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COMPARISON OF *WOLBACHIA* BACTERIAL DENSITY IN FEMALES OF FOUR THELYTHOKOUS STRAINS OF *TRICHOGRAMMA CORDUBENSIS* AND *T. EVANESCENS* (HYMENOPTERA, TRICHOGRAMMATIDAE)

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Сравнение плотности бактерий *Wolbachia* у самок четырех телитокичных линий *Trichogramma cordubensis* и *T. evanescens* (Hymenoptera, Trichogrammatidae). Паскаль К., Пинтюро Б., Катчадориан К., Гренье С., Боллан П., Робен К., Валлье А. — Эндосимбиотические бактерии рода *Wolbachia* заражают различных артропод и нематод, оказывая различный эффект на их репродуктивные особенности. Бактерии рода *Wolbachia* вызывают телитокию у яйцеедов рода *Trichogramma*. Использована техника Dot-blot для сравнения плотности симбионта *Wolbachia*, с помощью выделения гена *wsp* у симбионта *Wolbachia* и гена 18S у *Trichogramma*. Экспериментально установлено, что плотность *Wolbachia* у двух видов, *Trichogramma cordubensis* Vargas et Cabello и *T. evanescens* Haliday, одинаковая.

Ключевые слова: Hymenoptera, *Trichogramma*, яйцееды, Bacteria, плотность *Wolbachia*, дот-блоттинговый анализ, эндосимбионты, ген *wsp*, ген 18S, телитокия.

Comparison of *Wolbachia* Bacterial Density in Females of Four Thelythokous Strains of *Trichogramma cordubensis* and *T. evanescens* (Hymenoptera, Trichogrammatidae). Pascal C., Pintureau B., Katchadourian C., Grenier S., Bolland P., Robin C., Vallier A. — The endosymbionts of the genus *Wolbachia* infect numerous arthropods and nematods, and often cause different effects on the reproduction of these hosts. The endosymbiotic bacteria *Wolbachia* induces the thelytokous mode of reproduction in the egg parasitoids of the genus *Trichogramma*. The Dot-blot technique was performed to compare the symbiont *Wolbachia* density using the *wsp* gene of *Wolbachia* and the 18S gene of *Trichogramma*. It was established that *Wolbachia* density is not different in two host species, *Trichogramma cordubensis* Vargas et Cabello and *T. evanescens* Haliday.

Key words: Hymenoptera, *Trichogramma*, egg parasitoids, Bacteria, *Wolbachia* density, dot-blot analysis, endosymbionts, *wsp* gene, 18S gene, thelytoky.

Introduction

The micro-wasps of the genus *Trichogramma* Westwood are egg parasitoids of various insect species, used in the biological control of many lepidopterous pests of crops (Smith, 1996). They show bisexual reproduction (a non-fertilized egg develops into a haploid male and a fertilized egg develops into a diploid female) or, more rarely, a thelytokous mode of reproduction. In this case, a non-mated female produces only daughters. Such a phenomenon is induced in most of the *Trichogramma* species by the presence of Bacteria of the genus *Wolbachia* (Pintureau et al., 2001) which stops chromosome segregation during anaphase of the first mitotic division and causes diploidisation leading to the development of females (Stouthamer, Kazmer, 1994).

Wolbachia are endosymbiotic bacteria belonging to the Rickettsiaceae family of the α subdivision of Proteobacteria (Williams et al., 1991). These bacteria are mostly present in the reproductive tissues of their hosts (arthropods and filarial nematodes) and also in the somatic tissues (Dobson et al., 1999). They are vertically transmitted from mothers to daughters but, occasionally, can also be horizontally transmitted between individuals, even those belonging to different species. According to the first mode of transmission,

which is the most frequently observed, the bacteria are exchanged from one generation to the next by the oocytes (Louis et al., 1993). The second mode of transmission was indicated by the lack of congruence between the phylogenies of the *Wolbachia* and their hosts (Johanowicz, Hoy, 1996). *Wolbachia* induce different reproductive alterations in Arthropods: cytoplasmic incompatibility, thelytokous parthenogenesis, male feminization, male killing (O'Neill et al., 1997) and an increase in fecundity (Girin, Boulétreau, 1995). These alterations further the spread of the symbiont over generations.

Nevertheless, the intensity of an effect induced by *Wolbachia* in a host may be variable, being notably influenced by the bacterial density, the host genotype and the degree of co-adaptation between the host and its symbiont (Boyle et al., 1993; Bourtzis et al., 1996). For example, old thelytokous females of *Trichogramma* produce more and more males (Jardak et al., 1979), probably in response to a decrease in the density of *Wolbachia*.

The aim of the present study was to improve our understanding of the relationships between the intensity of the thelytokous mode of reproduction and the *Wolbachia* density in *Trichogramma*. More precisely, the objective was to compare the bacterial density in two species showing different *Wolbachia* prevalence, using four populations from different origins that all show 100% thelytoky. Differences in density would indicate that host genomes have variable abilities in regulating the symbiont population and, possibly, the level of thelytokous reproduction. This work was carried out with the Dot-blot technique (four Dot-blots were achieved) enabling us to make an estimation of the bacterial density within the whole *Trichogramma* body.

Material and methods

Biological materials

Four thelytokous strains including only females and belonging to two *Trichogramma* species were studied (tabl. 1). The Grey strain of *T. cordubensis*, including individuals with the «dark body» mutation (*db*) (i. e. body with grey and black zones instead of yellow and dark zones in the wild form, black eyes instead of red), was selected from the MB35 strain.

All the strains were reared on UV-irradiated *Ephestia kuehniella* Zeller (Lep.: Pyralidae) eggs previously glued with arabic gum solution onto cardboard strips (4.8 × 0.8 cm). These strips were placed in glass tubes (8 cm in length × 1 cm in diameter) where *Trichogramma* adults were present, together with a diluted honey drop as food. The rearing was performed at 23°C (70 ± 5% RH, 16: 8h L: D) allowing a development duration of about 14 days.

Adults, used to extract the DNA, were collected by phototactic migration to guarantee the fresh condition of the material and to avoid *Wolbachia* contamination by the *E. kuehniella* eggs which are also infected. Several successive migrations were performed from the same rearing tube to collect the maximum of individuals.

DNA extraction

The DNA extraction is difficult in very minute insects, such as *Trichogramma*. The classical technique using phenol, chloroform and isoamylic alcohol does not allow the extraction of good quality DNA, probably due to the high concentration of red pigments from *Trichogramma* eyes. We thus used the Promega "Genomic DNA Purification kit" for vegetal DNA, following the manufacturer's specifications but with some modifications to optimize the technique. Modifications concerned the incubation time (more than 12 hours instead of 15 min) and the incubation method (with stirring instead of no stirring). The extraction was performed on the homogenate of about 300 *Trichogramma* adults stored at –80°C (about 2.4 mg). The DNA samples were maintained at 4°C for 24 hours prior to storage at –20°C.

The Dot-blot technique requires good quality DNA and in sufficient quantities. An estimation of the quality and quantity was thus carried out by means of an electrophoretic migration on a 1% agarose gel in TAE buffer (40 mM Tris-acetate; 1 mM EDTA) followed by comparison with a control of known size and concentration (DNA Ladder 1Kb, Gibco BRL). This method allows the differentiation of the samples and was used to prepare different DNA quantities for the Dot-blot 1. Nevertheless, afterwards, such an estimation of the DNA quantity appeared not to be very reliable. We then attempted to use the spectrometry technique

Table 1. Thelytokous *Trichogramma* strains in which Dot-blots were performed
Таблица 1. Линии телитокических *Trichogramma*, в которых был проведен дот-блот акнализ

Species	Strain	Geographic origin	Date of collection	Host
T. cordubensis Vargas & Cabello	MB35	Mora, Alentejo, Portugal	1992	Noctuidae
	Grey	Mutant obtained from MB35	Obtention in 1994	
	1032	São Jorge, Azores, Portugal	June 1992	?
T. evanescens Westwood	M36	Cagnes-sur-mer, Alpes-Maritimes, France	August 1982	Noctuidae

but it did not provide good results from our samples, probably because of the presence of proteins and red pigments from *Trichogramma* eyes. This is why, for the Dot-blots 2, 3 and 4, we decided to analyse four dilutions of the raw solution of total DNA extracted.

Estimation of the relative density of Wolbachia using Dot-blot

The Dot-blot technique allows a rough estimation of the relative frequency of one DNA sequence among a heterogeneous population of DNA sequences. The DNA extracted from each sample was diluted in 400 µl of SSC 6X buffer (SSC 20X: 0.3 M Na-citrate; 3 M NaCl). It was then denatured at 100°C for 10 min, and quickly cooled on ice. The samples were transferred to the Dot-blot device, in a line on nylon membranes (Hybond, Amersham) based on two sheets of Watman 3M paper previously moistened in the buffer SSC 6X. After the vacuum was created, the samples took on a disc shape of about 7 mm in diameter. The nylon membrane was then treated with a denaturing solution (0.4 N NaOH; 1 M NaCl) for 10 min, and with a neutralisation solution (0.5 M Tris-HCl pH 7.5; 1.5 M NaCl) for 5 min. Finally, it was deposited on dry filter paper and heated at 80°C for 2 hours to fix the DNA. The membranes can be stored at room temperature, or at 4°C when longer storage is required.

To estimate the ratio of *Wolbachia* DNA to host DNA, the same membrane was successively hybridized with two radioactive probes, *wsp* for *Wolbachia* and 18S for *Trichogramma*. Each radioactive probe was directly labelled by PCR (50 µCi of radioactive dCTP ^{32}P) and purified. PCR were performed on the total DNA of the Grey strain of *T. cordubensis* with the specific primers *wsp*: 81F 5' TGG–TCC–AAT–AAG–TGA–TGA–AGA–AAC–3' and 691R 5'–AAA–AAT–TAA–ACG–CTA– CTC–CA–3', or 18S: 185F 5'–ATG–CTT–GTC–TCA–AAG–ATT–AAG–C–3' and 185R 5'–GGA–GCT–GGA–ATT–ACC–GCG–G. The purification was carried out on a Sephadex G50 column.

To saturate the membrane, a pre-hybridization at 50°C for 6 hours was performed in a solution including DNA from herring and salmon sperm denatured at 95°C (1% solution), 10% Denhardt 50X, 25% SSC 20X, 0.2% SDS and 50% formamide. The hybridization with the *wsp* probe (radioactive and denatured) was then performed overnight at 50°C in the same solution. To eliminate the non-specific interactions, washings were carried out in different baths of SSC (three concentrations: 2X, 1X, 0.1X) containing 0.1% SDS, for 15 min at 55°C for each concentration. In each Dot-blot, a non-symbiotic species (*T. brassicae* Bezdenko, B strain) was used as a negative control of the *wsp* probe activity.

The estimation of the radioactive DNA quantity was carried out with a Storm apparatus (Molecular Dynamics, USA) which allows rapid radioactive detection and linear quantification on five orders of magnitude. The data collection and analysis were performed using the ImageQuant software.

Before hybridizing the radioactive probe 18S, the membrane was de-hybridized by two successive washings in 0.2 M NaOH solution, at 42°C for 10 min, and by one washing in SSC 2X solution for 15 min. The estimation of the quantity of DNA was performed with the same method from the 18S gene of *Trichogramma* and from the *wsp* gene of *Wolbachia*. Each replicate from each sample was then characterized by its ratio of intensities *wsp*/18S.

A data filtration test was performed in order to eliminate the values of low quality. Hence, the correlation was calculated between three (Dot-blot 1) or four (other Dot-blots) intensities of *wsp* spots and three or four intensities of corresponding 18S spots. Dot-blots were thus performed using three or four quantities of total DNA from each sample: three quantities of DNA (0.50, 0.75 and 1 µg in Dot-blot 1) or four volumes of DNA solution (3, 6, 9 and 12 µl in Dot-blot 2; 1, 3, 6 and 9 µl in Dot-blot 3; 5, 7, 10 and 20 µl in Dot-blot 4). Samples associated with non-correlated ($p > 0.05$) intensities were rejected because the absence of correlation indicates technical problems (pipette handling, signal saturation, ...). The different ratios of intensities obtained in each validated sample were considered as «replicates» to calculate the means, although variability shown by these replicates does not reflect biological variability since only one DNA extraction was possible from one sample.

Results

The DNA quantity in the samples, estimated on agarose gel to be about 2.5 µg in 50 µl (i. e. 0.05 µg/µl), was sufficient to provide very clear spots.

Dot-blot 1

The Dot-blot was performed using three DNA quantities (0.50, 0.75 and 1 µg) of each sample (strain 1032 of *T. cordubensis* and strain M36 of *T. evanescens*). The two coefficients of correlation between the *wsp* and 18S spot intensities were significant ($p < 0.05$) (tabl. 2) and all the data were thus kept to compare the strains.

The «t» test showed that the two *Trichogramma* strains studied are not infected by different *Wolbachia* densities (fig. 1). Therefore, the bacterial density does not appear to be different in the two species *T. cordubensis* and *T. evanescens*. Moreover, it does

not seem to have any relation to the group of species, since *T. cordubensis* belongs to the *minutum* group and *T. evanescens* to the *evanescens* group (Pintureau, 1994), two groups, however, closely related and merged in the *exiguum* section by J. D. Pinto (1998). Finally, the *Wolbachia* density does not seem to have any relation to the prevalence of infection in the species, since *T. cordubensis* is completely infected (all the examined populations and individuals are infected) whereas *T. evanescens* is only partially and rarely infected (only some populations and individuals are infected). An intermediate prevalence is observed in other *Trichogramma* species when the infection is partial but frequent (most examined populations and individuals are infected) (Pintureau et al., 2002). Nevertheless, the present comparison is based on a low number of replicates and can only provide preliminary conclusions requiring further confirmation in the subsequent Dot-blots.

Dot-blot 2

The Dot-blot was performed using four volumes of DNA solution (3, 6, 9 and 12 µl) of each sample (strains MB35, Grey and 1032 of *T. cordubensis,* and strain M36 of *T. evanescens*). Among the 9 coefficients of correlation between the *wsp* and 18S spot intensities, 8 were significant ($p < 0.05$) (tabl. 3) and an obvious majority of the data was thus kept to compare the strains. Among all the 4 Dot-blots performed, the Dot-blot 2 included the highest number of replicates.

According to the ANOVA, no differences exist between three of the four *Trichogramma* strains studied (fig. 1). Only the strain Grey of *T. cordubensis* differs from the other strains by a higher bacterial density. Therefore, this result confirms the absence of any difference in *Wolbachia* density between the two species *T. cordubensis* and *T. evanescens*, between the *minutum* and the *evanescens* groups of *Trichogramma*, and between completely infected species and rarely infected species. The recorded difference between strains of *T. cordubensis* requires confirmation in the subsequent Dot-blots. Such variability in the bacterial density would not be in relation to the geographic origin since the two different strains Grey and MB35 come from the same location (Alentejo, Portugal), and the two similar strains MB35 and 1032 come from Alentejo and Azores, respectively.

Dot-blot 3

This Dot-blot was performed using four volumes of DNA solution (1, 3, 6 and 9 µl) of each sample (strains MB35, Grey and 1032 of *T. cordubensis,* and strain M36 of *T. evanescens*). All 4 coefficients of correlation between the *wsp* and 18S spot intensities were significant ($p < 0.05$) (tabl. 4) and all the data were thus kept to compare the strains. The ratio of intensities *wsp*/18S is obviously higher in this Dot-blot than in

Table 2. Analysis of the Dot-blot 1 performed with one thelytokous strain of *T. cordubensis* and one thelytokous strain of *T. evanescens*

Таблица 2. Дот-блот анализ 1, проведенный с одной телитокической линией *T. cordubensis* и одной телитокической линией *T. evanescens*

Species	Strain	Quantity (µg) of DNA	Spot intensity		Coefficient of correlation *wsp*-18S	Ratio of intensities *wsp*/18S
			wsp	18S		
T. cordubensis	1032	0.50	82810	123 566	0.99*	0.67
		0.75	107364	208 140		0.52
		1.00	143768	290 815		0.49
T. evanescens	M36	0.50	65803	128 832	0.99*	0.51
		0.75	85836	163 951		0.52
		1.00	93765	189 143		0.50

* $p < 0.05$

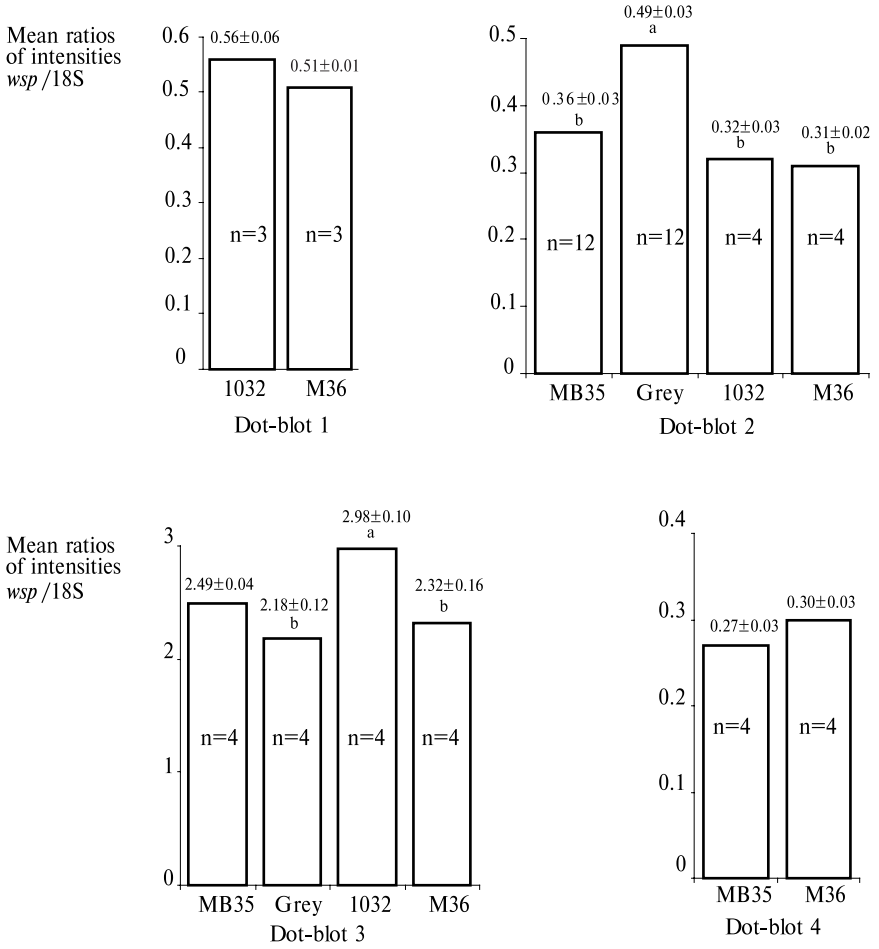

Fig. 1. Estimation of the *Wolbachia* density in three strains of *Trichogramma cordubensis* (MB35, Grey and 1032) and one strain of *T. evanescens* (M36). n – number of spots per strain in each Dot-blot performed. Means were compared by t-tests (Dot-blots 1 and 4; p = 0.422 and 0.524, respectively) or ANOVAs (Dot-blots 2 and 3; p = 0.003 and 0.002, respectively). In Dot-blots 2 and 3, means followed by the same letter are not significantly different, according to the Fisher PLSD test (p > 0.05).

Рис. 1. Плотность *Wolbachia* в трех линиях *Trichogramma cordubensis* (MB35, Grey and 1032) и одной линии *T. evanescens* (M36). n – количество пятен на линию в каждом дот-блоте. Средние значения сравнивались с применением t-тестов (дот-блоты 1 и 4; p = 0,422 и 0,524 соответственно) или ANOVA (дот-блоты 2 и 3; p = 0,003 и 0,002 соответственно). В дот-блотах 2 и 3, средние значения, следующие за соответствующей буквой, существенно не отличаются, согласно тесту PLSD Фишера (p > 0,05).

the preceding ones. The reason is a lengthening of the hybridization period with the *wsp* probe but not with the 18S probe.

According to the ANOVA, no differences exist between three of the four *Trichogramma* strains studied (fig. 1). Only the strain 1032 of *T. cordubensis* differed from the other strains with a higher bacterial density. Therefore, like the Dot-blot 2, this result confirms the absence of any difference in *Wolbachia* density between the two species studied, belonging to two different groups of *Trichogramma* and to two different categories of symbiont prevalence. On the other hand, the difference between strains of *T. cordubensis* recorded in Dot-blot 2 is not the same as the difference recorded in Dot-blot 3, where a logical explanation appears in relation to the geographic distribution (Azores vs. continental Portugal): Grey > 1032 = MB35 in Dot-blot 2 vs.

Table 3. Analysis of the Dot-blot 2 performed with three thelytokous strains of *T. cordubensis* and one thelytokous strain of *T. evanescens*

Таблица 3. Дот-блот анализ 2, проведенный с тремя телитокическими линиями *T. cordubensis* и одной телитокической линией *T. evanescens*

Species	Strain	Volume (µl) of DNA solution	Spot intensity		Coefficient of correlation *wsp*-18S	Ratio of intensities *wsp*/18S
			wsp	18S		
T. cordubensis	MB35	3	47037	99330	0.92*	0.47
		6	46636	153048		0.30
		9	49756	213568		0.23
		12	51896	259104		0.20
	MB35	3	29146	76783	0.96*	0.38
		6	32037	112400		0.29
		9	43274	145794		0.30
		12	45966	177374		0.26
	MB35	3	35936	68600	0,64	
		6	53016	173623		
		9	40230	187874		
		12	46977	208520		
	MB35	3	15415	30148	0.98*	0.51
		6	22329	40884		0.55
		9	33733	74505		0.45
		12	38034	99226		0.38
	Grey	3	63973	131459	0.99*	0.49
		6	77375	179641		0.43
		9	96214	247896		0.39
		12	118076	305095		0.39
	Grey	3	39635	73615	0.92*	0.54
		6	65807	128562		0.51
		9	81689	142302		0.57
		12	80118	181742		0.44
	Grey	3	50854	68911	0.97*	0.74
		6	56514	118953		0.48
		9	64070	134461		0.48
		12	70030	171283		0.41
	1032	3	40173	107586	0.89*	0.37
		6	50777	186547		0.27
		9	83680	228310		0.37
		12	79407	287685		0.28
T. evanescens	M36	3	33588	89735	0.99*	0.37
		6	40284	123948		0.33
		9	51711	185929		0.28
		12	58112	214594		0.27

* $p < 0.05$

1032 > Grey = MB35 in Dot-blot 3. Such an inconsistency does not lead to an obvious conclusion, and it is preferable to temporarily consider that the bacterial density can only be occasionally higher than the density recorded in the strain MB35, for some unknown reason.

Dot-blot 4

The Dot-blot was performed using four volumes of DNA solution (5, 7, 10 and 20 µl) of each sample (strain MB35 of *T. cordubensis,* and strain M36 of *T. evanescens*). Half of the 4 coefficients of correlation between the *wsp* and 18S spot intensities were significant ($p < 0.05$) (tabl. 5) and half of the data were thus kept to compare the strains.

Table 4. Analysis of the Dot-blot 3 performed with three thelytokous strains of *T. cordubensis* and one thelytokous strain of *T. evanescens*

Таблица 4. Дот-блот анализ 3, проведенный с тремя телитокическими линиями *T. cordubensis* и одной телитокической линией *T. evanescens*

Species	Strain	Volume (µl) of DNA solution	Spot intensity		Coefficient of correlation *wsp*-18S	Ratio of intensities *wsp*/18S
			wsp	18S		
T. cordubensis	MB35	1	51851	21152	0.99*	2.45
		3	92991	37809		2.46
		6	102816	39169		2.62
		9	124085	50878		2.44
	Grey	1	97128	52498	0.99*	1.85
		3	143824	65776		2.19
		6	212246	87504		2.43
		9	264105	116641		2.26
	1032	1	74107	23158	0.99*	3.20
		3	104929	38133		2.75
		6	223513	77667		2.88
		9	192490	62433		3.08
T. evanescens	M36	1	88848	46175	1.00*	1.92
		3	194440	87279		2.23
		6	346697	130286		2.66
		9	295819	118852		2.49

* $p < 0.05$

According to the «t» test, no differences exist between the two *Trichogramma* strains studied (fig. 1). This result confirms the absence of any difference in *Wolbachia* density between the two species *T. cordubensis* and *T. evanescens*, and between the strains MB35 and M36 (Dot-blots 2 and 3).

Discussion and conclusions

The *Wolbachia* density does not seem to be different in *T. cordubensis* and *T. evanescens*. Therefore, this density is not a species specific character or even a species group specific character. In *T. evanescens*, where the infection prevalence is clearly lower than in *T. cordubensis*, the restricted proportion of infected individuals is thus not caused by a poor installation of *Wolbachia* in the infected individuals. The restriction of prevalence could therefore come from other factors, such as the existence of more or less resistant host genotypes as shown in *Culex pipiens* L. (Berticat et al., 2002) or *Drosophila* sp. (McGraw et al., 2002). The host genome is also known to regulate the *Wolbachia* effect, as shown with the level of cytoplasmic incompatibility in *Nasonia* sp. (Bordenstein, Werren, 1998), or the induction of the thelytokous mode of reproduction in *Trichogramma* sp. (Pintureau et al., 2000).

In *T. cordubensis*, some differences between the strains were recorded, but these differences were inconsistent since, according to the experiment, they concerned either the Grey strain or the 1032 strain and the other strains. Moreover, the Grey strain is derived from the MB35 strain and so the difference recorded between them is very surprising. It is thus difficult to state that actual differences in *Wolbachia* density exist in natural populations of this species. Similarly, M. E. Clark and T. L. Karr (2002) did not record differences in symbiont density between two *Drosophila simulans* Sturtevant strains, although S. P. Sinkins et al. (1995) recorded differences between *Aedes albopictus* (Skuse) strains.

In species with a high symbiont prevalence, a co-adaptation between *Wolbachia* and *Trichogramma* is expected, leading to a lower physiological cost and density of bacteria. Our results did not confirm this hypothesis, but they could be preliminary.

Table 5. Analysis of the Dot-blot 4 performed with one thelytokous strain of *T. cordubensis* and one thelytokous strain of *T. evanescens*

Таблица 5. Дот-блот анализ 4, проведенный с одной телитокической линией *T. cordubensis* и одной телитокической линией *T. evanescens*

Species	Strain	Volume (μl) of DNA solution	Spot intensity		Coefficient of correlation *wsp*-18S	Ratio of intensities *wsp*/18S
			wsp	18S		
T. cordubensis	MB35	5	32016	118837	0.43	
		7	25595	183513		
		10	29090	137165		
		20	43320	201647		
	MB35	5	45294	132107	0.92*	0.34
		7	26583	89406		0.30
		10	31017	133735		0.23
		20	61385	282810		0.22
T. evanescens	M36	5	34763	94109	0.99*	0.37
		7	35377	115363		0.31
		10	36862	125343		0.29
		20	52879	229319		0.23
	M36	5	31919	81222	0.83	
		7	33205	80864		
		10	45121	141516		
		20	40697	175020		

* $p < 0.05$

Indeed, to better describe the variability of *Wolbachia* density in the *Trichogramma* genus, or to confirm the absence of variability, other species and populations have to be analysed. In addition, the methods used in the work to measure this density need to be improved, and other methods such as quantitative PCR and confocal microscopy techniques will probably be required for better reliability (Sinkins et al., 1995; Noda et al., 2001; Berticat et al., 2002; Clark, Karr, 2002; Kondo et al., 2002).

F. Gressent provided important advice for using the Storm apparatus. Strain 1032 was provided by P. Garcia (Univ. Azores, Portugal), strains M36 by J. Pizzol (INRA-Antibes, France) and strain MB35 by I. Silva (Univ. Wageningen, The Netherlands). L. Neto (Univ. Algarve, Portugal) obtained the mutant strain Grey. All the strains were reared by A. Clavel (INRA/INSA de Lyon).

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