Symbiosis, 34 (2003) 231–251
Balaban, Philadelphia/Rehovot

Symbiosis in the Dryophthoridae Weevils (Coleoptera, Curculionoidea): Morphological Variability of Symbiotic Intracellular Bacteria

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Received February 13, 2003; Accepted May 10, 2003

Abstract

Dryophthoridae weevils are phytophagous insects thriving on a broad host-plant spectrum. Some, such as *Sitophilus* spp. or *Cosmopolites sordidus*, are major agricultural pests of cereals and bananas, respectively. Previous studies have concluded that several Dryophthoridae species harbour intracellular bacteria (endosymbionts) in specialized organs named bacteriomes. In this work, we have demonstrated the presence of intracellular symbiosis in seven out of the eight Dryophthoridae species tested, and have assessed the morphological variability of these endosymbionts. Histological analyses of the endosymbionts in either the larval bacteriome or adult female ovaries reveal two fields of variability: physiologic (length variation during host development) and morphologic (both interspecific and intraspecific). These variations are expressed to different degrees in the species studied. Indeed, *Sitophilus granarius* displays much less pleiomorphism than *S. zeamais*. Such pleiomorphism may be a consequence of both genetic polymorphism and host/bacteria interaction.

Keywords: Intracellular symbiosis, insect, weevil, Dryophthoridae, endosymbiont, bacterial morphology

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0334-5114/2003/$05.50

1. Introduction

More than 500 species have been described within the Dryophthoridae weevil family previously named Rhynchophoridae (Alonzo-Zarazaga and Lyal, 1999). Some species are ubiquitous, such as *Sitophilus oryzae* L. and *S. zeamais* Mots, whereas others, such as *Trigonotarsus rugosus* Boisduval (Australia) are restricted to limited geographic areas. Moreover, feeding habits and ecological niches are highly variable in the Dryophthoridae family. Most species, like *Cosmopolites sordidus* Germar, which feeds on the trunk base of banana trees, or *Rhynchophorus palmarum* L., which thrives on palm trees, develop on monocot plants. In contrast, some larvae develop within the seeds of cereals (e.g., *S. oryzae, S. zeamais* and *S. granarius*) or other plants like tamarind (*S. linearis*), while other species, like *Sipalinus gigas* Fab, develop in decaying wood. The size range of such larvae is quite large, extending from 3–4 mm for *S. vateriae* Marshall to 120 mm for *R. palmarum.*

Intracellular symbiosis has been previously described in several species of Dryophthoridae by Buchner (1965). It is characterized by the presence of a unique larval bacteriome, a structure that is always located between the digestive track and the fat body (Fig. 1). The bacteriome organ is located at the junction between foregut and midgut, but is never directly connected to the digestive track (Nardon, 1971; Nardon et al., 2002).

In *Sitophilus* spp., the bacteriome disintegrates during larval metamorphosis, and bacteriocytes migrate to occupy the mesenteric caeca that develop in the adult (in Nardon and Grenier, 1988). In the young (e.g., one month old) female, bacteria are also found in an "apical" bacteriome located at the apex of each of the four ovarioles. Nevertheless, bacterial transmission occurs exclusively through the germinal line, and the oocytes become permanently infected with the symbiotic bacteria (Nardon and Wicker, 1981; Heddi et al., 1999). Such a symbiotic interaction has also been described in *Sitophilus granarius* (Mansour, 1930), *S. oryzae* (Pierantoni, 1927), *S. zeamais* (Musgrave and Homan, 1962), *Cosmopolites sordidus,* and *Metamasius hemipterus* (Nardon et al., 1985). Interestingly, *Sitophilus linearis,* which lives on the nutritionally rich seeds of *Tamarindus indica,* is completely devoid of intracellular bacteria (Delobel and Grenier, 1993).

During 30 years of investigations, no significant variability has been reported in the morphology of larval or adult bacteriomes themselves; however, variation among the endosymbionts has been noted. The aim of this work is to describe the inter- and intraspecific differences that occur between endosymbionts within the Dryophthoridae, as well as to identify morphological differences that occur during the larval development of a given host species.

2. Material and Methods

Morphological analysis

We have analyzed a total of 45 strains comprising eight different species (Tables 1 and 2). Phylogenetic analysis, based on 16S-rDNA sequence comparisons, revealed that Dryophthoridae endosymbionts belong to the γ3-protebacteria and are very closely related to the Enterobacteriaceae. Dryophthoridae endosymbionts can be divided into three monophyletic groups that seem to have been acquired at different times during the evolutionary history of their host: (1) *Sitophilus* sp. group, (2) *Trigonotarsus-Diocalandra* group, and (3) *Metamasius-Cosmopolites-Sphenophorus* group (Lefèvre et al.,

Table 1. Origin of species and strains of studied Dryophthoridae.

Species and strain	Origin	Food
Metamasius hemipterus L.	Guadeloupe	Sugar cane
Cosmopolites sordidus Germar	Guadeloupe	Sugar cane
Trigonotarsus rugosus Boisduval	Queensland (Australia)	*Xanthorrhea* sp.
Sphenophorus striatopunctata Goeze	Lyon (France)	*Sirpus lacustris*
Sitophilus granarius L.		
AG	Jura (France)	Wheat
Brayard	Jura (France)	Wheat
Chambe	Jura (France)	Wheat
Gilles	Jura (France)	Wheat
GRA	Jura (France)	Wheat
Lapins	Jura (France)	Wheat
Mâcon	Mâcon (France)	Barley
Sitophilus rugicollis Casey	Chandigarh (India)	*Eugenia jambolana*
Sitophilus oryzae L.		
Australie	Queensland (Australia)	Wheat
Bénin	Ina (Benin)	Sorghum
Bouriz	Grocer's Lyon	Rice
Chine	Guangzhou	Vermicelli
Chaver	Grocer's Lyon	Rice
Françoise	Grocer's Lyon	Long grain rice
Guad 1 and 2	Guadeloupe	Maize
GV (Lab strain)	Lyon (France)	Wheat
Sfr (Lab strain)	Lyon (France)	Wheat
SSO (Lab strain)	Slough (England)	Wheat
Tunisie	Nasrallah (Tunisia)	Wheat
W	Grocer's Lyon	Wheat

Table 2. Origin of the studied strains of *Sitophilus zeamais*.

Strains	Geographic origin	Food
Abruzzes	Italy	Maize
Arau	South Brazil	Araucaria
Basmati	Lyon (France)	Basmati rice
Bic	Bouake (Ivory Coast)	Maize
Burki	Burkina Faso	Maize
Cameroun	Yaoundé (Cameroun)	Maize
Chariz	Grocer's Lyon	Long-grain rice
Congo 1	Nsango (Congo)	Maize
Congo 2	Missingha (Congo)	Maize
Congo 3	Knasso (Congo)	Maize
Congo 4	Laudima (Congo)	Paddy rice
Dax (Lab strain)	Bordeaux (France)	Wheat
FB	Réunion Island	Rice
Gérard	Réunion Island	Maize
Guyane	French Guiana	Sorghum
Lagoa	Acores	Maize
Mex2	Puebla (Mexico)	Maize
Mex5	San Pedro (Mexico)	Maize
Syrie	Damas (Syria)	Maize
Thai	Thailand	Maize
ZMA	Slough (England)	Maize

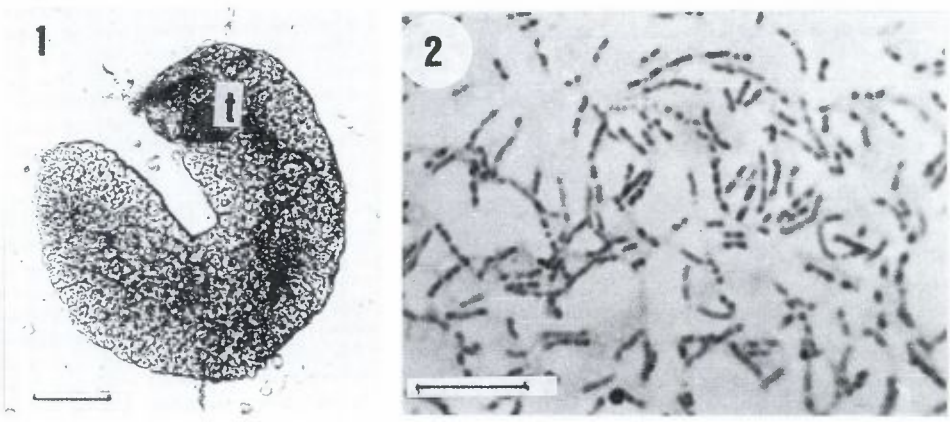


Figure 1. Larval bacteriome of *Sitophilus oryzae* (SFr strain) separated from the intestine (3rd larval instar). Trachae are clearly visible (t). Vital observation. Scale bar = 200 µm.

Figure 2. Chromatin granules in symbiotic bacteria of *Sitophilus oryzae* (Sfr strain) larvae. Ziehl fuchshine. Scale bar = 4 µm.

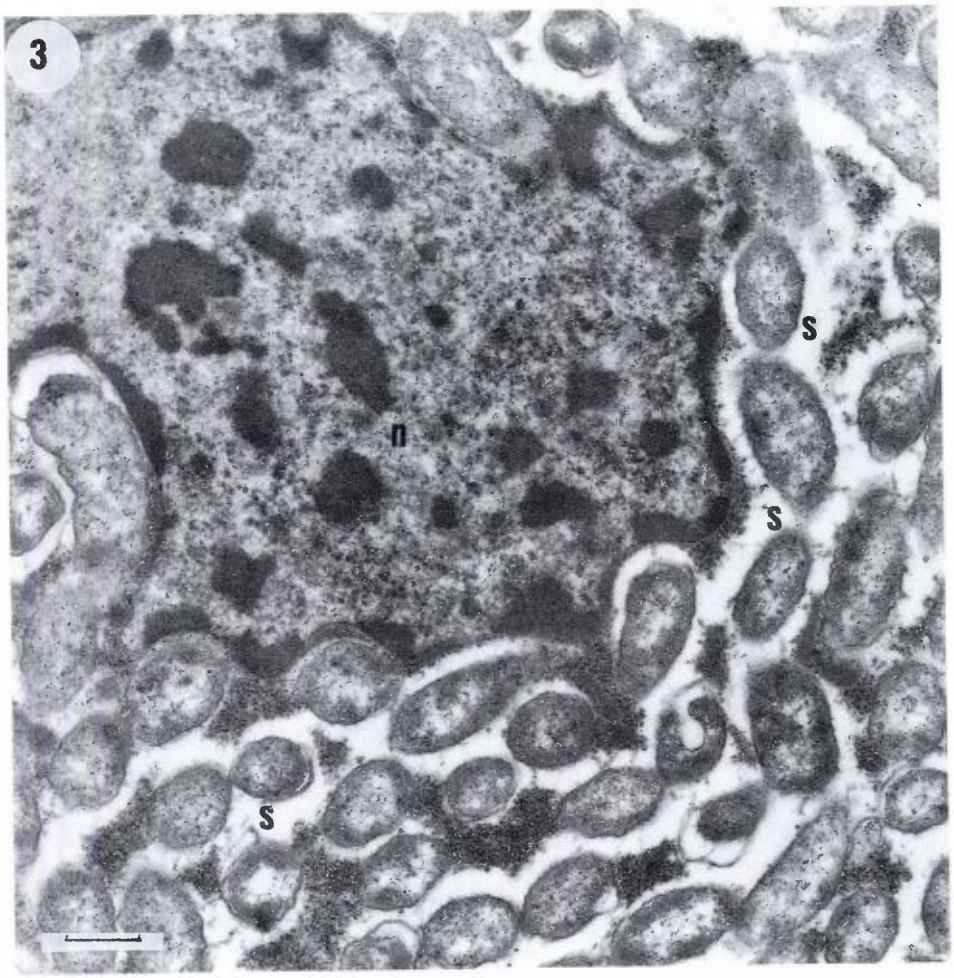


Figure 3. *Sitophilus granarius* (Mâcon strain) larval bacteriome: contact of bacteria with the nucleus envelope of a bacteriocyte. Scale bar = 0.5 µm.

personal communication). Except for those of *Trigonotarsus rugosus*, observed in histological sections, endosymbionts were described either in ovaries or in larval bacteriomes. Ovaries or larval bacteriomes were dissected under the microscope and squashed in a drop of Yeager's solution [200 mM NaCl, 20 mM KCl, 3.5 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 0.1 mM NaH_2PO_4, 5 mM glucose, 2 mM levulose, and 40 mM maltose] by compression between two microscopy slides. At least 10 squashes per strain were examined. Squashes were observed after drying the sample at 60°C on a heating block, and staining by standard

microbiology techniques. The stains included: Gram, Wirtz-Conklin (for the spores), Fite, Cambre and Turner, Stamp, Ziehl-Nielsen, fuchshin and methylene blue (capsule detection), toluidine blue (1% w/v, pH=5.4), Feulgen (chromatin granules) or crystal violet (in Bourdon and Marchal, 1973).

Transmission electronic microscopy was performed on samples fixed with glutaraldehyde/osmic acid as previously described (Nardon et al., 1992), and using a Hitachi Hu 12A microscope. Squashes were directly sputter-coated with gold and observed with a JEOL 35CF apparatus.

Counting the ovarian endosymbionts

The morphological type and number of endosymbionts were determined using tracings of photographic images. While not precise, it is a simple method that allows a dependable characterization of endosymbiont types. Almost all comparisons between symbiotic bacteria were performed on either old larvae or adult female ovaries. The age of the larvae was estimated by measuring the size of both the cephalic capsule, and the whole body.

Ovaries were chosen at nearly the same physiological state; i.e., at previtellogenesis and the onset of vitellogenesis. To count the bacteria, four ovaries were gently homogenized using a Potter tube containing 0.2 ml of Yeager's solution. The bacteria present in an aliquot of the homogenate were counted in a Thoma cell using phase contrast microscopy.

3. Results

General description of endosymbionts

Of the eight species analyzed in this work, *Sitophilus oryzae* is the best described (Table 1). The different types of insect species, or the different endosymbiont strains in the same host species, cannot be distinguished by staining techniques. Endosymbionts from larval bacteriomes and ovaries are Gram negative and do not form endospores (Nardon, 1971). Bacteria observed by phase-contrast microscopy exhibit a sort of twitching movement, however no flagellum could be observed using sputter-coated scanning microscopy analysis. Nucleoid regions were stained by some techniques, such as Stamp, Wirtz-Conklin and Feulgen (Fig. 2), that also specifically stain the nucleus of the host cells. The nucleoid regions may also reveal the presence of unseptated bacterial filaments.

No evidence of typical capsules was found, but a halo was often observed surrounding the endosymbionts (Fig. 4). This halo may result from the presence of mucus, possibly induced by the host/endosymbiont interaction.

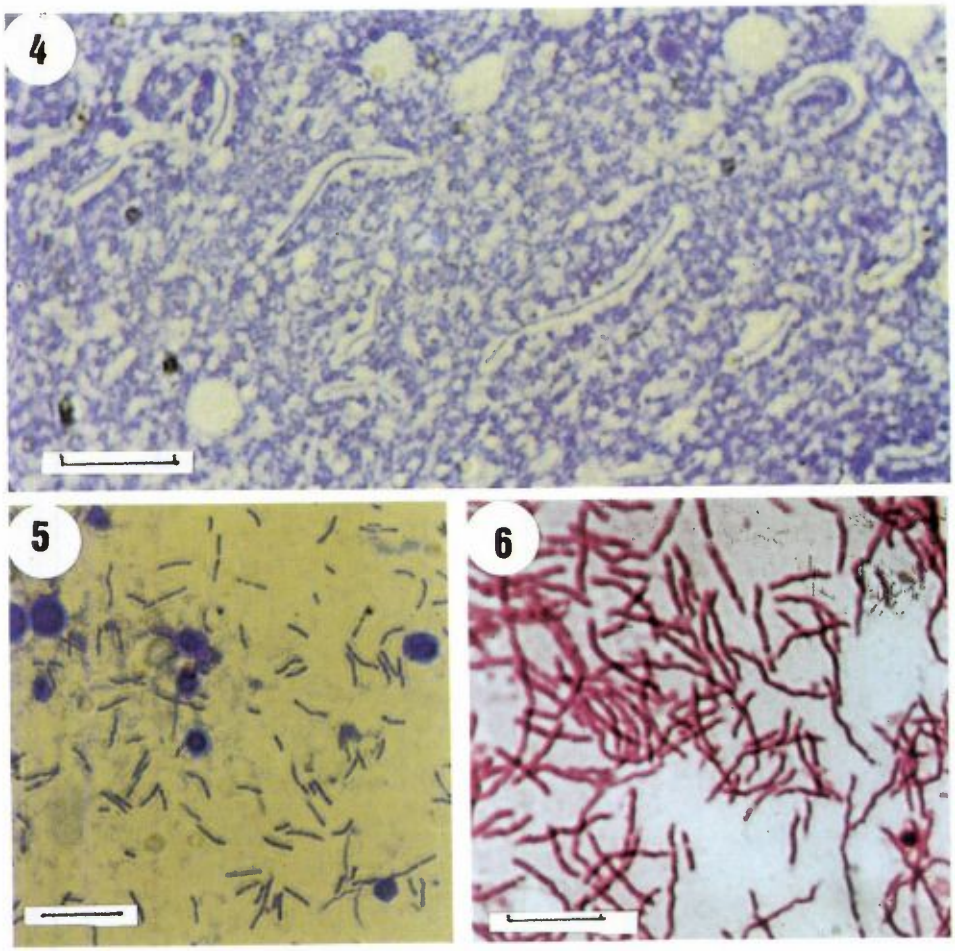
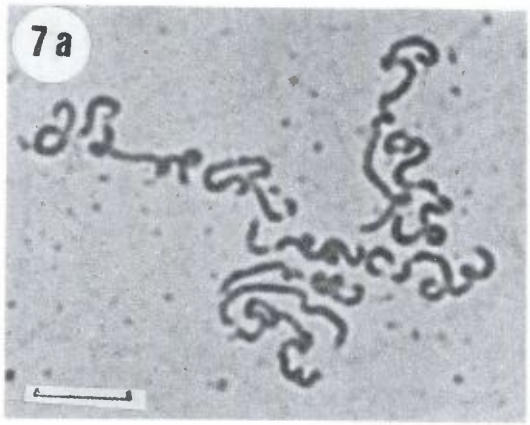


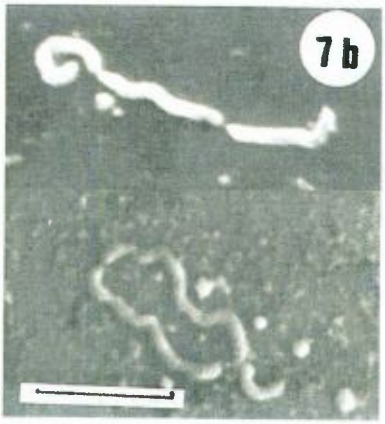
Figure 4. Ovarian endosymbionts in *Sitophilus granarius* (Mâcon strain). The halo is clearly visible. Crystal violet. Scale bar = 10 µm.

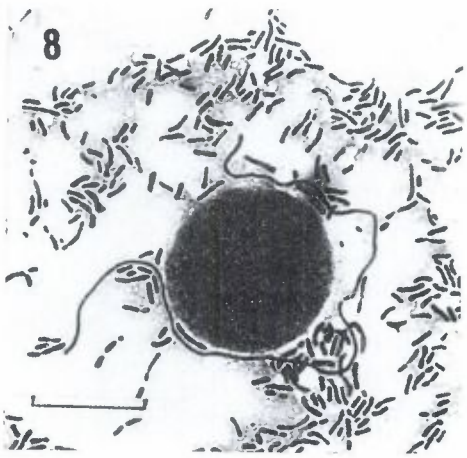
Figure 5. Squash of a young larva of *Sitophilus oryzae* (SFr strain). Crystal violet. Scale bar = 10 µm.

Figure 6. Squash of a bacteriome from a IV stage larva in *Sitophilus oryzae* (SFr strain) to compare with Figure 5. Stamp. Scale bar = 10 µm.

The Dryophthoridae endosymbionts analyzed in this work were not shown to be enclosed in a vacuole. This observation is still questionable in the *Metamasius* group of endosymbionts (Nardon et al., 1985). Instead they are free in the cytoplasm of the host cells (Fig. 12), where they may be protected by the







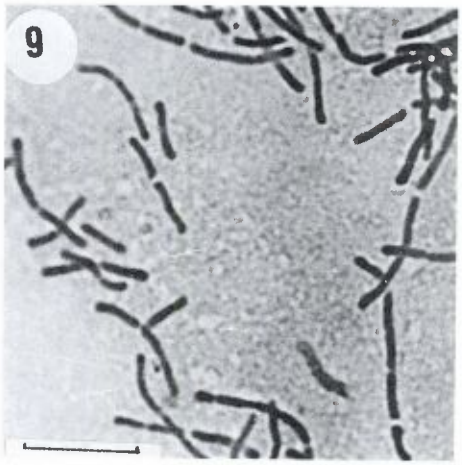


Figure 7. Ovarian endosymbionts of *Cosmopolites sordidus*. a: Crystal violet. Scale bar = 5 µm. b: scanning. Division of the bacilli. Scale bar = 4 µm.

Figure 8. Ovarian endosymbionts of *Metamasius hemipterus*. The two morphotypes (long and short bacilli) and their contact with a nucleus. Crystal violet. Scale bar = 10 µm.

Figure 9. Larval endosymbionts of *Metamasius hemipterus*. Division of the bacilli. Crystal violet. Scale bar = 10 µm.

mucus-like material of the halo (Nardon et al., 1985). The absence of a vacuole might facilitate the bacterial movement inside the host cells.

Within the cytoplasm of the bacteriocyte the location of the bacteria is variable. Bacteria can be found surrounding the bacteriocyte nucleus (Figs. 3 and 8), and some appear attached to the nucleus in specific "loges" within the

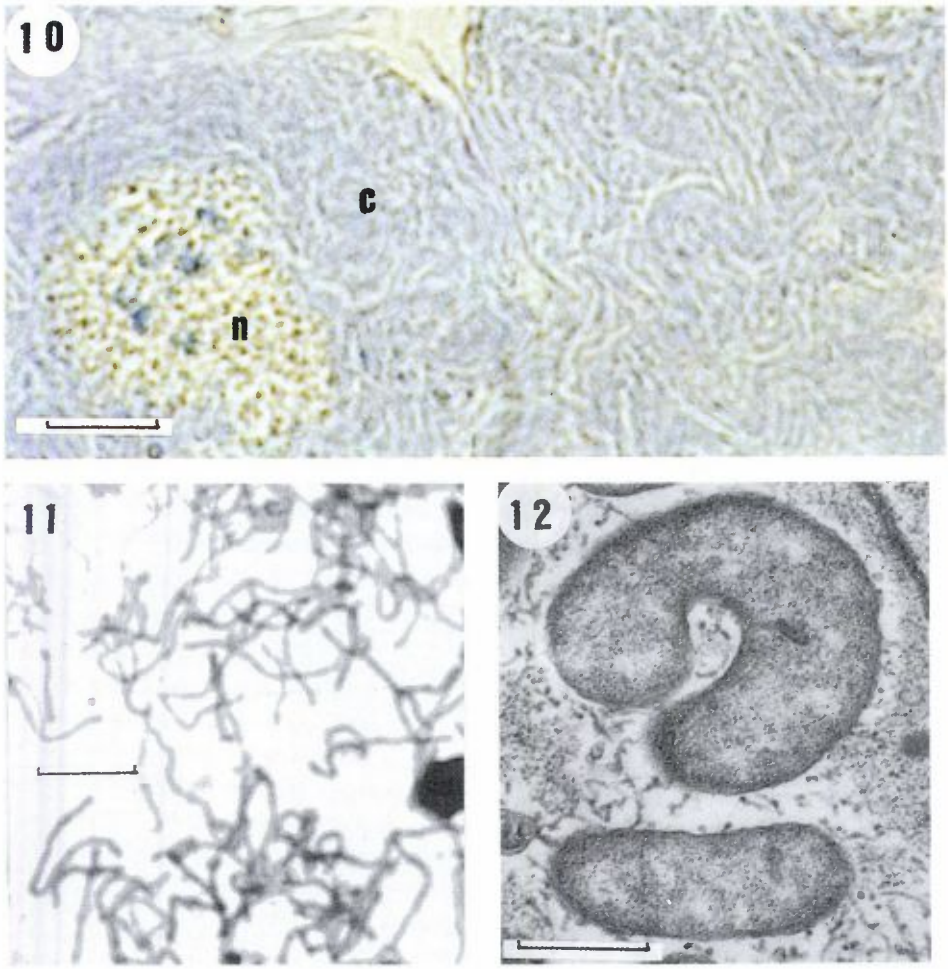


Figure 10. Larval endosymbionts of *Trigonotarsus rugosus*. Long and flexuous bacilli filling the cytoplasm (c) of the bacteriocytes. Ribonuclease, toluidine blue and safranine. n: nucleus. Scale bar = 20 µm.

Figure 11. Endosymbionts from squash of *Sitophilus rugicollis* ovaries. Crystal violet. Scale bar = 10 µm.

Figure 12. Larval endosymbionts of *Sitophilus zeamais* (ZMA strain). They are not included in vacuoles. Electronic microscopy. Scale bar = 0.5 µm.

nucleus membrane. When tissue retraction occurred during the fixation procedure, little filaments could be observed both between the bacteria and the nuclear membrane, as well as between the bacterial cells (Fig. 3). Such images suggest that there may be some direct interaction between the nucleus and the

bacteria. These observations were repeatedly made in *Cosmopolites sordidus*, *Sitophilus oryzae* and *S. zeamais* and, thus, may constitute a general characteristic among the Dryophthoridae.

Variable characters

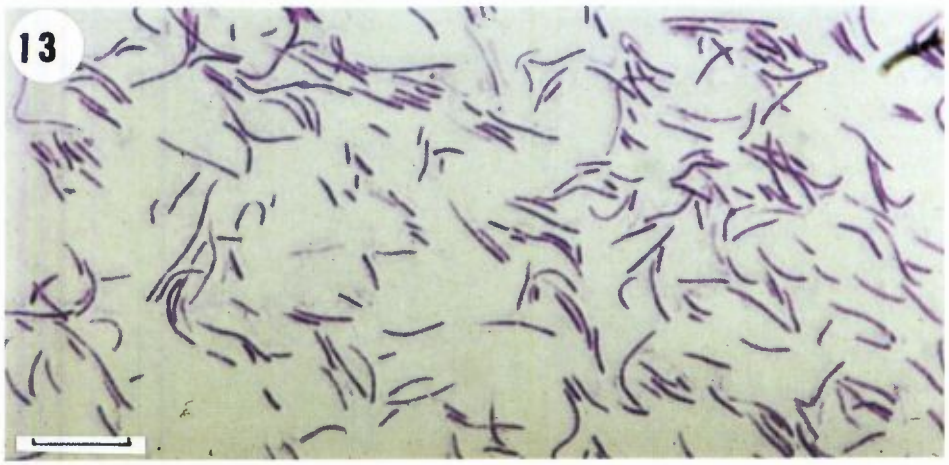
The endosymbiont number is highly variable in *S. oryzae* and is dependent both on biological parameters, such as diet (Delobel and Grenier, 1993), and on genetic factors (Nardon et al., 1998). Hence, within *S. oryzae* the strain GV revealed 75,500±5,900 endosymbionts per ovary, whereas strain W possessed significantly fewer (24,600±2,100) endosymbionts, even when both strains were reared on wheat grains under the same environmental conditions.

Cellular morphology is the most variable character observed in the endosymbionts, especially in the length and the degree of flexure of the rod-shape cell. Variability is observed both at the level of host-species and host-strain, and also exists between the different developmental stages of the host insect. Hence, in *S. oryzae*, bacteria from oocytes are longer than those from larval bacteriomes. In *S. oryzae*, endosymbionts of old larvae are longer than those of young larvae (Figs. 5 and 6). This age-dependant variability means that comparisons of symbiotic bacteria must be made at the same stage of host development.

Interspecific morphological variability

Metamasius hemipterus and *Cosmopolites sordidus*, both of these species live in the same ecological niche (i.e., within the trunks of banana trees), and their endosymbionts appear to belong to the same taxon (Lefèvre et al., personal communication). In *M. hemipterus*, the endosymbionts are 1 µm-wide rod-shaped, with a highly variable length (Figs. 8 and 9). In the ovaries, short (2 to 10 µm) forms predominate, but long (10 to 20 µm) forms exist as well, sometimes reaching 100 µm in length as a flexuous form. In young larvae (i.e., a cephalic capsule length of less than 2 mm), the bacteria are 5 to 15 µm long within the bacteriome. As the bacteriocyte grows the bacteria lengthen, reaching 100 and 200 µm during metamorphosis (Nardon et al., 1985).

In *Cosmopolites sordidus* the endosymbionts are always flexuous regardless of their location and the age of the host (Fig. 7a). Moreover, they always appear grouped in squashed samples, forming a sort of "colony" (Nardon et al., 1985). None of these characteristics was found among the endosymbionts of the other weevil species studied. Secretion of very abundant mucus by the endosymbionts might explain the apparent agglutination phenomenon. Division structures were also observed (Fig. 7b), as were endosymbionts with



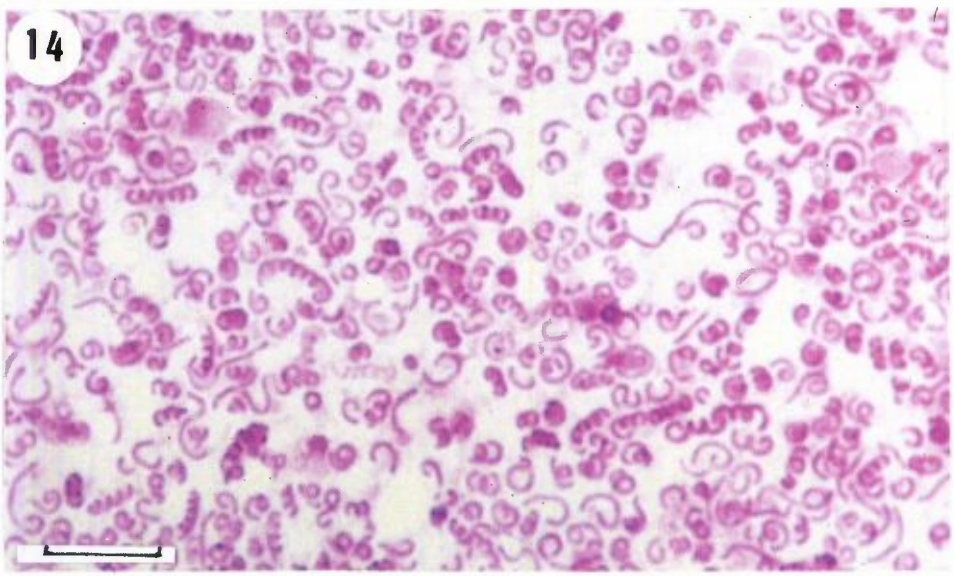


Figure 13. Larval endosymbionts of *Sitophilus oryzae* (Chaver strain). Gram. Scale bar = 10 µm.

Figure 14. Larval endosymbionts of *Sitophilus zeamais* (Chariz strain). The bacteria present numerous morphotypes. Safranine. Scale bar = 10 µm.

complex twisted shapes, resembling oriental letters (Fig. 7a). These forms were found in the same samples as simple C- or S-shape endosymbionts, which may illustrate the beginning stages of a spiraling process, such as seen in *S. zeamais*.

A variety of other forms have also been observed. In *Sphenophorus striatopunctata*, the rod-shaped endosymbionts are relatively short (2 to 10

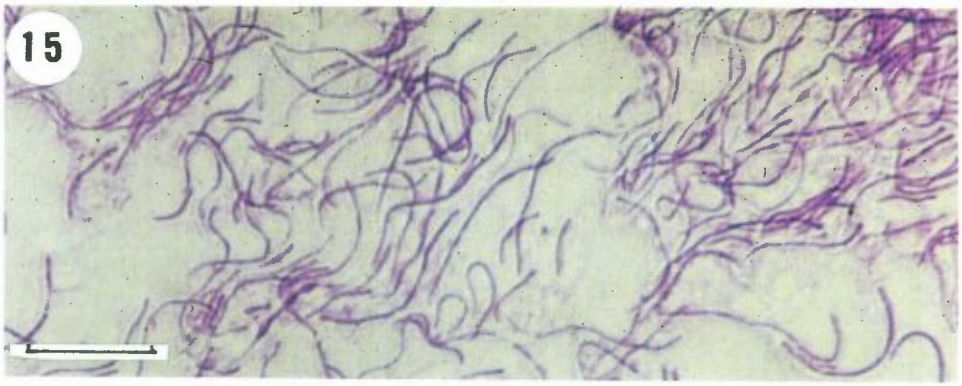


Figure 15. Larval endosymbionts of *Sitophilus oryzae* (Bénin strain). Here, the bacilli are longer than the bacteria of the Chaver strain. Crystal violet. Scale bar = 10 µm.

µm) and wide (1 µm), while in *Trigonotarsus rugosus*, the endosymbionts, which are clearly visible in histological sections (Fig. 10), are very long (10 to 25 µm), wide (1 to 2 µm) and twisted. In contrast, *Sitophilus rugicollis* endosymbionts are long (8 to 20 µm), thin (less than 1 µm in diameter), and flexuous (Fig. 11). In *Sitophilus granarius* the endosymbionts are very flexuous and occasionally form loops. They are 0.3 to 0.6 µm wide, and they often reach 25 µm in length. In general, there is a halo around these endosymbionts (Fig. 4). Rod-shaped cells resemble those of *S. oryzae* (see below). Finally, in *S. zeamais*, intermediary forms were found, ranging from short (more or less flexuous) to helicoidal cells with a variable number of turns (Fig. 19).

Intraspecific morphological variability

Morphological variability was studied only within *S. oryzae*, *S. zeamais* and *S. granarius*, the three species for which distinct strains worldwide are available.

Sitophilus granarius. We have studied seven strains of this flightless species, collected from distinct French populations occurring on isolated farms (Table 1). Endosymbionts of these strains were always found to be similar.

Sitophilus oryzae and *S. zeamais*. *S. oryzae* and *S. zeamais* are sibling species (Grenier et al., 2000). In *S. oryzae*, we have examined 13 strains (Table 1). Length and flexure of the endosymbionts are highly variable. Hence, the endosymbionts of *S. oryzae* strain Sfr, are 0.3 to 0.5 µm in diameter and moderately flexuous (Figs. 5 and 6). In the larval bacteriome, while these endosymbionts are usually between 4 and 6 µm long, individual cells may range from 1 to 20 µm in length. In contrast, long forms (15 to 25 µm) are more frequent

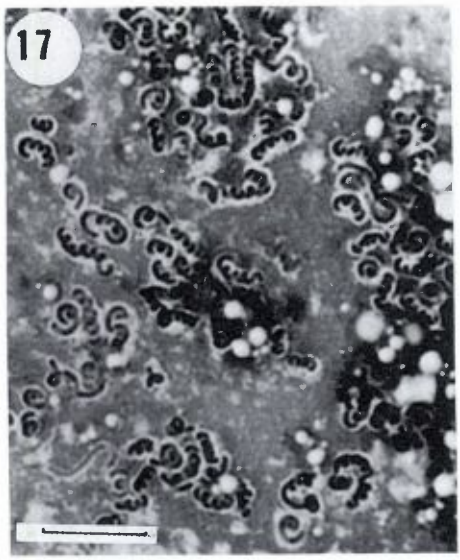


Figure 16. Ovarian endosymbionts of *Sitophilus oryzae* (Bouriz strain). Presence of a halo. Crystal violet. Scale bar = 10 µm.

Figure 17. Ovarian endosymbionts of *Sitophilus zeamais* (Mex II strain). Here the helicoidal morphotype is more numerous. Crystal violet. Scale bar = 10 µm.

Figure 18. Larval endosymbionts of *Sitophilus zeamais* (Dax strain). Here the long helicoidal morphotype is absent or rare. Crystal violet. Scale bar = 5 µm.

in the female ovaries, and some endosymbionts can reach 60 µm in length. To summarize our observations, in *S. oryzae* two endosymbiont types can be

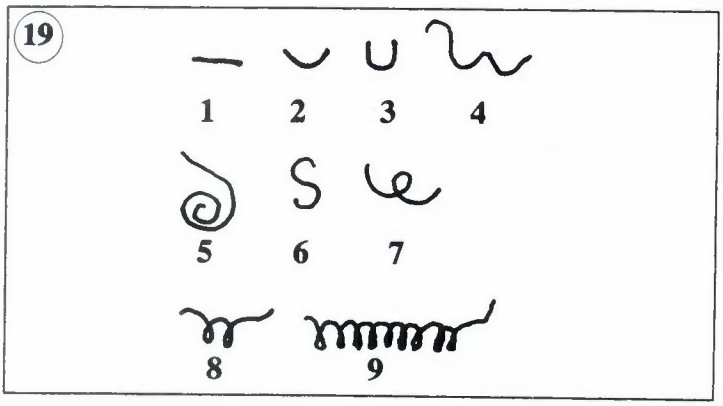


Figure 19. Endosymbionts of Dryophthoridae: 8 morphotypes frequently present in larval bacteriome or in ovaries of the adult female. 1: straight and short bacillus; 2: comma-shaped; 3: U-shaped; 4: long snake-like; 5: spiral-shaped; 6: S-shaped; 7: gamma-shaped with or without a tail; 8,9: helicoid (2 turn spiral to 18 turn spiral) with or without tail.

Table 3. Percentage of helicoid endosymbionts (at least one turn) in larval bacteriome (L) or in adult female ovary (F), in *Sitophilus zeamais*.

Strains		Flexuous bacteria	Bacteria with one turn at least	% of helicoids
Dax	L	104	26	20% (3)
Congo	F	158	47	23% (2)
Araucaria	F	51	20	28%
Lagoa	F	35	19	35%
Cameroun	L	271	186	41%
Burkina	L	16	12	43%
Chariz	F	1169	975	45% (1)
ZMA	L	218	240	52%
Mex II	L	130	147	53%
Bic	F	252	286	53%
Gerard	L	11	15	58%
Guyane	F	32	73	70%

(1) Numerous long helicoidal bacilli are present in this strain (15 to 17 turns) (Fig 19.8); (2) In this other strain short bacilli (Fig. 19.1) are not abundant while we observed flat spirals (Fig. 19.5). (3) Here, numerous commas (Fig. 19.2) but few helicoid.

distinguished: the majority resemble the SFr type (short rods that are moderately flexuous) as in Chaver (Fig. 13), while the Guad I and II, and SSO strains among others are longer and more sinuous as in the Benin (Fig. 15), Bouriz (Fig. 16), and Australie, GV and W strains. Other strains, like Mexique 1, Françoise or Tunisie, which have a very small number of endosymbionts, are more difficult to characterize.

Twenty one strains of *S. zeamais* were also studied (Table 2). Endosymbionts were most often very different from those of the sibling species *S. oryzae* and are quite heterogeneous in form (Fig. 14). From the simplest rod- and coma-shaped cells to the long coil-shaped forms, all the helicoidal intermediates were found (Figs. 14, 18 and 19). The helicoidal forms exhibit up to 15 to 18 turns (over a length of 20 µm) as in the Guyane and Chariz strains. Some strains do not exhibit any long helicoidal form (Abruzzes, Arau), whereas these forms are very abundant in Chariz or ZMA strains. Furthermore, some strains are characterized by bacteriocytes that contain a great number of endosymbionts (Chariz, Cameroun, Dax, Bic, FB, Mex 2), whereas others show only a small number (Lagoa, Syrie, Thai, Burki, Basmati, Arau). To summarize the diversity of forms encountered in our studies we have calculated the ratio of the endosymbiont number with at least one turn (Fig. 19) to the total number of endosymbionts (Table 3). This ratio varies between a low of 20% (Congo and Dax strains; Fig. 18) and a high of 70% (Guyane strain). Coiled forms are slightly predominant in Bic (53%; Fig. 17), Gérard (58%), Mex 2 (53%) and ZMA (52%) strains. There is no relevant qualitative difference between endosymbionts of the larval bacteriome and those from the adult female ovary in these species.

4. Discussion

Numerous recent studies have been dedicated to the extent and control of morphological variability in bacteria such as *Escherichia coli* (Addinall and Lutkenhaus, 1996), *Helicobacter pylori* (Andersen et al., 1997), and *Bdellovibrio bacteriovorus* (Gray and Ruby, 1989) among many others. In this work we extend these studies into the endosymbionts of the Dryophthoridae.

Morphotypes

Morphological data provide strong evidence of endosymbiont diversity within the Dryophthoridae family. These endosymbionts are characterized by both their size (length and thickness) and their flexuous degree that results from a variability in elongation and spiraling. During elongation, chromatin granules appear (indicating a possible ploidy increase) in *Sitophilus granarius*

as described by Musgrave and Singh (1965). Such a polyploidy was also observed in *Buchnera*, the primary endosymbiont of aphids (Komaki and Ishikawa, 2000). We hypothesize that the diversity of the endosymbionts is the consequence of specific interactions with the host, and subsequent modifications of bacterial gene expression.

S. zeamais is the species in which endosymbionts are the most polymorphic (Fig. 19). They can be divided in two categories: those with flexuous cells without loops or complete turns, and those with at least one complete turn (Fig. 19). These helicoidal shapes seem to be different from the shapes of other described bacteria, and might be specific to bacteria in an intracellular environment. The most similarly shaped Gram negative bacteria are *Rhodospirillum rubrum* or *Spirillum volutans*. However, these bacteria never exhibit complete loops, and instead are of the winding or sinusoidal type (in Prescott et al., 1995). Endosymbionts of *Cosmopolites sordidus* are also atypical in form, resembling oriental letters (Fig. 7). In contrast, the endosymbionts found in the other insect species described here are rod-shaped bacteria with a more or less flexuous shape.

Interaction between the host, the endosymbionts and the environment

The bacteriocyte-forming symbiosis constitutes a new biological unit called the "symbiocosm" (Nardon and Grenier, 1993). The symbiocosm can be considered as a micro-ecosystem that is subjected to selection pressure. The maintaining of the association probably results from coevolution between both partners, as a result of their mutual interaction as well as their interaction with the physicochemical environment. The effects of the environment may act not only on the endosymbionts directly, but also through changes in the physiology of the host. It has been shown that variations in the nutritional state of *Sitophilus* spp. can modify endosymbiont density (Delobel and Grenier, 1993). Endosymbiont density is also regulated by host genetic factors (Nardon et al., 1998). In this case, the bacteria react both to the external environment of the symbiocosm and to the intracellular environment. Modifications in the morphology similar to those of the endosymbionts, have been reported in other symbiotic models, such as when *Rhizobium* spp. become bacteroids within the root nodule of legume plants (Ausubel, 1984).

However, occurrence on the same plant in a similar environment does not result in a similarity in endosymbiont morphology. Indeed, while *C. sordidus* and *M. hemipterus* are closely related phylogenetically (O'Meara and Farrell, personal communication) and both live on banana tree trunks, their endosymbionts are morphologically very distinct (Figs. 7, 8 and 9). This is also

the case for *S. zeamais* and *S. oryzae*, the two sibling species living on wheat seeds (Figs. 13 and 14).

Cellular plasticity (pleiomorphy)

By chemically modifying their peptidoglycan envelope, bacteria can alter their morphology (Cooper, 1991) through molecular processes that are just now becoming known. Such a process occurs in *Symbiobacterium thermophilum* which cannot be cultivated in the absence of a *Bacillus* sp. (Ohno et al., 1999); nevertheless, in a conditioned culture medium, limited growth is observed. However, if culture is prolonged, the *Symbiobacterium* cell becomes elongated and filamentous, whereas the amount of DNA it contains remains unchanged. As a rule, when DNA synthesis is inhibited by antibiotic or gene mutation, bacterial division often ceases and the cell lengthens. The intracellular environment of the host bacteriocyte might produce a similar effect on its endosymbionts.

It is also possible to have different morphotypes occurring simultaneously when the bacteria undergo a developmental cycle. For instance, *Campylobacter jejuni* shows a very high cellular plasticity. The bacteria are spiral-shaped during the logarithmic phase of growth, lengthen during the stationary phase, and become rod-shape during the decline phase (Thomas et al., 1999). *Bdellovibrio bacteriovorus* is a bacterial predator of other Gram-negative bacteria (Ruby, 1989). It has a developmental cycle that begins once it enters the periplasm of the host bacterium. In the periplasm the *bdellovibrio* grows as a spiral filament that has a striking similarity to the endosymbionts of *Sitophilus zeamais* (Fig. 14). The *bdellovibrio* filament fragments into a number of vibrio-shaped cells that are finally released from the spent host cell.

Electronic microscopy studies of the endosymbionts of *Bemisia tabaci*, a member of a different family of insects (Homoptera Aleyrodidae), have revealed a geographical relationship between the morphology of the bacterial symbionts and the geographical origin of the host (Costa et al., 1995). Two types of endosymbionts are found in species originating from Florida: P (pleiomorphic bacteria) and C1 (rod-shape bacteria); however, in Porto Rico, three types were reported: P, C1 and C2 (coccoid). We show here a similar variation in endosymbiont morphology relative to the geographical origin of the host. However, no statistical correlation could be detected between strain variability and geographical origin, perhaps due to the small numbers of samples available.

Pleiomorphy and cellular growth

The degree of endosymbiont pleiomorphy observed in *Sitophilus zeamais* suggests a progression that may reflect different steps in bacterial growth (Fig. 19); i.e., the diversity of forms could each derive from another. Helicoidal shapes might result both from lengthening and spiraling, giving a more or less elongated shape with between 2 and 18 turns.

In young larvae of *Metamasius hemipterus* a majority of short forms in the vicinity of longer ones can be seen (Fig. 9). However the latter may fragment and give rise to the observed pleiomorphy. In some individuals, differential expression of genes controlling cell division might explain such pattern, which may respond to changes occurring during host development. Lengthening of rod-shape bacteria during larval development can be explained in the same way. Similar pleiomorphy was already observed in *E. coli* and the morphogenes were isolated (Lutkenhaus, 1990; Addinall and Lutkenhaus, 1996).

Endosymbiont pleiomorphy or plurisymbiosis in Dryophthoridae?

Many insects harbor several endosymbiont types (Buchner, 1965). Indeed, in the genus *Psylla*, one permanent endosymbiont (P) was described, as well as another non-obligate (or secondary) endosymbiont (S). These two species are localized in separate regions of the host and have been shown to have evolved independently (Thao et al., 2000).

Thus, the two morphotypes (such as those seen in *Sitophilus zeamais* and *Cosmopolites sordidus*) could either (1) represent two different species of endosymbionts (as described in Dash, 1975 or Campbell, 1992), or (2) are a single pleiotropic species. Until a single morphotype population from any of the Dryophthoridae species can be successfully purified and genetically characterized, these two possibilities remain unresolved.

Resolution of the question is even more difficult when comparing the two sibling species *S. oryzae* and *S. zeamais* (Grenier et al., 2000). Indeed, although the insect species are genetically very closely related, they are characterized by very different endosymbionts (Figs. 13, 14 and 15). Actually, this criterion is more appropriate than the morphogenetic one for *S. oryzae* and *S. zeamais* differentiation (Musgrave and Homan, 1962; Nardon and Wicker, 1981): more or less long bacilli in *S. oryzae*, and loop-shape, spiral-shape or helicoidal-shape in *S. zeamais*. However, a very small number (14 out of 1069, or 1.3%) of endosymbionts resembling those of *S. zeamais* were found in strain SSO of *S. oryzae* (S-shaped, U-shaped and short helix-shaped endosymbionts).

5. Prospects

This work highlights the high pleiomorphism present among the endosymbionts of the Dryophthoridae weevils. Hypotheses about the involvement of morphogenes, as well as intracellular living conditions, on bacterial morphology have been proposed based on the comparison of two sibling species, *Sitophilus oryzae* and *S. zeamais*. Work in progress concerning the phylogenetic position of Dryophthoridae endosymbionts, has revealed that they are γ3-proteobacteria and can be divided into three monophyletic groups. These groups may have been acquired at different times during the evolution of their hosts (Lefèvre et al., personal communication). The possible relationship between the age of the symbiosis and the degree of endosymbiont variability might contribute to understanding evolution of these intracellular bacterial associations.

Acknowledgments

We would like to thank E. Ruby for his helpful comments during the preparation of the manuscript. We also express our thanks to the colleagues who sent us the insects: G. Couturier (Brazil, Belem), A. Delobel (Africa, Senegal), G. Matthew (India, Mangalore), R. Oberprieler (Australia, Queensland), Pr. Pajni (India, Chandigarh). This work was supported by the French Society of Entomology (Legs Germaine Cousin).

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