

## **Wolbachia Infection in the *Chorthippus parallelus* Hybrid Zone: Evidence for Its Role as a Reproductive barrier**

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# Wolbachia infection in the *Chorthippus parallelus* hybrid zone: evidence for its role as a reproductive barrier

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## Abstract

*Chorthippus parallelus parallelus* (Cp) and *Chorthippus parallelus erythropus* (Cpe) are two grasshopper subspecies whose distributions overlap in the Pyrenees, where they form a hybrid zone at those points where the topography and their ecological requirements allow. This hybrid zone is a consequence of secondary contact between endemic Iberian (Cpe) and continental European populations (Cp) that expanded after the most recent glaciations from the refugia in which they had diverged genetically in allopatry.

The morphological, physiological, genetic and behavioral differences between these subspecies (and their natural and laboratory-reared hybrids) have been intensively studied in recent years, for which reason this hybrid zone is considered a singular model in evolutionary biology. These studies reveal a complex pattern of factors involved in the origin, structure and maintenance of the hybrid zone. They offer an excellent panorama of evolution "in action".

*Wolbachia* is a genus of obligate endosymbiont bacteria that induce changes in the reproduction of arthropods and nematodes. In previous studies we have reported the existence of these bacteria in individuals and populations of *Chorthippus parallelus*. Now we show that they produce a significant reproductive barrier in this hybrid zone, implying, as proposed elsewhere, that *Wolbachia* may be involved in speciation phenomena.

## Key words

Hybrid zones, *Chorthippus parallelus*, *Wolbachia*, speciation

## Introduction

The Pyrenean hybrid zone (HZ) of the orthopteroid *Chorthippus parallelus* (Acrididae: Gomphocerinae. Zetterstedt, 1821) is formed by Iberian endemism of *C.p. erythropus* (Cpe) with the continental European taxon *C.p. parallelus* (Cp) (Reynolds 1980). The multiple morphological, cytogenetic, ecological, ethological and molecular studies done in recent years have contributed valuable information about this model for the study of the processes involved in genetic divergence and speciation (Butlin 1998, Shuker *et al.* 2005a).

The origin and establishment of this HZ is attributed to the isolation experienced by the ancestral populations of this grasshopper that, because of the Quaternary glaciations, formed refugia in the southernmost zones of the European continent: the Iberian, Italian and Balkan peninsulas. The retreat of the ice allowed these organisms to recolonize habitats from the south towards the north. During this isolation and slow overall advance, allopatric genetic divergence between the Iberian populations and those of the rest of Europe took place, generating a panorama of "refugia into refugia" with a discontinuous distribution of Iberian populations (Bella *et al.* 2007). The barrier of the Pyrenees has prevented gene flow and maintained this separation between the two taxa, except in those regions where the topographic characteristics allow the formation

of the aforementioned HZ: both ends of the mountainous barrier, and the areas and cross-sectional valleys that link Spain and France (Cooper *et al.* 1995, Hewitt 2004). The laboratory-produced F1 hybrids between the two pure subspecies strictly obey Haldane's rule, producing viable but sterile hybrid heterogametic X0 males (Hewitt *et al.* 1987, Bella *et al.* 1990). Current natural hybrids and populations are fit after the approximately 9,000 generations that have arisen since the origin of the HZ, during which recombination has broken down the gene complexes underlying the epistatic incompatibilities generated in allopatry (see also Shuker *et al.* 2005b).

The significance of an HZ (as a region where genetically different taxa hybridize and leave viable descendants) to this type of study is beyond dispute, but this example is especially relevant: we have recently noticed that *Wolbachia* (Rickettsiales) infects *C. parallelus* individuals inside and outside the HZ (Martínez *et al.* 2009, Zabal-Aguirre *et al.* 2010). A previous report had already detected this bacterium in the gut of *C. parallelus* (Dillon *et al.* 2008).

*Wolbachia* are gram-negative bacteria described as obligate endosymbionts of an enormous variety of invertebrates, such as insects, spiders, mites, crustaceans and nematodes. Transmission seems to be fundamentally maternal and it is accepted that up to 75% of invertebrate species can be infected (Hilgenboecker *et al.* 2008).

The interactions of this bacterium with the host are complex, potentially ranging from mutualism to pathogenesis. The "reproductive parasitism" that it often shows is remarkable, altering the reproduction of its host to facilitate its own transmission by the following mechanisms: i) Cytoplasmic Incompatibility (CI): crosses between infected males and uninfected females yield fewer or no descendants. This pattern is associated with the absence of fertilization or the death of the embryos. ii) Induction of Parthenogenesis: infected females are able to reproduce asexually from unfertilized ova. iii) Feminizing Effect: females carrying *Wolbachia* mainly yield females by selective death of male embryos, or infected male embryos develop like functional, morphologically normal females (Werren 1997, Moran *et al.* 2008, Serbus *et al.* 2008).

In this article we bring up to date our results concerning the distribution of *Wolbachia* in populations of *C. parallelus* inside and outside the HZ, as well as those about the phylogeny of the bacterium. We also present introductory results from experimental crosses set up to ascertain effects on the fecundity of infected individuals and on spermatogenesis.

Our findings are evidence of *Wolbachia*'s capacity to induce or reinforce genetic divergence between organisms and contribute to the continuing debate about a model of "speciation by infection" (Wade 2001).

### Distribution of *Wolbachia* in populations of *Chorthippus parallelus*

In a previous article (Zabal-Aguirre *et al.* 2010) we reported that populations of *C. parallelus* are infected to varying degrees by two strains of *Wolbachia* belonging to the B and F supergroups, as identified by Bayesian likelihood inference, based on their 16S rDNA genes (Giordano *et al.* 1995, Lo *et al.* 2002). Four categories of individuals and therefore of populations, are possible: infected by the B strain, by the F strain, co-infected (BF) and uninfected (0). The analysis of almost 5,000 individuals from 16 populations inside and outside the HZ enabled us to confirm that all the populations are infected by this bacterium, although to varying degrees. In general, we did not detect any statistically significant differences in patterns of infection between the sexes in these populations.

These differences in the proportions of the different types of infection (B, F, BF and O) from population to population, reveal three geographic patterns of distribution of the infection: northern, Iberian and hybrid. Northern-European locations show a low prevalence of infection and their individuals are mainly infected by the B strain. In Iberian populations, most grasshoppers carry *Wolbachia*, but have different proportions of B, F, BF and uninfected individuals. There is a north-south gradient between these two patterns along the HZ, from low to high frequencies of infection, but it is noticeable that in the hybrid population of Sallent de Gállego, which is geographically close to the middle of the HZ, a new pattern appears that is characterized by an extremely high degree of co-infection with the two *Wolbachia* types in its individuals. This co-infection peak in Sallent de Gállego was interpreted as existing through strong positive selection for individuals harboring both bacterial supergroup infections (Zabal-Aguirre *et al.* 2010); its cline coincides with a cytogenetic marker associated with the X-chromosome (Gosálvez *et al.* 1988, Serrano *et al.* 1996).

Our current results are consistent with those of the previous study. At present, we have data from approximately 7,000 individuals from 20 populations inside and outside the HZ. Updating our analyses confirms the existence of the aforementioned three biogeographical patterns, and provides further evidence for the

presence of an infection area in the HZ for this north-south cline, between the two patterns generated by the frequencies of the types of the *Wolbachia* infection (Fig. 1, Table 1). This "hybrid zone pattern" also emerges from the phylogenetic analysis of these data (see below), highlighting the different, specific interaction between the bacterium and the hybrid grasshoppers.

### Phylogeographic analysis of European populations of *Wolbachia*

A wide-ranging survey of *Wolbachia*-infected European populations of *C. parallelus* has been carried out in recent years. The bacteria from infected individuals from 14 populations from Spain, UK, Italy and France (Martínez-Rodríguez *et al.* in prep.; data not shown) have been characterized for their 16S rDNA, *wsp* gene, and a further five loci that make up a "Multi Locus Sequence Typing System" (MLST) specific to this bacterium (Baldo *et al.* 2006).

MLST genes (*coxA*, *fbpA*, *hcpA*, *ftsZ* and *gatB*) and *wsp* (*Wolbachia* surface protein) sequences in infected *C. parallelus* individuals were amplified and analyzed following the methods of Baldo *et al.* (2006). Identical nucleotide sequences at a given MLST gene of different samples were assigned the same arbitrary and unique allele number. A sequence type (ST) is characterized by the combination of the five MLST allele numbers: each ST corresponds to a different "strain". On the other hand, a ST complex is defined as a group of STs sharing at least three alleles, as defined above. This analysis allows us to discriminate the *Wolbachia* variability described in *C. parallelus* in two B- and 12 F- strains, and at least four ST- complexes.

The topology of the phylogenetic tree was constructed by Bayesian inference using the MrBayes 3.1 program (Ronquist & Huelsenbeck 2003) on the basis of concatenated MLST genes sequences. The GTR+G+I model of sequence evolution was selected using hierarchical likelihood ratio tests available in the jModeltest 3.7 program (Posada 2008). The two independent runs performed, each with four chains, reached convergence of the split frequencies after 10<sup>6</sup> generations and a sample frequency of 100. The first 2,500 trees were discarded as burn-in, and the 50% majority rule was applied to the remaining set of trees.

**Table 1.** Infection frequencies in the *C. parallelus* populations analyzed, with their location and altitude.

Population	B-infected	F-infected	Coinfected	Uninfected	Number of individuals	Coordinates		Altitude (m)
Sabiñánigo	0.05	0.91	0.05	0.00	22	42°31'06"N	0°20'51"W	766
Biescas	0.00	0.56	0.41	0.03	34	42°37'31"N	0°19'39"W	848
Escarrilla	0.04	0.55	0.27	0.14	78	42°43'54"N	0°18'39"W	1,130
Sallent de Gállego	0.23	0.02	0.74	0.01	127	42°45'57"N	0°20'33"W	1,343
Corral de Mulas	0.12	0.15	0.32	0.41	123	42°47'09"N	0°23'34"W	1,569
Portalet	0.08	0.03	0.18	0.72	120	42°48'03"N	0°24'54"W	1,708
Cabaña Antigua	0.10	0.01	0.20	0.69	99	42°48'51"N	0°25'05"W	1,695
Cabaña Tourmont	0.18	0.08	0.01	0.73	84	42°49'11"N	0°24'21"W	1,625
L'Hermine	0.09	0.10	0.18	0.63	96	42°51'46"N	0°23'30"W	1,209
Soques	0.16	0.04	0.21	0.60	82	42°50'07"N	0°23'02"W	1,416
Gabas	0.19	0.00	0.03	0.78	91	42°53'60"N	0°25'60"W	1,020
Arudy	0.24	0.09	0.05	0.62	66	43°07'00"N	0°25'60"W	400
Navafría	0.06	0.44	0.25	0.24	108	40°59'02"N	3°49'00"W	1,710
Becedas	0.07	0.34	0.20	0.39	83	40°24'18"N	5°38'17"W	1,097
Bubión	0.00	0.89	0.08	0.04	53	36°57'01"N	3°21'22"W	1,300
Epping Forest	0.40	0.00	0.00	0.60	15	51°39'36"N	0°03'00"E	107
Massif Centrale	0.30	0.10	0.00	0.60	10	44°28'43"N	3°45'35"E	1,088
Col de Larche	0.23	0.00	0.00	0.77	22	44°24'60"N	6°52'60"E	1,942
L'Argentera	0.34	0.06	0.03	0.57	35	44°14'00"N	7°23'00"E	877
Valdieri	0.21	0.00	0.00	0.79	33	44°12'00"N	7°21'60"E	1,007

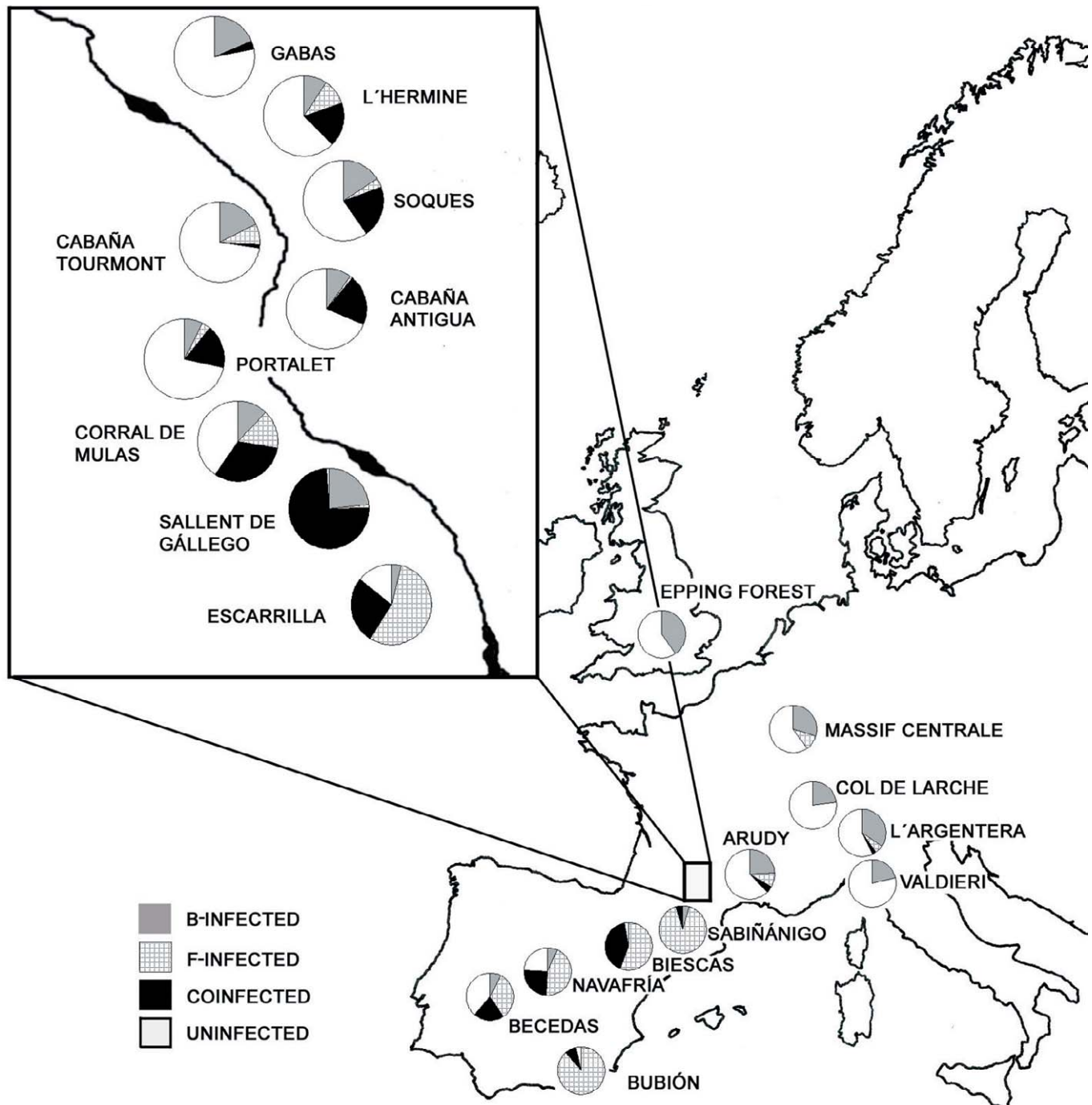


Fig. 1. Map showing the *Wolbachia* infection in sampled populations. Note the south-north gradient. Actual frequencies are reported in Table 1.

The allelic profiles obtained are associated with the geographical distribution of the *C. parallelus* populations analyzed and concord with the northern, southern, and HZ patterns described above. However, we intend to extend this analysis with data from other European populations — from Norway, Germany, Italy and Slovenia; in doing so, we are open to the possibility that other ST complexes may appear.

The B supergroup (ST1 complex) homogeneously infects both subspecies. The F supergroup is more variable than the B, with three ST complexes: ST2 infects pure *Cpp* populations and some hybrid populations on the other edge of the HZ; ST3 infects some

populations in the center of the HZ; the ST4 complex infects pure and some hybrid populations on the edge of the HZ (Fig. 2a).

*Wsp* typing reveals a similar pattern (Fig. 2b). To date, six *wsp* types have been reported in pure (*Cpe* and *Cpp*) and hybrid populations. The Bayesian tree based on *wsp* sequences showed that the different lineages found in *C. parallelus* are clustered in three groups: *wsp* 1, 2 and 3 (F supergroup) in *Cpe* and hybrid populations; *wsp* 4 and 5 belong to the F supergroup, but they only appear in *Cpp* populations. However, *wsp* 6 clearly groups with the other *Wolbachia* that make up the B supergroup.

The analysis of allelic profiles in the ST3 complex reveals that



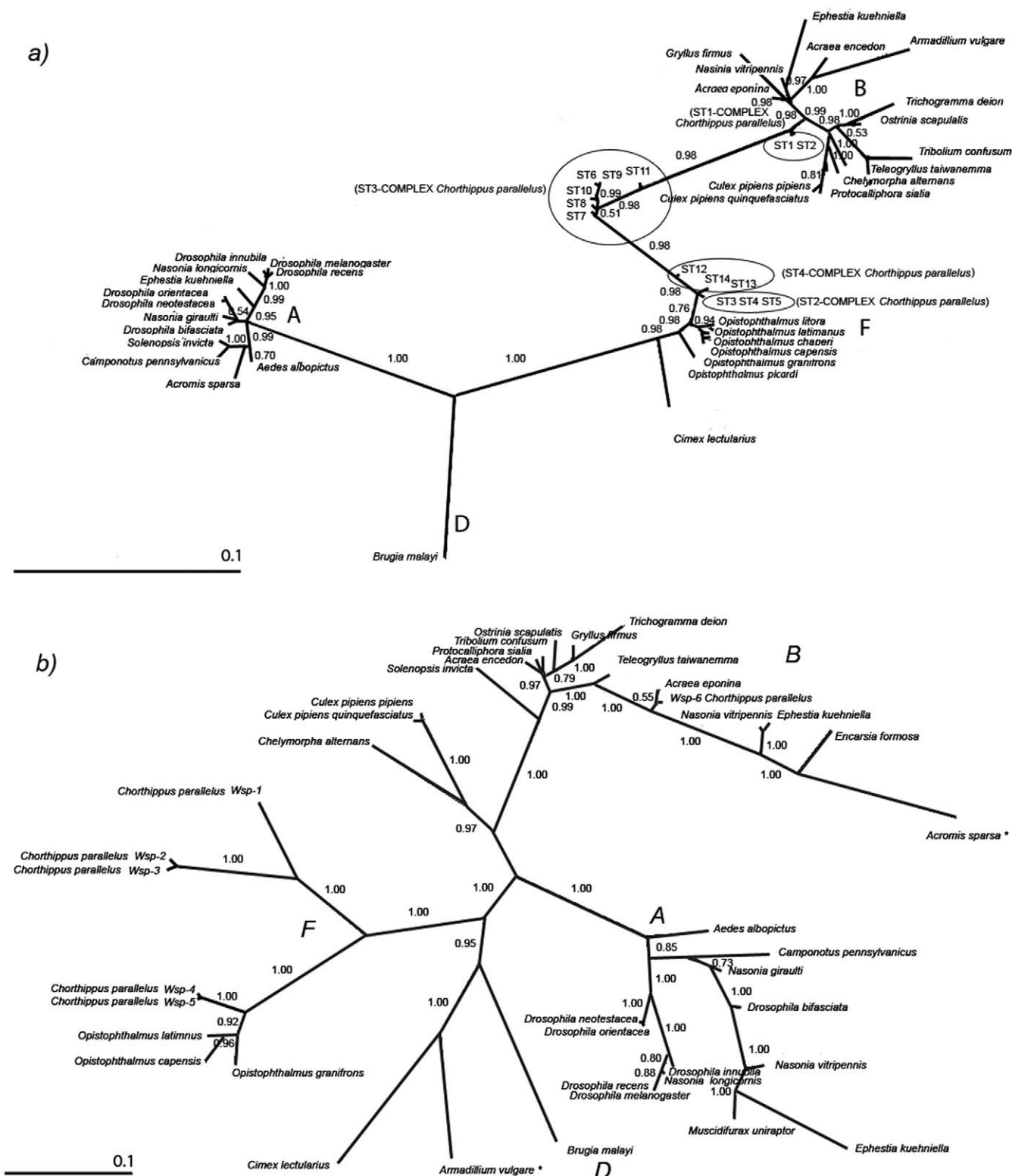
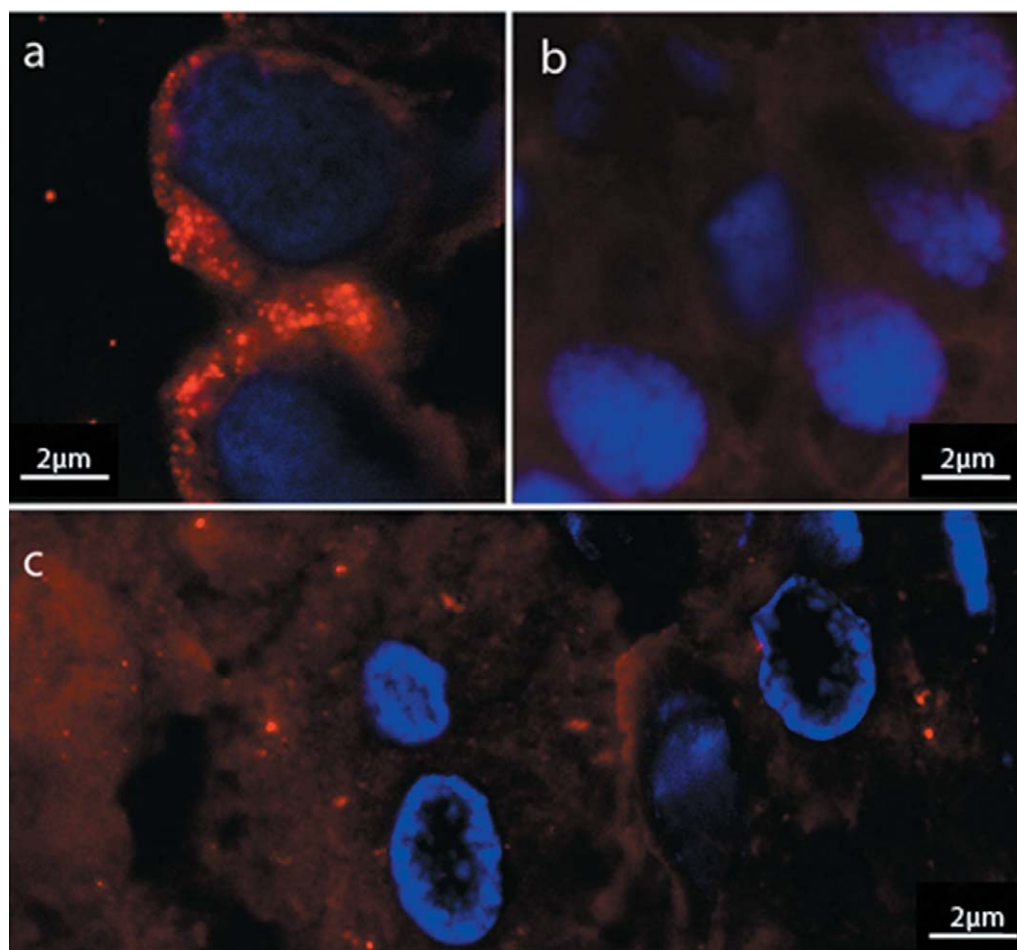


Fig. 2. a) Bayesian likelihood inference based on concatenated sequences from the Multilocus Sequence Typing system (MLST), following Baldo *et al.* (2006). A-F denote *Wolbachia* supergroups. Species names indicate the infected hosts. The ST1 complex belongs to the *Wolbachia* B supergroup. The ST2 and ST4 complexes are clearly ascribed to the F supergroup of the bacterium. However the ST3 complex shows an intermediate position, interpreted as indicating recombination between the F and B supergroup complexes. b) Bayesian likelihood inference based on *wsp* sequences, also showing a three-group distribution: *wsp*-1, 2, 3, 4 and 5 belong to the F supergroup, while *wsp* 6 is clearly grouped with the other *Wolbachia* that make up the B supergroup. Asterisks indicate discordances (discrepancies) between the MLST and the *wsp* supergroup classification.



**Fig. 3.** Whole-cell *in situ* hybridization of *Wolbachia*-infected *C. parallelus* female individuals in paraffin-embedded tissue sections. a) Infected individual showing the cytoplasmic distribution of the infection (red signal) in germinal cells (egg tubes). *Wolbachia* do not appear in the nuclei counterstained with DAPI (blue). b) Uninfected individual (control). No hybridization appears. c) Accessory gland epithelium infected by *Wolbachia*. For color version, see Plate I.

some alleles of *ftsZ*, *coxA* and *hcpA* genes belong to the B supergroup, despite being characterized in F supergroup individuals by 16SrDNA, *fbpA*, *gatB* and *wsp* genes. These variant alleles constitute the ST3 complex and only appear in the center of the HZ.

The variations in the ST3 complex are interpreted as evidence of bacterial recombination between F and B supergroups (see also Werren & Bartos 2001). It is striking that they occur exclusively in the Pyrenean HZ between two subspecies of this grasshopper, and where their Iberian and northern infection patterns overlap (see above). Again, this is coincident with the hybrid area where we find the aforementioned HZ pattern for infection frequencies (the Sallent de Gállego hybrid population), supporting the idea of specific *Wolbachia* infection dynamics for the hybrids and the HZ (Zabal-Aguirre *et al.* 2010). No evidence for bacterial recombination has been found in populations outside the HZ.

#### Effects of *Wolbachia* on *Chorthippus parallelus* individuals and populations

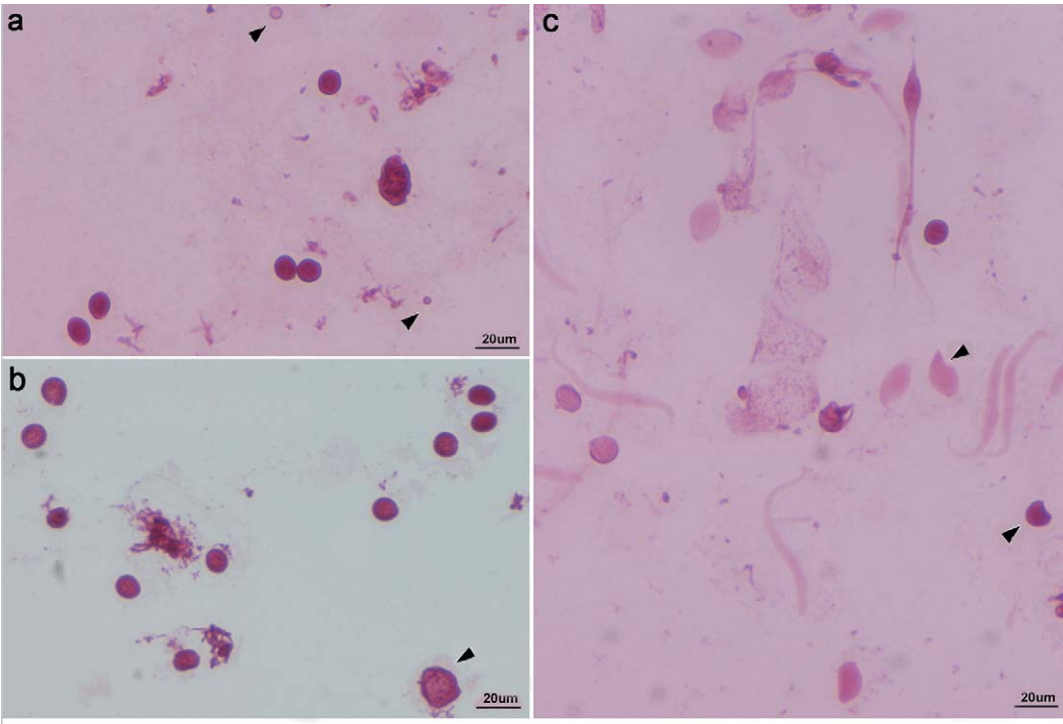
Whole-cell hybridization (Amann *et al.* 1990, 1995; García-Moyano *et al.* 2007) allows *in situ* localization of *Wolbachia* in squashed and even in paraffinized tissues of *C. parallelus* (Martínez *et al.* 2009). Short fluorescent monolabelled oligonucleotides target bacterial RNA for *in situ* hybridization in host cells and tissues. The large quantity of bacterial mRNA per cell produces an intense signal, which not only allows us to detect the bacteria, but also provides information about the distribution of the infection and its density.

In this study Martínez *et al.* (*loc. cit.*) showed that *Wolbachia* is

widely distributed in different tissues of the infected individuals of *C. parallelus*, showing high bacterial density in male and female gonads. Differences in bacterial density among tissues and individuals were also detected, but not quantified. *Wolbachia* is transmitted through the egg cytoplasm, which explains the high bacterial density in female gonads. However, high bacterial densities in male gonads may be related to the sperm modification by *Wolbachia* proposed by the “modification and rescue” model (Werren 1997, Charlat *et al.* 2001, Serbus *et al.* 2008). *Wolbachia* infection in the testes of *C. parallelus* seems to be homogeneous and all follicles are infected (Fig. 3). However, there is a notable cytoplasmic redistribution of bacteria during male meiosis: a progressive polarized organization occurs, with bacteria disappearing and aggregating at one of the poles of the prespermatid cell, finally disappearing in mature spermatid cells. The proposed sperm modifications by the “modification and rescue” model must therefore occur at the beginning of meiosis (Martínez *et al.* 2009).

The consequences of infection of male gametogenesis in *C. parallelus* have been quantified by analyzing the proportion of abnormal spermatids (morphology and size: aberrant, micro- and macro-spermatids) in pure populations of both subspecies and from infected and uninfected individuals from the hybrid Pyrenean population of Portalet (Fig. 4) (Bernal *et al.* in prep). Testicular follicles were squashed and stained with orcein and observed under light microscopy (Zeiss Standard, 40X). 1,500 spermatids were scored from each individual before their infection status was determined. The preliminary results obtained indicate an increasing frequency of abnormal spermatids, ranging from uninfected pure to infected hybrid individuals: more than 30,000 spermatids from 20 individu-

Fig. 4. Arrows indicate orcein-stained micro- (a), macro- (b) and morphologically abnormal (c) spermatids from *C. parallelus* individuals infected by *Wolbachia*. Observations made under light microscopy (40×). For color version, see Plate I.



als from the corresponding categories (pure infected/uninfected individuals and infected/uninfected hybrids) were recorded and their abnormal spermatids scored. Pure uninfected individuals (independent of the subspecies) show 0.7% abnormal spermatids, which is comparable to the standard for other grasshoppers (Teruel *et al.* 2009). Uninfected hybrids display 2% abnormality, indicating some sort of genomic conflict and consistent with the idea of this HZ as a “tension zone”, *i.e.*, one maintained by a balance between the dispersal of individuals and selection operating against them (see Hewitt 1990, Shuker *et al.* 2005a, but also Shuker *et al.* 2005b). *Wolbachia*-infected pure individuals of *Cpp* and *Cpe* have more abnormal spermatids (3.2%), while this figure reaches 6% in hybrid-infected individuals. These differences are all statistically significant (data and analyses not shown).

This confirms that *Wolbachia* induces modifications in the male germinal cells of grasshoppers and reveals the existence of synergistic effects of the “hybrid” and “infected” traits: either of them separately induces irregular spermatids and, in conjunction, noticeably increase the frequency of irregularity. It is clearly difficult to judge the real effect of this production of aberrant spermatids on the fertility and fecundity of the affected individuals, since we cannot simply assume a direct relationship leading to a real reduction in the number of their descendants. Nevertheless, it should also be remembered that spermatogenesis is a complicated process and that the journey from spermatid to sperm is a long one. The percentage of cells not completing this process cannot be any lower than the numbers described here, but we cannot discount the possibility that the effects of *Wolbachia* on the sperm continue, even increasing this frequency of abnormal mature germ cells. There is other evidence of the effect of *Wolbachia* on cell division and sperm formation (Wade & Chang 1995, McGraw *et al.* 2001, Rancès *et al.* 2008, Serbus *et al.* 2008).

In any case, this draws attention to a possibly important effect of *Wolbachia* on the dynamics of the HZ: the data presented below on the effect of the infection on the number of offspring arising from certain crosses support this idea, demonstrating the existence

of uni- and bidirectional cytoplasmic incompatibility (CI) in the HZ. As stated above, *Wolbachia* infection in many organisms produces an alteration that reduces or impedes reproduction when the uninfected female is crossed with an infected male. This is called unidirectional CI. Similarly, interference through bidirectional CI has been described, wherein both the male and the female are infected by distinct bacterial strains (Moran *et al.* 2008, Serbus *et al.* 2008). Both reproductive alterations are present in our system (Table 2).

In order to ascertain the effects of *Wolbachia* in the HZ, we have analyzed the descendants of crosses between individuals from the Pyrenean hybrid population of Portalet. Regarding the *Wolbachia* infection, in this population all the possible types of individuals are represented (B- or F-infected, co-infected and uninfected) (Zabal-Aguirre *et al.* 2010). To ensure that the females were virgin, we initiated crosses with larvae. The crosses were blind, *i.e.*, we did not know the infection status of the parents. This required us to establish a large number of crosses to ensure that all possibilities were sufficiently represented. As controls, we also made inter- and intrapopulational crosses with individuals from the pure populations of Escarrilla (*Cpe*) and Gabas (*Cpp*).

**Table 2.** Types of crosses and presumed cytoplasmic incompatibilities (uni- and bidirectional) derived from the infection status of the parental individuals. Key: c, compatible cross; UCI and BCI, unidirectional and bidirectional cytoplasmic incompatibility, respectively.

♀ \ ♂					
		O	B	F	BF
O		c	UCI	UCI	UCI
B		c	c	BCI	BCI
F		c	BCI	c	BCI
BF		c	c	c	c



Finally, the descendants of 141 valid crosses between pure and hybrid individuals of *Cp* of the different types of infection were scored (close to 800 egg pods, more than 6,000 eggs) and the parents typed by the 16SrDNA and nested PCR analyses following Zabal-Aguirre *et al.* (2010) (Zabal-Aguirre *et al.* in prep.; data and analyses not shown).

To estimate possible *Wolbachia*-induced CI, the embryo proportions (number of embryos/number of eggs) from the crosses were compared. To check the potential effects of *Wolbachia* infection on female fecundity, we measured the number of eggs (quantity) and embryo proportions (quality) in females with different infection statuses from those crosses defined as compatible in Table 2.

The controls made with pure *Cpp* and *Cpe* individuals (collected in pure populations from both ends of the HZ) yielded no significant differences among intra- (*Cpp* × *Cpp* and *Cpe* × *Cpe*) or intersubspecific crosses (*Cpp* × *Cpe* and *Cpe* × *Cpp*). No significant differences between compatible pure and hybrid crosses were found. Nevertheless, in crosses susceptible to unidirectional CI (female uninfected × infected male, Table 2) there was a significant 32.5% reduction in the number of embryos obtained. A smaller reduction (16.4%) was found in the crosses susceptible to bidirectional CI. No significant differences in female fecundity were found with respect to infection status or pure/hybrid condition.

These results indicate the existence of cytoplasmic incompatibility in the HZ of *C. parallelus*. Other models show higher rates of incompatibility, but we should emphasize that these studies have usually been conducted under laboratory conditions. Studies performed in nature usually give rise to lower rates of incompatibility than those done in the laboratory (see Hoffmann *et al.* 1998). Our data come from experimental crosses set up in the field, a setting that more closely matches the natural conditions and mechanisms operating in these organisms within the HZ.

### **Wolbachia as a reproductive barrier in the *Chorthippus parallelus* hybrid zone**

We now have distinct lines of evidence that *Wolbachia* markedly affects the dynamics of the *C. parallelus* HZ: the biogeographical distribution of bacterial strains and infected individuals and the phylogenetic data clearly indicate that the Pyrenean HZ is a "hot spot", with its own peculiar pattern of frequencies (the so-called "Sallent de Gállego pattern") and evidence of bacterial recombination events that appear to be exclusive to the contact zone (Martínez-Rodríguez, in prep.). Moreover, the analysis of spermatid cells clearly denotes that *Wolbachia* modifies the sperm of the infected individuals of *C. parallelus*. Considering the biogeographical distribution of the infection in these subspecies by itself, suggests the influence of the bacterium in the HZ. The remarkable observation that infected natural hybrid individuals have a significantly higher frequency of abnormal spermatids firmly supports this hypothesis. The demonstrated existence of the two types of cytoplasmic incompatibility operating in the HZ demonstrates *Wolbachia* to be an effective reproductive barrier in this case. Our current efforts attempt to model the influence of the distinct strains of the bacteria in the HZ, examining in greater depth the mechanisms that operate in *Wolbachia* and analyzing its maternal and horizontal transmission in our model.

The role of *Wolbachia* in inducing or reinforcing genetic divergence is a stimulating topic that has only recently attracted the attention of researchers. This bacterium has been proposed as a possible speciation agent (Werren 1998; Telschow *et al.* 2005, 2007), in the context of the "infectious speciation" model (Wade 2001). Some evidence for this exists in the wasp *Nasonia* (Werren 1997, Borden-

stein *et al.* 2001, Bordenstein 2003) and the ant *Acromyrmex* (Van Borm *et al.* 2001). Our results contribute to the controversy in the form of data derived from natural populations of this well-known grasshopper.

The *C. parallelus* HZ is evidently the result of complex interactions involving genomic, genetic, cytogenetic, ethological and ecological factors, amongst others. We do not propose that the *Wolbachia* infection entirely explains the origin and maintenance of the HZ. However, we now know that we have to consider this new factor, since its influence is not negligible. These results raise other questions, a major one being whether the infection is recent or ancient. The answer is central to assessing the role of the bacterium in this HZ: in the case of *Nasonia*, for example, *Wolbachia* has a fundamental, but not unique, role in its reproductive isolation. However, the interactions between the nucleus, cytoplasm and bacteria are crucial and complex, establishing different cytotypes as a result of the relations between the nuclear, mitochondrial and bacterial DNAs (Bordenstein *et al.* 2001, 2003; Ellison *et al.* 2008). These cytotypes are those that interact, cooperate or compete, and ultimately explain the complicated circumstances in which we find *Wolbachia*.

The *C. parallelus* HZ joins the list of systems in which *Wolbachia* contributes to a reproductive barrier, in this case between the two subspecies *Cpp* and *Cpe*, and in a manner that we can quantify in nature. Other studies currently underway will allow us to assess the extent of this effect and its influence on the origin and maintenance of an HZ whose character is turning out to be even more fascinating than we had ever thought.

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