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The endosymbionts *Wolbachia* and *Cardinium* and their effects in three populations of the predatory mite *Neoseiulus paspalivorus*

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Abstract Whereas endosymbiont-induced incompatibility is known to occur in various arthropod taxa, such as spider mites, insects and isopods, it has been rarely reported in plant-inhabiting predatory mites (Acari: Phytoseiidae). Recent cross-breeding studies with the phytoseiid mite *Neoseiulus paspalivorus* De Leon revealed a complete post-mating reproductive isolation between specimens collected from three geographic origins—Northeast Brazil (South America), Benin and Ghana (West Africa)—even though they are morphologically similar. We carried out a study to assess to what extent these populations exhibit genetic differences and whether endosymbionts are involved in the incompatibility. First, we used the mitochondrial cytochrome oxidase I (COI) gene to assess genetic diversity among the three populations. Second, we used a PCR-based method to check for the presence of *Wolbachia* and/or *Cardinium* in these populations, and we determined their phylogenetic relationships using specific primers for *Wolbachia* and *Cardinium* 16S rDNA genes. Third, we also conducted a test using an antibiotic (tetracycline) in an attempt to eliminate the symbionts and evaluate their effects on the reproductive compatibility of their host. Based on the DNA sequences of their COI genes, specimens of the three populations appear to be genetically similar. However, the 16S rDNA gene sequences of their associated endosymbionts differed among the three populations: the Benin and Brazil

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populations harbour different strains of *Wolbachia* symbionts, whereas the Ghana population harbours *Cardinium* symbionts. In response to antibiotic treatment females of each of the three populations became incompatible with untreated males of their own population, similar to that observed in crossings between females from one geographic population and males from another. Compatibility was restored in crosses involving uninfected Brazil females and uninfected Benin males, whereas the reciprocal crosses remained incompatible. *Cardinium* symbionts seem to be essential for oviposition in the Ghana population. It is concluded that their associated bacterial symbionts are the cause of the post-mating reproductive isolation previously observed among the three geographic populations. This insight is relevant to biological control of coconut mites for which *N. paspalivorus* is an effective predator, because introducing one geographic strain into the population of another (e.g. in field releases or mass cultures) may cause population growth depression.

Keywords Phytoseiidae · Cytoplasmic incompatibility · Geographic populations · COI · Endosymbiotic bacteria · 16S rDNA

Introduction

In the two past decades, our knowledge of the endosymbiotic bacteria and our understanding of the reproductive abnormalities they cause in their hosts have increased considerably, thus opening avenues for new research (Duron et al. 2008). Yet, little attention has been paid to the association of these endosymbiotic bacteria with natural enemies used as biological control agents and the consequences of this association for the outcome of classical biological pest control (Stouthamer et al. 2000; Zindel et al. 2011).

Predatory mites (Acari: Phytoseiidae) are widely used for biological control of plant-feeding mites. A large body of literature on the role of endosymbionts in plant-feeding mites has accumulated in the last 15 years (i.e., Breeuwer 1997; Gotoh et al. 2003, 2005a, b, 2007; Vala et al. 2000); however, very little is known about the presence and the role of endosymbionts in plant-inhabiting phytoseiid mites that represent their most important natural enemies (Hoy and Cave 1988; Enigl and Schausberger 2007). In this article, we focus on the predatory mite *Neoseiulus paspalivorus* De Leon, a candidate predator for the control of the coconut mite, *Aceria guerreronis* Keifer, a key pest of coconut palms (Moraes et al. 2004; Lawson-Balagbo et al. 2008; Reis et al. 2008; Negloh et al. 2011). Crossing experiments between populations from three geographic regions—Northeast Brazil (South America), Benin and Ghana (West Africa)—yielded no F1 offspring or only a few sterile males and thus revealed a complete post-mating and bidirectional incompatibility (Famah Sourassou et al. 2011).

It is well known that post-zygotic incompatibility can result from genetic incompatibility (Navajas et al. 1999; Uesugi et al. 2003; Gotoh et al. 2005b; Pryke and Griffith 2008; Koevoets and Beukeboom 2009), or from cytoplasmic incompatibilities due to intracellular bacteria such as *Wolbachia* and *Cardinium* (O'Neill and Karr 1990; Breeuwer 1997; Gotoh et al. 2007). These two bacterial symbionts infect a wide range of arthropods and are known to cause reproductive abnormalities in their host, including cytoplasmic incompatibility, feminization, parthenogenesis, hybrid breakdown and male killing (Rigaud et al. 1997; Hoffmann and Turelli 1997; Hurst et al. 1999; Vala et al. 2000; Charlat et al. 2001; Gotoh et al. 2007; Wu and Hoy 2012; Zhu et al. 2012). Cytoplasmic incompatibility, the

most common effect of *Wolbachia* and *Cardinium* infections, is a sterility phenomenon that has been described first in insects and mites (Wade and Stevens 1985; Hoffmann and Turelli 1988, 1997; O'Neill 1989; Breeuwer and Werren 1990; Gotoh et al. 2007; Wu and Hoy 2012; Zhu et al. 2012). Cytoplasmic incompatibility can be either unidirectional or bidirectional. In the former, crosses between populations are incompatible in one direction, but the reciprocal cross matings are fully compatible, as are matings between infected (by the same strain) individuals. In contrast, bidirectional incompatibility usually occurs when the male and female carry different strains of *Wolbachia* (Laven 1959, 1967; Breeuwer and Werren 1990; Bordenstein et al. 2001; Bordenstein 2003; Telschow et al. 2005, 2007). Incompatibilities have been reported in crosses between populations of some phytoseiid species, but with unidirectional incompatibilities being most commonly observed (Hoy and Cave 1988; Johanowicz and Hoy 1998; Noronha and Moraes 2002, 2004; Wu and Hoy 2012). However, the role of endosymbionts in causing these incompatibilities in phytoseiid species is still unclear, except in the case of *Metaseiulus occidentalis* Nesbitt (Acari: Phytoseiidae) (Hoy and Cave 1988; Wu and Hoy 2012).

In this study, our aim is to determine to what extent the three populations of *N. paspalivorus*—Northeast Brazil (South America), Benin and Ghana (West Africa)—exhibit genetic differences and whether endosymbionts are involved in reproductive isolation between these geographic populations. First, we assessed genetic diversity between the three populations based on the variability in the mitochondrial cytochrome oxidase I (COI) gene. Second, we used the 16S ribosomal DNA primers to detect the presence of two endosymbiotic bacteria, *Wolbachia* and *Cardinium,* in the three populations and to analyse the phylogenetic relationships of these bacteria. Third, we performed an antibiotic test to determine whether any of these bacteria is associated with cytoplasmic incompatibility in the host, because some *Wolbachia* strains do not induce incompatibility in their host (Giordano et al. 1995; Navajas et al. 1999; Gotoh et al. 2005b).

Materials and methods

Populations studied and laboratory rearing

The three laboratory-reared populations—from Benin, Ghana and Brazil—used in the present study were the same as those used by Famah Sourassou et al. (2011). In addition, specimens collected from weeds in Benin were included in genetic analyses for comparative reasons. Details on the locality and date of collection, host plant and the number of colonizing females are presented in Table 1. The various populations were maintained in rearing units consisting of a black PVC tile (4 × 4 × 0.1 cm) placed on top of a foam pad (4 × 4 × 1 cm) resting in a Petri dish (14.5 cm diameter and 1 cm high). The edges of the tile (and part of the foam pad) were covered with a band of tissue paper. To prevent mites from escaping, distilled water was supplied to the Petri dish on a daily basis to keep the foam pad and the tissue paper wet. A small tuft of hydrophobic cotton wool covered by a piece of transparent plastic was placed in the center of each rearing unit to serve as a resting place and as an oviposition substrate for the predators. All colonies were maintained in a room at 25–27 °C, 70–90 % relative humidity and a 12–12 h light–dark cycle. At three-day intervals the colonies were provided with a fresh supply of prey consisting of eggs of *Tetranychus urticae* Koch and inevitably also the immatures that emerge from them.

Table 1 Collection localities, host plant and date of collection of four geographic populations of *Neoseiulus paspalivorus*

Country	Locality	Host plant	Latitude	Longitude	Collection date	No. colonizing females
Benin	Ouidah	*Cocos nucifera*	06°21′66N	02°09′76E	October 2005	52
Benin	Ouidah	*Cyperus esculentus*	06°21′66N	02°09′76E	October 2010	–
Ghana	Winneba	*Cocos nucifera*	05°22′90N	00°38′68W	December 2008	11
Brazil	Itamaraca	*Cocos nucifera*	07°46′05S	34°52′08W	September 2006	5

Genetic analysis

Two or three adult females of each colony were used for molecular analysis. DNA was extracted using the Chelex method (Walsh et al. 1991). Mites were crushed using Precellys 24 (Brevet Bertin Technology, France) in a sterile tube containing 100 µl of 5 % chelex solution and 4–5 sterile beads. Tubes were then vortexed, after which 5 µl of proteinase K (20 mg/ml) was added. Tubes were subsequently incubated at 56 °C for 1 h, heated at 95 °C for 8 min, and then briefly centrifuged and stored at −20 °C until later use.

The mitochondrial COI gene is well suited for population and species identification (Hebert et al. 2003; Tixier et al. 2010), and has been successfully used in the phylogenetic studies of Tetranychidae and Phytoseiidae (Navajas et al. 1996, 1998; Toda et al. 2000; Hinomoto et al. 2001, 2007; Navajas and Boursot 2003; Ramadan et al. 2004; Tixier et al. 2006a, b; Famah Sourassou et al. 2012).

A fragment of the mitochondrial COI was amplified and sequenced using primers specifically designed for tetranychid mites (5′-TGATTTTTTGGTCACCCAGAAG and 5′-TACAGCTCCTATAGATAAAAC; Navajas et al. 1996). The amplification reaction was carried out in a 25 µl reaction mixture containing 11.4 µl of sterile water, 2.5 µl 10X Super Taq Buffer (HT BioTechnology, Cambridge, UK), 5 µl dNTP mix (1 mM), 1.25 µl bovine serum albumin (10 mg/ml), 1.25 µl $MgCl_2$ (25 mM), 0.2 µl of each primer (10 µM), 0.2 µl of Super Taq (5 U/µl), and 3 µl of DNA template. The PCR amplification (PTC-200 thermal cycler, BioRad), was initiated with a 4-min incubation at 95 °C, followed by 35 cycles, each with 1 min at 94 °C, 1 min at 48 °C and 1 min at 72 °C, and then an extension for 4 min at 72 °C. PCR products were purified using the Invitek DNA extraction kit (Invitek, UK) and the sequence was determined by the ABI Prism Big Dye Terminator Sequencing method (Applied Biosystems, The Netherlands). Quality check of sequence chromatograms and construction of contigs were performed with BioEdit version 5.0.6 (Hall 1999). Sequences were aligned with ClustalW (as implemented in BioEdit), and a construction of the distance matrix and the Neighbour-Joining Tree (with 1,000 bootstraps) using the Jukes-Cantor model (Jukes and Cantor 1969) was performed with MEGA® software version 5.

Screening for endosymbionts

DNA was extracted from a pool of 8–10 adult females of each population using the same method as described above. A PCR-based approach was used to check for the presence/absence of the two symbionts in each population. For detection of *Wolbachia* and *Cardinium*, the 16S *Wolbachia* rDNA primers (76F-5′-TTGTAGCCTGCTATGGTATAACT and 1012R-5′-GAATAGGTATGATTTTCATGA; O'Neil et al. 1992), and the 16S *Cardinium* rDNA primers (CLO-f1–5′-GGAACCTTACCTGGGCTAGAATGTATT and

CLO-r-5′-GCCACTGTCTTCAAGCTCTACCAAC; Kurtti et al. 1996) were used. Twenty-five µl PCR reactions were set up using 11.4 µl H_2O, 1.25 µl $MgCl_2$ (50 mM), 1.25 bovine serum albumin (10 mg/ml), 2.5 µl PCR buffer, 5 µl dNTPs mix (1 Mm each), 0.2 µl each primer (20 µM each) (HT Bio Technology), 0.2 µl Taq polymerase (5 U/µl) and 3 µl of DNA extract. For *Cardinium*, neither $MgCl_2$ nor bovine serum albumin was added. PCR amplification was carried out in a PTC-200 thermal cycler (BioRad). The thermal profile used for 16S *Wolbachia* was: 1 min at 94 °C, followed by 35 cycles each of 40 s at 94 °C, 30 s at 48 °C and 1 min at 72 °C, and 94 °C. For the *Cardinium* 16S rDNA (CLO-f/r), the thermal conditions were as follow: 2 min at 94 °C, followed by 34 cycles of 40 s at 94 °C, 30 s at 51 °C and 45 s at 72 °C, 5 min at 72 °C, and a final extension for 10 min at 10 °C. PCR products (2 µl) were visualized on 1 % agarose gel stained with ethidium bromide in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA pH 8.0). Eggs and immatures of *T. urticae* used as prey for rearing these populations in our laboratory, as well as all stages of *A. guerreronis*, the natural prey of the predatory mite under study, were also tested for the occurrence of endosymbionts. PCR products were purified using the Invitek DNA extraction kit (Invitek, UK) and sequenced along both strands by the ABI Prism Big Dye Terminator Sequencing method (Applied Biosystems, The Netherlands). Sequence alignment and construction of contigs were performed using the software BioEdit 5.0.6 with ClustalW.

Phylogenetic analysis of the 16S rDNA sequences

A NCBI BLAST search on January 15, 2012 reported our 16S *Wolbachia* rDNA sequences to be most similar with the existing 16S *Wolbachia* rDNA sequences in the NCBI database. A high similarity score (98–100 %) was observed with the first fifty displayed 16S *Wolbachia* rDNA sequences in the NCBI database. Representative *Wolbachia* 16S rDNA sequences were retrieved from the NCBI database. These sequences, in addition to the ones obtained in this study, were aligned by the aid of ClustalW (Thompson et al. 1994), whereupon short entries were discarded resulting in a global alignment of approximately 695 bp, consisting of 31 sequences retrieved from the GenBank and of the 3 sequences obtained in this study. This final data set of 34 sequences was used for phylogenetic analysis. A BLAST search for the *Cardinium* 16S rDNA sequence in the NBCI also showed a similarity score of 95–97 % with the existing 16S *Cardinium* rDNA sequences. For the phylogenetic analysis of the 16S *Cardinium* rDNA sequence obtained in this study, the same procedure was used, and a global alignment of 395 bp was obtained, consisting of 27 sequences retrieved from the NCBI database and of the unique sequence obtained in the present study. Phylogenetic trees based on maximum likelihood (ML) method were constructed in MEGA5 using the Juke-Cantor evolution model with 1,000 bootstrap replications.

Antibiotic treatment and crossing experiments

Our objective here was to determine whether the symbionts found are associated with cytoplasmic incompatibility of their host. Based on the fact that cytoplasmic incompatibility is typically observed between uninfected females and infected males (Stouthamer et al. 1999), we performed a test in which tetracycline-treated females from each population were mated with untreated males of the same population. To obtain virgin females, gravid females were removed from the colony and confined in a rearing unit as described above. Twenty-four hours later, eggs laid were transferred to a separate experimental unit and reared individually to adulthood on a diet consisting of all stages *A. guerreronis*. To administer tetracycline to

females, new experimental units were placed on a cotton bed soaked with tetracycline solution (0.5 % w/v in 10 % glucose solution) in Petri dishes similar to the ones described above (ten discs per Petri dish). In addition, 3–5 droplets of the tetracycline solution were deposited on the PVC disk representing the experimental unit. The experimental units did not harbour any food source. In this way, each female was maintained on tetracycline for 24 h, thereafter mated with untreated males taken from the same colony as that of the female. Female F1 progeny from this cross was used directly in crossing experiments of Benin and Ghana populations. In case of the Brazilian population F1, females were treated once more with antibiotics and mated with males from the infected base population. Progeny from this cross were then used in the crossing experiments. This additional step in the Brazilian population was necessary to reach incompatibility between treated females and untreated males of their base colony. Tetracycline-treated females of the three populations were PCR-checked for the efficiency of the antibiotic treatment.

To test whether compatibility can be restored in inter-population crosses, both males and females of each population were tetracycline-treated for three successive generations using the same procedure as described above. Individuals that were produced after the last treatment were kept individually for 1 week before the crossing experiments. Inter-population crosses were set up as a single pair mating between tetracycline-lines of the Benin and Brazil populations. Each pair was observed daily and the number of eggs laid was recorded for a period of 10 days. Per cross all eggs produced from all mating pairs were pooled in a single arena.

Statistical analysis

A single-factor ANOVA (SAS 2005) was used to test the effects of crossing types on the fecundity within each population, and means were compared using the Student–Newman–Keuls multiple range test (SNK) at $P < 0.05$. A Fisher Exact test was performed within each population to determine whether there was difference in the female progeny (sex-ratio) produced in the crossings involving infected females and those involving tetracycline (symbiont-free) females.

Results

Genetic analysis

We amplified approximately 500 bp of the COI gene from individuals from each of three *N. paspalivorus* populations. A 455-bp section was aligned and used for further analyses. Similar nucleotide composition was observed for all populations studied (30.7 % Adenine, 43.5 % Thymine, 14.3 % Guanine, and 11.4 % Cytosine). A BLAST search in the GenBank database showed that the sequences aligned with others COI sequences of Phytoseiidae. There were no nucleotide differences between the Benin and Brazil populations, and 0.2 % nucleotide diversity between the Ghana population and the former two populations.

Detection of endosymbionts

The Benin and Brazil populations were PCR-positive for the 16S *Wolbachia* rDNA gene, whereas the Ghana population was positive for the 16S *Cardinium* rDNA gene. This

suggests the presence of *Wolbachia* symbionts in both Benin and Brazil populations, and the presence of *Cardinium* symbionts in the Ghana population. However, the bands displayed in those populations were always weak compared with those of positive controls, *Bryobia* sp. (for *Wolbachia*) and *Brevipalpus* sp. (for *Cardinium*), for each of which a single mite was used to extract DNA. This could be due to low bacterial densities or inefficient PCR. Moreover, neither *Wolbachia* nor *Cardinium* was detected in the prey used for rearing those populations in the laboratory (eggs and immatures of *T. urticae*), as in their natural prey (all stages of *A. guerreronis*) collected from Ouidah, Benin. This rules out the possibility that positive PCRs in the predatory mites was due the presence of bacteria in their digestive tract.

Phylogenetic relationships of the 16S rDNA genes

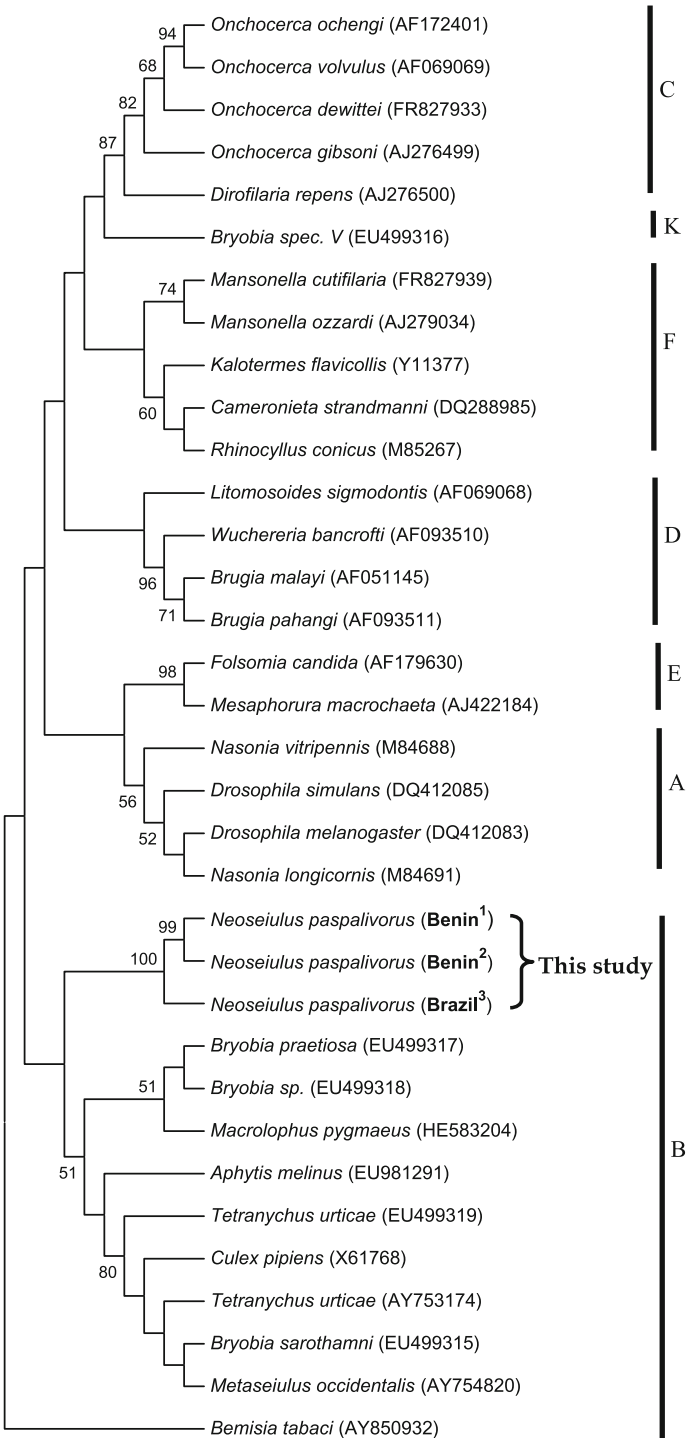
After alignment and trimming of approximately 1,000 bp of the 16S *Wolbachia* rDNA and 500 bp of the *Cardinium* 16S rDNA gene, the final dataset contained 879 bp of the former gene and 450 bp of the latter gene. The nucleotide sequences of the 16S *Wolbachia* rDNA gene amplified from the two Beninese populations—from weeds and coconut fruits—were 100 % identical. However, the nucleotide sequence of the 16S *Wolbachia* rDNA gene obtained from the Brazil population collected from coconut fruits differed by 4.5 % from those nucleotides of the two Benin populations. Sequences were deposited in GenBank under the following accession numbers: *Wolbachia* symbiont of *N. paspalivorus* from Benin collected on coconut and on weeds: KF135424 and KF135425 respectively; *Wolbachia* symbiont of *N. paspalivorus* from Brazil collected coconut: KF135426.

The phylogenetic tree based on the 16S *Wolbachia* sequences indicated that the symbionts identified in the Beninese and Brazil populations formed a monophyletic group with other 16S rDNA *Wolbachia* present in GenBank. Moreover, there was a clear differentiation, with 100 % bootstrap support, between the 16S *Wolbachia* rDNA sequences from the two Benin populations together, and that from the Brazil population (Fig. 1).

In the phylogenetic analysis of the 16S *Cardinium* rDNA (Fig. 2), the symbiont detected in the Ghana population of *N. paspalivorus* was clearly placed in the same group with other *Cardinium* symbionts present in GenBank. The *Cardinium* in the Ghana population of *N. paspalivorus* is closely related to the *Cardinium* symbionts from other phytoseiid mites. Sequence of the *Cardinium* symbiont of *N. paspalivorus* from Ghana collected on coconut was deposited in GenBank under the accession number KF135427.

Cytoplasmic incompatibility

Both Benin and Ghana populations showed the typical pattern of unidirectional incompatibility: all eggs produced in crosses between uninfected females and untreated males failed to hatch (Table 2). In the Brazil population, on the other hand, cytoplasmic incompatibility was expressed as a significant reduction in egg production in crosses between uninfected females and infected males compared to the other cross combinations: the few eggs that were produced hatched and 46 % developed into adult females. All other possible cross combinations showed similar egg production, hatchability and survival rates. Not all cross combinations with the Ghana population could be made because antibiotic treatment of infected females from the Ghana base population yielded very few offspring. Therefore all female offspring that was obtained after treatment of the parental generation was only combined with infected males, which is the expected incompatible cross. A PCR


Onchocerca ochengi (AF172401)
Onchocerca volvulus (AF069069)
Onchocerca dewittei (FR827933)
Onchocerca gibsoni (AJ276499)
Dirofilaria repens (AJ276500)
Bryobia spec. V (EU499316)
Mansonella cutifilaria (FR827939)
Mansonella ozzardi (AJ279034)
Kalotermes flavicollis (Y11377)
Cameronieta strandmanni (DQ288985)
Rhinocyllus conicus (M85267)
Litomosoides sigmodontis (AF069068)
Wuchereria bancrofti (AF093510)
Brugia malayi (AF051145)
Brugia pahangi (AF093511)
Folsomia candida (AF179630)
Mesaphorura macrochaeta (AJ422184)
Nasonia vitripennis (M84688)
Drosophila simulans (DQ412085)
Drosophila melanogaster (DQ412083)
Nasonia longicornis (M84691)
Neoseiulus paspalivorus (Benin[1])
Neoseiulus paspalivorus (Benin[2])
Neoseiulus paspalivorus (Brazil[3])
This study
Bryobia praetiosa (EU499317)
Bryobia sp. (EU499318)
Macrolophus pygmaeus (HE583204)
Aphytis melinus (EU981291)
Tetranychus urticae (EU499319)
Culex pipiens (X61768)
Tetranychus urticae (AY753174)
Bryobia sarothamni (EU499315)
Metaseiulus occidentalis (AY754820)
Bemisia tabaci (AY850932)
94
68
82
87
74
60
96
71
98
56
52
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100
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D
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A
B

◀**Fig. 1** Phylogenetic tree of 16S rDNA sequences of *Wolbachia* based on ML procedure in MEGA5. *Numbers* on the *nodes* indicate bootstrap values (%, values above 60 are displayed). *Wolbachia* strains are designated by their host names (*in parentheses*) and bear GenBank accession number. (1) Benin population collected from coconut; (2) Benin population collected from weeds; (3) Brazil population collected from coconut

check (from a pool of ten individuals) for *Wolbachia* and *Cardinium* confirmed that all uninfected females used in the crosses were free of endosymbionts.

In addition, we carried out crosses between uninfected Brazil and Benin populations. Previous crossing experiments between mites from the Brazil and Benin populations did

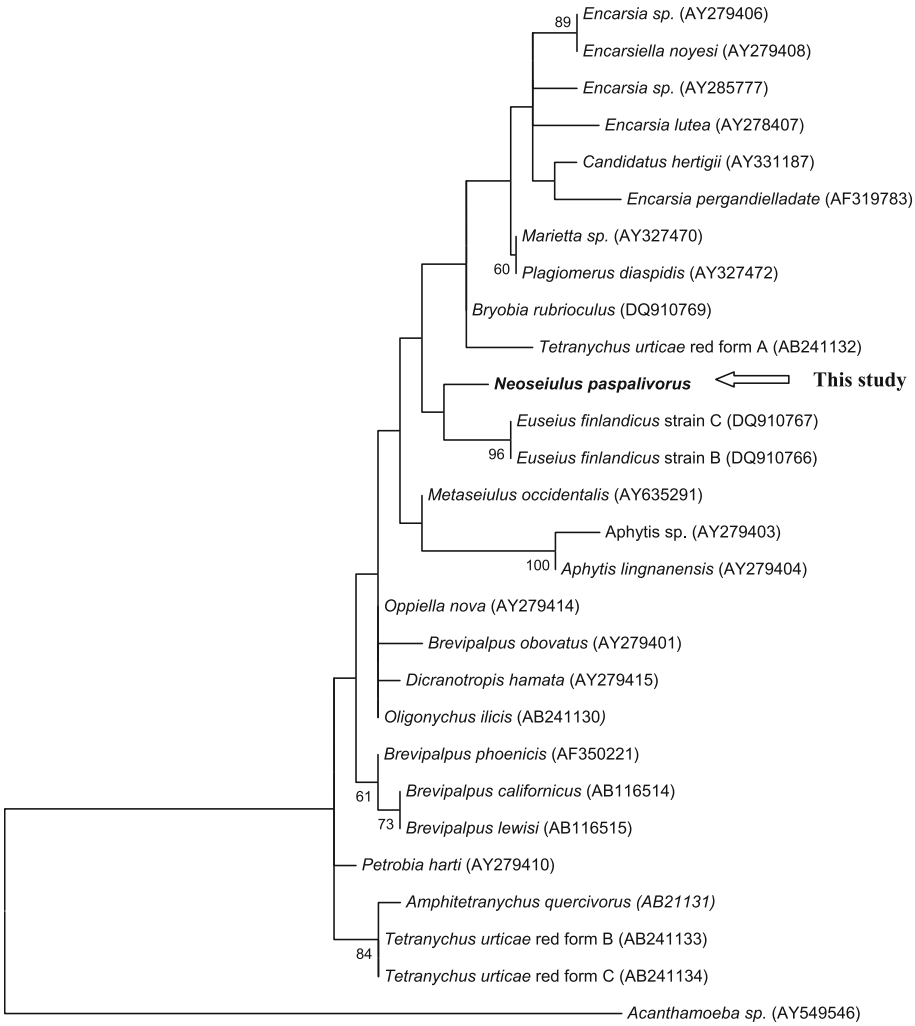

Fig. 2 Phylogenetic tree of 16S rDNA sequences of *Cardinium* based on maximum likelihood procedure in MEGA5. *Numbers* on the *nodes* indicate bootstrap values (%, values above 60 are displayed). *Cardinium* strains are designated by their host names (*in parentheses*) and bear GenBank accession number. *Arrow* indicates *Cardinium* sequence obtained in this study from Ghana population collected from coconut

Table 2 Cytoplasmic incompatibility patterns in intra-population crosses between uninfected (U) and infected (I) individuals of *Neoseiulus paspalivorus*

Cross (♀ × ♂)	N	% gravid ♀	Fecundity (eggs/♀/day)	% of egg hatchability	% survival	Sex ratio (% ♀)
Benin population						
I × I	10	100	1.4 ± 0.02a	100	100	70.0
U × I	10	100	1.1 ± 0.08b	0	–	–
U × U	10	100	1.2 ± 0.09b	100	100	52.6
I × U	8	100	0.9 ± 0.08c	100	100	69.4
Brazil population						
I × I	10	100	1.4 ± 0.09a	100	100	70.8
U × I	13	92.3	0.2 ± 0.03b	100	100	54.0
U × U	10	100	1.7 ± 0.31a	100	100	58.0
I × U	10	100	1.6 ± 0.06a	100	100	60.0
Ghana population						
I × I	10	100	1.2 ± 0.07a	100	100	69.1
U × I	10	100	0.7 ± 0.08b	0	–	–

Note that all eggs per cross were pooled for egg hatch, survival and sex ratio measurements. Percentage survival is individual survival from hatching to adulthood. Sex ratio is the percentage female offspring. Not all cross combinations within the Ghana population were done because tetracycline-treated females produced very few offspring. Mean ± SE followed by different letters are significantly different (SNK test, $P < 0.05$)

Table 3 Compatibility patterns of crosses between infected (I) and uninfected (U) individuals of the Benin (Be) and Brazil (Br) populations of *Neoseiulus paspalivorus*

Cross Female × Male	N	% gravid ♀	Fecundity (eggs/♀/day)	% egg hatchability	Survival	Sex ratio (% ♀)
$U^{Br} \times U^{Be}$	15	100	1.3 ± 0.12a	96	100	78.8
$U^{Be} \times U^{Br}$	16	100	0.2 ± 0.02b	0	–	–
$I^{Br} \times I^{Be}$	30	83	0.5 ± 0.03*	7.6	100	0
$I^{Be} \times I^{Br}$	30	93	0.6 ± 0.05*	0	–	–

For comparison, crosses between infected individuals from the base populations are shown, but were done in a previous study (Famah Sourassou et al. 2011). Mean ± SE followed by different letters within column are significantly different (SNK test, $P < 0.05$). Statistical testing only includes crosses from this study

* Famah Sourassou et al. (2011)

not produce any offspring (Famah Sourassou et al. 2011). At that time the presence of *Wolbachia* in these populations was not known. The crossing experiments between uninfected individuals of the two populations revealed a different pattern (Table 3). Crosses between uninfected Brazil females and Benin males were fully compatible producing on average 1.3 eggs per day with 96 % egg hatch rate. The reciprocal cross between Benin females and Brazil males, however, did not differ from earlier crosses between infected individuals (Famah Sourassou et al. 2011); females produced on average 0.2 eggs per day and all eggs failed to hatch.

Discussion

The key results of this study demonstrate that all three geographic populations of *N. paspalivorus* used in the present study represent a single species composed of at least three isolated populations, and that the post-mating reproductive isolation observed between these populations is due to their associated endosymbiotic bacteria.

The three populations are morphologically identical (Famah Sourassou et al. 2011) and the genetic dissimilarity between the three populations based on the mitochondrial COI gene is low, <0.2 %, and far less than intra-specific genetic distances reported in other mite species such as *Kampimodromus* sp. (1.4 %) (Tixier et al. 2008) and *Euseius* sp. (0–5 %) (Okassa et al. 2009). Moreover, pre-zygotic isolation does not appear to explain the absence of offspring in crosses between these three populations of *N. paspalivorus*. The proportions of gravid females in inter-population matings were as high as the proportions of gravid females in the intra-population matings (Table 3; Famah Sourassou et al. 2011). In pseudo-arrhenotokous mites, females only become gravid with eggs after copulation and successful sperm transfer (see Toyoshima et al. 2000). Strikingly, crosses between the populations under study produced no offspring (Table 3; Famah Sourassou et al. 2011).

The two Benin populations of *N. paspalivorus* from weeds and coconuts had identical *Wolbachia* 16S rDNA sequences, whereas the Brazil population had a different sequence, as supported by 100 % bootstrap value in the phylogenetic analyses (Fig. 1). Thus the Benin and Brazil mite populations are infected with different *Wolbachia* strains. Although not all individuals of each population were checked, results from crossing experiments conducted by Famah Sourassou et al. (2011), as well as from those conducted in the present study indicate that all individuals of each population are probably infected. There were no outliers in crossings between populations, in which altogether forty to sixty individuals of each population were tested: all intra-population-crosses were compatible, whereas all inter-population crosses were incompatible (Famah Sourassou et al. 2011). Similarly, no outliers were found in crossings conducted in the present study.

Both *Wolbachia* and *Cardinium* symbionts can cause unidirectional incompatibility in all three respective populations of *N. paspalivorus* (Table 2). Alternatively, the *Cardinium* symbiont in the Ghana population may be essential for host fitness (Bandi et al. 2001; Vavre et al. 2002; Pannebakker et al. 2007), or may be particularly affected by the antibiotic, explaining why treated females from the Ghana base population yielded very few offspring.

Interestingly, the timing of incompatibility differs between populations. In the *Wolbachia* infected Benin and *Cardinium* infected Ghana populations, incompatibility was expressed early in offspring development resulting in shrivelling of the eggs, whereas in the *Wolbachia* infected Brazil population incompatibility resulted in very low fecundity, similar as observed in *M. occidentalis* (Wu and Hoy 2012). Host-symbiont interactions can vary as a result of genetic differences of both host and symbiont (Bordenstein and Werren 2003). The *Wolbachia* infections in the Brazil and Benin populations are, at least based on 16S gene, different, but also the two mite populations appear to be genetically different, as reciprocal crosses between uninfected individuals from both populations did not yield offspring (Table 3). One possible mechanistic explanation for the difference in timing of incompatibility is that *Wolbachia* concentrations in the Benin population are lower than in the Brazil population. As a result, incompatibility is less severe in incompatible crosses that involve males with the lower bacterial density (Breeuwer and Werren 1993, Engelstaedter et al. 2007). Regardless of whether *Wolbachia* concentrations actually differ, host-symbiont interactions can vary due to genetic differences between *Wolbachia* and/or hosts (see Engelstaedter and Hurst 2009).

In addition, infected females produced a more female biased sex ratio than uninfected females in compatible crosses, as in *Wolbachia* infected females. An increase in the proportion of male offspring was detected in crosses involving treated females. Female bias sex ratio shift is one of the common effects of *Wolbachia* infection (i.e. Rigaud et al. 1997; Breeuwer 1997; Kajeyama et al. 2002). From an evolutionary point of view, symbionts that are maternally transmitted are expected to bias sex ratios toward the transmitting—female—sex.

Bidirectional incompatibility was observed in reciprocal crosses between mites from the Benin and Brazil populations that are both infected with *Wolbachia*. Bidirectional reproductive incompatibility often occurs in crosses between populations harboring different *Wolbachia* strains. Our finding of different bacterial symbionts in the three populations is consistent with this hypothesis. Compatibility was restored in crossings between tetracycline-treated females of the Benin population and tetracycline-treated males of the Brazil population. However, compatibility could not be restored in the reciprocal cross between tetracycline-treated males from Benin and tetracycline-treated females from Brazil. A possible explanation is that cyto-nuclear incompatibilities may exist between the Benin nuclear genome and the Brazil cytoplasm. Such cyto-nuclear incompatibilities have been reported between closely related species of the parasitoid wasp *Nasonia* (Breeuwer and Werren 1995; Koevoets et al. 2011), and between populations of the spider mite *Tetranychus quercivorus* Ehara and Gotoh (Gotoh et al. 2005a, b).

We have only studied three mite populations, and found that they are infected with two types of symbionts that have effects on sex ratio and incompatibility. It would be interesting to determine the infection patterns throughout the geographic range of *N. paspalivorus* and determine the nature of host-symbiont interactions.

Although morphologically and genetically similar, geographic populations of the predatory mite *N. paspalivorus* have developed reproductive isolation (Famah Sourassou et al. 2011), which is associated with (and perhaps also due to) endosymbiotic bacteria found in them. Evidently, this isolation has not (yet) led to morphological differences (Famah Sourassou et al. 2011) and to genetic differences at the level of the mitochondria. It is yet to be investigated whether these populations differ in other traits that are relevant to biological control of coconut mites on palm trees. Another practical implication could be that introducing an incompatible strain from one geographic area into the habitat of another strain may initially create a depression in population growth due to failures in producing viable offspring. The same may occur when different strains are mixed to initiate a mass rearing of the predatory mites. These are all good reasons to check for endosymbionts in biocontrol agents and carefully study their role in reproductive isolation between populations, as well as the genetic differentiation that is expected to arise from this.

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