

In situ observation of the *Cardinium* symbionts of *Brevipalpus* (Acari: Tenuipalpidae) by electron microscopy

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Abstract *Brevipalpus* (Acari: Tenuipalpidae) mites are important pests on a variety of host plant species. The mites damage their hosts directly by feeding and some species also serve as vectors of plant viruses. Among more than 200 described *Brevipalpus* species, three are recognized as vectors of plant viruses: *B. phoenicis*, *B. californicus* and *B. obovatus*. These species occur worldwide in subtropical and tropical regions. *Brevipalpus* mites reproduce mostly by thelytokous parthenogenesis and this condition was attributed to a bacterial endosymbiont, recently characterized as a member of the genus *Cardinium*. The same symbiont infects many other arthropods and is capable of manipulating their host reproduction in various ways. Generally the presence of *Cardinium* is determined by molecular, PCR based, techniques. In the current work we present visual evidence for the presence of these bacteria by transmission electron microscopy as a complement of previous detection by PCR. *Cardinium* is easily identified by the presence of a unique array of microtubule-like structures (ML) in the cell. Symbionts have been observed in several organs and eggs from different populations of all three *Brevipalpus* species known as vector of plant viruses. *Cardinium* cells were always immersed directly within the cytoplasm of

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infected cells. Bacteria were observed in all females of all instars, but were absent from all males examined. Females from some *Brevipalpus* populations were observed to be uninfected by *Cardinium*. This observation confirmed previous PCR-based results that these populations were aposymbiotic. The observed distribution of the bacteria suggests that these bacteria could have other functions in the mite biology beside feminization.

Keywords False spider mites · *B. phoenicis* · *B. californicus* · *B. obovatus* · Aposymbiont

Introduction

The presence of an endosymbiont of the Bacteroidetes phylum and its role in the feminization of *Brevipalpus phoenicis* (Geijskes) (Acari: Tenuipalpidae) were first described by Weeks et al. (2001). Since then, this bacterium, now classified as *Cardinium*, has been found in many arthropods (Weeks et al. 2003; Zchori-Fein et al. 2004), including three other *Brevipalpus* species, *B. obovatus* Donnadieu, *B. lewisi* McGregor and *B. californicus* (Banks) (Weeks et al. 2003; Novelli et al. 2005; Chigira and Miura 2005). The presence of the symbiotic bacterium has been determined through PCR using primers that amplify the 16S rDNA gene from all known Eubacteria or using sequence specific primers for *Cardinium* (Weeks et al. 2001; Weeks et al. 2003; Zchori-Fein et al. 2004; Chigira and Miura 2005; Novelli et al. 2005).

Natural populations of *Brevipalpus* are made up mostly of haploid females which reproduce by thelytokous parthenogenesis. Occasionally, males are present, and although they mate with females, fertilization is probably not effected (Pijnacker et al. 1981). Antibiotic treatment provided strong evidence that *Cardinium* is responsible for the feminization; untreated females produce almost exclusively females while antibiotic treated females produce significantly more males (Weeks et al. 2001; Chigira and Miura 2005; Groot and Breeuwer 2006). However, aposymbiotic populations of *B. phoenicis* were discovered that are thelytokous and haploid. It has been suggested that in those mites, genes responsible for feminization of unfertilized haploid eggs may have been transferred from the symbiont to the host genome (Groot and Breeuwer 2006). Although parthenogenetic reproduction may be an evolutionary disadvantage by limiting the amount of genetic variation, some species of *Brevipalpus* seem well adapted for the environmental variation. Specifically, *B. phoenicis*, *B. californicus* and *B. obovatus* have been reported from nearly 1,000 different plant species belonging to 139 families (Childers et al. 2003). In addition, transplantation experiments of *B. phoenicis* to alternative host plant species led Groot et al. (2005) suggest that the model of frozen niche variation, which postulates that different clones are specialized to different niches, best explains the clonal adaptation of these mites. *Brevipalpus* mites are also currently attracting interest because of their role as vector of several plant viruses. These viruses characteristically induce localized lesions in infected plants (Kitajima et al. 2003). Among them citrus leprosis is economically the most important viral disease of citrus industry in the Americas. In the Brazilian state of São Paulo alone, expenditures for chemical control of *B. phoenicis*, the vector of the causal virus, have been estimated to be up to US\$ 80 million/year (Rodrigues et al. 2002, 2003).

PCR has been efficient to detect *Cardinium* in *Brevipalpus* mites (Weeks et al. 2001; Chigira and Miura 2005; Novelli et al. 2005; Groot 2006) but it does not provide information regarding the distribution of the bacterium within the host organism. On the other hand *Cardinium* has a unique structural feature among bacteria, the presence of microtubule-like

material (ML) (Zchori-Fein et al. 2004; Bigliardi et al. 2006), which permits its discrimination from other bacterial types in sections.

To explore such a peculiarity, an electron microscopic study was undertaken in sections of three *Brevipalpus* species (*B. californicus*, *B. obovatus* and *B. phoenicis*) to confirm the presence of *Cardinium* in different instars and observe its distribution among different organs. Here we report the results of such studies which confirm the presence and absence of *Cardinium* bacteria as determined by PCR-based techniques. In addition we describe the distribution of the bacteria among distinct organs and tissues of the host, as well as in eggs. Finally, we provide details on the typical ML of these symbionts.

Materials and methods

Mite populations

The populations of *Brevipalpus* species studied are listed in Table 1. Most of them were collected in Brazil, but samples from The Netherlands, Costa Rica, Colombia and Japan are included. Some samples came from populations collected in Brazil and The Netherlands, maintained on bean (*Phaseolus vulgaris* L.) at the University of Amsterdam. Presence or absence of *Cardinium* in these populations was previously assessed by PCR (Novelli et al. 2005, 2006; Groot 2006).

Electron microscopy

Adult mites of all populations (Table 1) were fixed in a solution of 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, with 1.8% (w/v) sucrose (R. Dallai. Personal communication), post fixed in 1% osmium tetroxide, dehydrated in acetone and embedded in Spurr's low viscosity epoxy medium. To enable penetration of the fixative and infiltration of the embedding medium, a small portion of either the anterior part of the propodosoma or the posterior end of the hysterosoma was cut off with a razor blade under a binocular. Sections were made in a Leica Ultracut UC2 microtome equipped with a diamond knife. Thin sections were collected on copper grids, stained with 3% uranyl acetate and Reynold's lead citrate (Maunsbach and Afzelius 1999) and examined in a Zeiss EM 900 transmission electron microscope. To orient the localization of the organs, semi-thins sections (1–2 μm) were mounted on glass slides and stained with basic 1% toluidine blue. All the chemicals used were purchased from Electron Microscope Sciences (Hartfield, PA, USA). Males, larvae, protonymphs, deutonymphs and aposymbiotic adults (Table 1) of some populations were also included in these studies. At least six mites per population and instars were examined.

Results and discussion

Because of the cuticle, tissue preservation and cutting properties of the processed mites were poor if entire mites were used. The opening made by the cutting off mite's extremities resulted in good fixation and infiltration of the resin and in good quality sections.

Cardinium was the only bacterium (Figs. 1 and 2) found in *Brevipalpus* mites, confirming data of Groot (2006). In sections the bacterial cell profile varied from oval/elliptical to sausage-shaped. The largest cells were 4–5 μm long and 0.5–1 μm wide. *Cardinium* cells

Table 1 List of the *Brevipalpus* mite populations studied and the results of electron microscopic examination

Mite species and source plant	Geographic Origin	Results	
		PCR	TEM
<i>B. phoenicis</i>			
<i>Citrus sinensis</i> -A,P,D ^a	1*	+ ^b	+ ^c
<i>C. sinensis</i> -M ^a	1	– ^b	–
<i>C. sinensis</i> -A	2	– ^c	–
<i>C. sinensis</i> -A	3	+ ^c	+
<i>C. sinensis</i> -A	4	+ ^c	+
<i>C. sinensis</i> -A	5	+ ^c	+
<i>Wisteria floribunda</i> -A	6	– ^c	–
<i>Passiflora edulis</i> f. <i>flavicarpa</i> -A	6	nt ^d	+
<i>Clerodendron x speciosum</i> -A	6	+ ^c	+
<i>C. thomsonae</i> -A	6	+ ^c	+
<i>C. splendens</i> -A	6	+ ^c	+
<i>Coffea arabica</i> -A	7	+ ^c	+
<i>C. arabica</i> -A	8	+ ^c	+
<i>C. arabica</i> -A	9	+ ^c	+
<i>Malvaviscus arboreus</i> -A	10	nt	+
<i>C. sinensis</i> -A,P,D	11a	+ ^c	+
<i>C. sinensis</i> -M	11a	–	–
<i>C. sinensis</i> -A	11b	+ ^c	+
<i>B. obovatus</i>			
<i>Solanum violaeifolium</i> -A,P,D	6	+ ^c	+
<i>Cestrum nocturnum</i> -A	1	+ ^c	+
<i>Malpighia glabra</i> -A	11c	– ^b	–
<i>Strongylodon macrobotrys</i> -A	11d	– ^b	–
<i>B. californicus</i>			
Several orchid species-A	6	+ ^c	+
<i>Cymbidium</i> sp.-A	12	nt	+
<i>Rhododendron</i> sp.-A	11e	+ ^b	+

^a A-female, adult; P-protonymph; D-deutonymph; M-male

^b PCR carried out at the University of Amsterdam, The Netherlands (Groot 2006)

^c PCR carried out at the Centro APTA Citros, Cordeirópolis, SP, Brazil (Novelli et al. 2005, 2006)

^d nt PCR not tested

^e –absence and + presence of *Cardinium* in sectioned mites

* 1-Centro APTA Citros, Cordeirópolis, SP, Brazil; 2-Vila Fão, RS, Brazil; 3-FCAV/UNESP, Jaboticabal, SP, Brazil; 4-CORPOICA, Villavicencio, Colombia; 5-Universidade de Costa Rica, San Jose, Costa Rica; 6-ESALQ, Piracicaba, SP, Brazil; 7-Lavras, MG, Brazil; 8-São Paulo, SP, Brazil; 9-Instituto Agronomico, Campinas, SP, Brazil; 10-Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil; 11-populations maintained on bean (*Phaseolus vulgaris*) plants at the University of Amsterdam (Groot 2006) and collected at: a. Viçosa, MG, Brazil; b. Piracicaba, SP, Brazil; c. Pantanal, MS, Brazil d. Arnhem, The Netherlands; e. Viçosa, MG, Brazil; 12-University of Okayama, Kurashiki, Japan

were enclosed by the plasma membrane and a thin, Gram negative type cell wall and contained small ribosomes and fibrillar DNA as other prokaryotes. The most characteristic feature of *Cardinium*, and unique among bacteria, is the presence of a bundle of thin tubular structure, generally arranged perpendicularly to the lateral part of the bacterial body (Fig. 2). These tubules were associated with a plate-like structure as originally described by Zchori-Fein et al. (2004) and detailed by Bigliardi et al. (2006) and referred to as microtubule-like structures (ML). In cross section these ML had a uniform diameter of ca. 12–15 nm, and were up to 100 nm long as seen in longitudinal sections. Counts made in

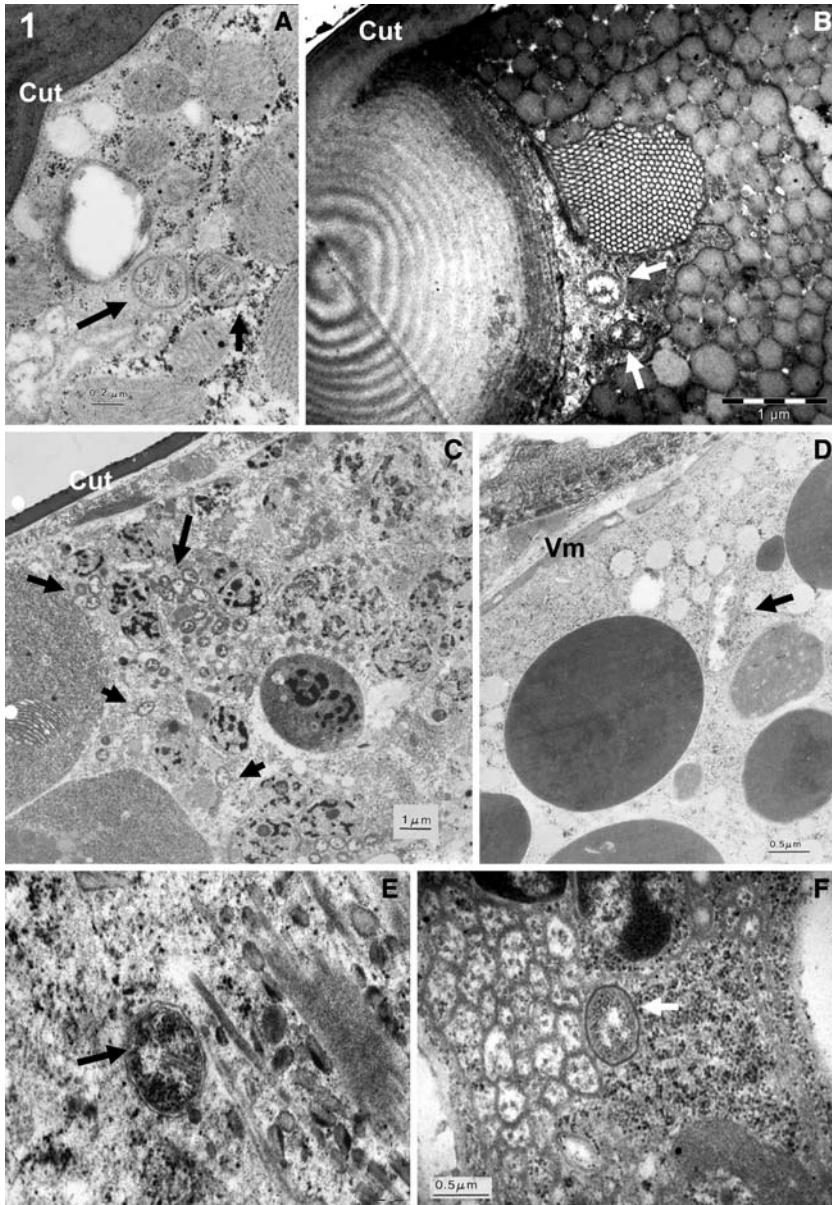


Fig. 1 Transmission electron micrograph of sections of three *Brevipalpus* mite species (*B. phoenicis*, *B. obovatus* and *B. californicus*) indicating the presence of *Cardinium* symbionts in different organs of adult females. (A) Symbionts in the epidermis of *B. californicus* collected from orchid. (B) *Cardinium* can be seen within the eye complex of *B. phoenicis* infesting *Clerodendron thomsonae*. (C) Numerous bacteria are present in the ovary, within the oocytes in *B. phoenicis* infesting *Passiflora edulis* f. *flavicarpa*. (D) A single bacterium in an egg within the ovary of *B. obovatus* collected from *Solanum violae-folium*. (E) *Cardinium* in the epithelial cell of branched digestive tube (cecum) of a *B. phoenicis* infesting *Citrus sinensis*. (F) Symbiont in a nerve cell surrounding the nervous ganglion in *B. obovatus* collected from *Cestrum nocturnum*. Cut—cuticle; Vm—vitelline membrane. Arrows point to the bacterial cells

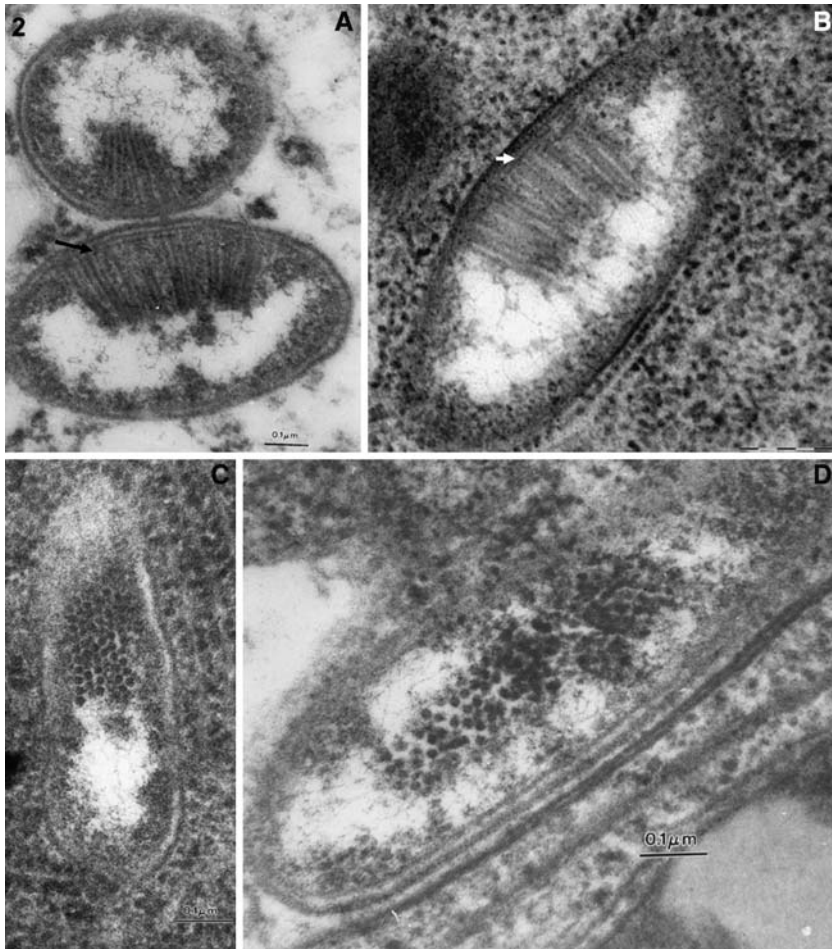


Fig. 2 Details of the microtubule-like structures (ML) in the cells of *Cardinium* from adult *Brevipalpus* mites. (A and B) Longitudinal sections of the ML arranged perpendicularly onto the cell membrane. A layer is visible adjacent to the insertion of the ML on the cell membrane (arrow), respectively from *B. phoenicis* infesting *Clerodendron x speciosum* and *B. obovatus* colonizing *S. violaefolium*. (C and D) Cross section of bundles of the ML, showing their tubular nature and the ordered array, respectively in *B. phoenicis* collected from *C. sinensis* and *C. thomsonae*

cross sections revealed up to 50 of MLs/cell. The ultrastructural features of the *Cardinium* found in *Brevipalpus* were essentially similar to the detailed description made by Bigliardi et al. (2006) in the vine leafhopper *Scaphoideus titanus* Ball except for the diameter of the MLs which was rather uniform in *Brevipalpus*. The function of the ML is still unknown. Bigliardi et al. (2006) suggested that they may serve as cytoskeleton, have a role in cell transport or in communication between cell membrane and cell wall. These ML seem to be different from the recently described filament bundles in *Caulobacter crescentus* using electron cryotomography (Briegel et al. 2006) that are thinner and have varied location in the bacterial cell.

Groot (2006) mentions at least two different types of *Cardinium* in *Brevipalpus* based on molecular data. One of them is present in *B. phoenicis* and *B. obovatus* and the other, in

populations of *B. californicus*. No morphological difference in the morphology of the *Cardinium* was observed in the different specimens studied here.

Cardinium cells were detected in every part of the *Brevipalpus* body including the legs and palps. However, even without critical morphometric analysis, the examination of a large number of sections of different organs of the three *Brevipalpus* species indicated that the number and distribution of *Cardinium* was highly variable between individuals including different instars, and in the inner organs. The symbionts were located in most wide array of tissues (Alberti and Coons 1999), including epidermis, muscle, eye, fat body, digestive system epithelium, nervous cells (ganglia and innervations), tracheal cells, prosomal gland, ovarium and developing eggs (Fig. 1). This is in contrast to *S. titanus* where the presence of symbionts was restricted to the salivary gland and reproductive system (Bigliardi et al. 2006). *Cardinium* cells were absent in males, confirming PCR assays by Weeks et al. (2001). The symbionts appeared immersed directly in the cytoplasm of the infected cells (Figs. 1 and 2). This is a different situation in comparison with *Wolbachia* symbionts which consistently are located within a vacuole, separated from the cytoplasm by a membrane (Louis and Nigro 1989).

Electron microscopy revealed that *Cardinium* bacteria were present in all three *Brevipalpus* species studied. However, in three populations of *B. phoenicis* no symbiont could be observed confirming PCR data (Groot 2006; Novelli et al. 2006). One of these populations was collected from *Wisteria floribunda* DC found in Piracicaba, SP, Brazil. Another was collected from *Citrus sinensis* (L.) Osbeck at Vila Fão, RS, Brazil. The third population was collected from *Strongylydon macrobotrys* A. Grey in The Netherlands. The latter population though morphologically is *B. phoenicis* type 2, based on the idiosomal reticular ornamentation (Mesa Cobo 2005), is more related to *B. obovatus* based on mitochondrial DNA markers and might actually be a fourth species (Groot 2006). No similar analysis was made with other two aposymbiotic populations.

Because *Cardinium* is widespread within the mite body (Fig. 1) it is likely that most of the mite cells, including ovary, become infected during early embryogenesis, though uninfected cells may appear and later be infected through cell-to-cell movement of the bacterium. The appearance of the rare males in natural population could be due to the production of some bacteria-free egg cells. This situation is enhanced with tetracycline treatment which kills *Cardinium* in females and significantly increases the number of males among the offsprings (Weeks et al. 2001). This led to the notion that infection by *Cardinium* results in the feminization of the haploid *Brevipalpus*. The finding of female-only, aposymbiotic populations seem enigmatic but Groot and Breeuwer (2006) suggest as an explanation that the gene(s) of the symbiont responsible for the feminization may have been transferred to the mite genome. What the effect would be of a reinfection of such population, a possibility that exists because evidence of horizontal transmission of *Cardinium* was presented (Groot 2006), has to be assessed. Whether or not *Cardinium* has a role in the transmission of the viruses by *Brevipalpus* mites is under investigation.

One advantage of the visualization of the *Cardinium* in situ was to determine that the symbiont is widespread within the body of the examined *Brevipalpus* species, a situation that is difficult to conclude through PCR based technique or by light microscopy. On the other hand, this fact raises some questions regarding the role of the symbiont in the biology of the mite host. If the symbiont is involved only in feminization or related function, it would be expected to be restricted to the host reproductive system as is commonly observed for *Wolbachia* symbionts (Stouthamer et al. 1999) and also noticed for *Cardinium* in the cicadellid *S. titanus* (Bigliardi et al. 2006). But being present everywhere in the mite body, the possibility of some additional role should be considered, for it is likely that

maintaining these bacteria is of high metabolic cost to the host. A critical comparison of the physiology and behavior of normal and aposymbiotic populations may provide some answers. For instance, Weeks and Stouthamer (2004) showed that infection with *Cardinium* had a positive effect on the fecundity of the predatory mite *Metaseiulus occidentalis*.

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