allow testing the effect of these parameters on the CI-induced *Wolbachia* spread and on the progression of the nuclear genetic signatures of its hosts.

Using genetic tools, we demonstrated herein that hybridization between the 2 lineages “*dilatatus*” and “*petiti*” gave rise to fertile hybrids further than F1. Microsatellite-specific markers showed that this hybridization was asymmetrical, leading to an increase in the nuclear genetic signature of “*dilatatus*” at the expense of that of “*petiti*.” Concomitantly, we also monitored an increase in *Wolbachia* frequency, due to both strong CI penetrance and a perfect vertical transmission. Asymmetry in gene flow between the 2 taxa, the perfect transmission *Wolbachia* and CI led to the production of hybrids with nuclear genomes dominated by “*dilatatus*” alleles and with cytoplasm hosting *w*Pet *Wolbachia* and “*petiti*” mitochondrial genome. These results document a rapid experimental introgressive acquisition of *Wolbachia* between 2 closely related taxa. Introgressive acquisition of *Wolbachia* could be thus a major mechanism that may explain why host and *Wolbachia* cladogenesis is not conserved at the macro-evolutionary scale although the perfect vertical transmission is observed at each generation.

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Data Availability

The full dataset is available as Supplementary Material 3.

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

Conflict of interests

The authors declare that they have no conflict of interest.

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