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Occurrence of Endosymbiosis in Dryophthoridae Weevils: Cytological Insights into Bacterial Symbiotic Structures

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Abstract

To gain a better understanding of the occurrence of intracellular symbiosis in the Dryophthoridae family, nine species worldwide were analyzed in relation to the presence/absence and the structure of bacteriomes, the specialized organs harbouring the symbiotic bacteria. Staining protocols have indicated the presence of bacteriomes in seven species: *Sitophilus rugicollis*, *Diocalandra frumenti*, *Sphenophorus striatopunctata*, *Sipalinus gigas*, *Trigonotarsus rugosus*, *Dynamis borassi* and *Rhynchophorus palmarum*. Strong evidence also suggests the presence of intracellular bacteria within *Sitophilus vateriae*. However, *Sitophilus linearis* has been shown to be completely devoid of intracellular endosymbionts. In all symbiotic species, the larval bacteriome does not exhibit any diversity with regard to the form and the location, always in the anterior part of larvae. It is delimited by a thin membrane, it contains an important tracheal network and it is attached to the intestine at many sites. At least two cell types have been observed within the bacteriome: bacteriocytes, the polyploid cells harbouring bacteria and, between them, small flat cells forming the interstitial tissue. Taken together, these results argue in favor of a common origin of the symbiotic structures in the Dryophthoridae family.

Keywords: Intracellular symbiosis, Dryophthoridae, endosymbionts, bacteriome, bacteriocytes

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1. Introduction

Symbiosis is no longer considered as a biological curiosity, and its role in physiology and evolution of organisms is now recognized (Nardon and Grenier, 1989, 1993; Margulis, 1993). Among Coleoptera three types of symbiosis are found: (i) ectosymbiosis with fungi, like in *Ambrosia* beetles; (ii) endosymbiosis limited to the gut lumen as it is in Scarabaeidae; and (iii) intracellular endosymbiosis, where the symbionts are housed in specialized cells (bacteriocytes), often grouped in compact organs: the bacteriomes (Buchner, 1965; Nardon and Nardon, 1998).

In this work, we have investigated the occurrence of symbiosis in the Dryophthoridae family. According to the current classification (Alonzo-Zarazaga and Lyal, 1999), the Dryophthoridae family includes the species belonging to the former family of Rhynchophoridae. It comprises more than 500 species represented by about 140 genera. These insects are of great agronomic importance because many of them are cosmopolitan pests of cereals, palm trees and banana trees. From an ecological point of view, the Dryophthoridae develop principally on monocotyledons. Insects feed on leaves, stipes and even roots. Nevertheless, some species such as *Sipalinus gigas* and *Trigonotarsus rugosus* are adapted to feed on decaying wood. Only insects from the *Sitophilus* genus feed on various seeds of mono- or dicotyledonous trees (Delobel and Grenier, 1993).

Several cases of cellular endosymbiosis are already known in this family: in *Sitophilus oryzae* (L.) (Pierantoni, 1927; Nardon and Grenier, 1988); *S. granarius* (L.) (Mansour, 1930); *S. zeamais* (Mot.) (Musgrave and Homan, 1962); *Cosmopolites sordidus* (Germar) and *Metamasius hemipterus* (L.) (Nardon et al., 1985). In all these species, symbiotic bacteria are located in a larval bacteriome. During metamorphosis, the larval bacteriome disappears and bacteriocytes are either destroyed or migrate to the anterior mesenteric ceca of male or female adults. Females possess a bacteriome at the apex of each of their four ovarioles but the intracellular symbionts are transmitted vertically through oocytes only. Intracellular bacteria remain in the germ cells, as cellular organelles (Nardon and Nardon, 1998).

The physiological role of these intracellular symbionts is currently poorly understood. In the case of *Sitophilus* species thriving on cereal seeds, an unbalanced diet, the bacteria supply the insect with vitamins (Wicker, 1983), interact with amino acid metabolism (Gasnier-Fauchet and Nardon, 1986) and increase mitochondrial oxidative phosphorylation (Heddi et al., 1993). However, the role of endosymbionts is not understood in most species. In the present study, we looked for symbiosis in nine new species of Dryophthoridae, originating from different places worldwide, trying to find a relationship between environment and symbiosis. This work is a part of a more general study

of the Dryophthoridae family, including the molecular phylogeny of insects and of their endosymbionts.

2. Materials and Methods

The presence of "integrated" bacteria living in specialized cells, the bacteriocytes, can be detected in three ways: (i) cytological search of bacteriocytes in the larvae, (ii) histological search of bacteriocytes in the anterior mesenteric ceca of adults and (iii) search of bacteria in squashes of adult ovaries or anterior mesenteric ceca of male or female adults.

Fixation

The search for bacterial symbionts was essentially performed on larvae since bacteriomes are larger in larvae than in adults. Insects were collected by ourselves or sent by colleagues from different countries, either alive or immersed in fixative fluid. The alcoholic formula of Bouin's fluid (Dubost-Brazil, in Gabe, 1968) was used for the big larvae. The smaller larvae were fixed in NMB's fluid (Nardon Modified Bouin), a formula finalized in our laboratory as follows: 20 ml of a 1.5% picric acid solution in 80° ethanol, 5 ml of 40% neutral formalin solution (addition of $CaCO_3$ to commercial formalin), 2 ml of glacial acetic acid and H_2O qsp 50 ml.

In order to facilitate penetration of the fixative, the larvae were pierced or even cut in two pieces. Fixation time increases with the size of the larvae from one to several days. It is worth noting that NMB does not induce an over-fixation and the larvae can be maintained in it for several months. In the adults the appendages and wings were removed, and the body was cut between the thorax and abdomen in order to facilitate the penetration of the fixative fluid.

Softening and inclusion

After fixation, the larvae (or adults) were washed in 70° ethanol (2 or 3 baths), dehydrated in 95° and 100° ethanol and immersed in butanol-1 (2 or 3 baths) for several months. The integument was softened in butanol-1 for easy sectioning (6 µm), and the pieces were embedded in paraffin. From butanol, tissue can be directly immersed in paraffin.

To detect bacteriocytes and bacteria, various stains were used. Among the classical stainings, Gram, toluidine blue (TB) and Feulgen were used. It is also advisable to combine the last two stainings (i.e. TB+Feulgen). The duration of 5N HCL hydrolysis must be determined for each larva (8 to 12 min). We have

Table 1. List of the studied insects with their geographical origin and diet

Name	Geographical origin	Plant host	Stage
Sipalinus gigas Fab.	Australia (Queensland)	Decaying wood	2 larvae: 15 and 26 mm long
Diocalandra frumenti Fab.	Australia (Queensland)	*Ravenea rivularis* leaves (Arecaceae)	3 larvae: 6–7 mm; 1 nymph
Sitophilus vateriae Marshall	India (Mangalore)	*Vateria indica* grains (Dipterocarpaceae)	5 adults 3.5 mm
Sitophilus rugicollis Casey	India (Chandigarh)	*Eugenia jambolana* (Myrtaceae)	Currently reared in the lab on maize
Sphenophorus striato-punctata Goeze	France (Lyon)	*Sirpus lacustris* (Poaceae)	2 adult females
Trigonotarsus rugosus Boisduval	Australia (Queensland)	*Xanthorrhea* sp. Trunk (Xanthorrheaceae)	2 larvae: 2.5 and 3 cm long
Dynamis borassi Fab.	Brazil (Belem)	Palm tree (Arecaceae)	1 big larva: cephalic capsule 7 mm width
Rhynchophorus palmarum L.	Guadeloupe	Palm tree (Arecaceae)	2 big larvae: 4 cm long, 12 and 11.5 cm long
Sitophilus linearis Herbst.	Africa (Senegal)	*Tamarindus indica* grains (Sesalpiniaceae)	Currently reared in the lab on the same grains

also finalized new staining protocols, such as RTF (Ribonuclease, toluidine blue, Feulgen) or RTS (Ribonuclease, TB, safranin).

RTF: After removal of paraffin and hydration of the sections, a RNase treatment (0.4 mg/ml in H_20) was applied at 37°C for 30 min to 1 hour. Sections without RNase treatment were used as control. Water rinsing was followed by TB staining (1‰, pH=5.4 for about 2 min). A brief water rinsing is followed by acid fuchsin staining (1‰ in 100° ethanol for a few seconds up to one minute). Mounting was performed after a brief 100° alcohol rinsing, 3 baths in tert-butanol and 2 in xylene or toluene.

RTS: The protocol is the same as RTF except that safranin was used instead of acid fuchsin (for a few seconds). Smears or squashes of ovary or intestine were dessicated on a heating block at 60°C. Just before staining the slides were dipped in toluene in order to eliminate the small lipid droplets. Then the slides were dipped in absolute ethanol and fixed (facultative), for several minutes with Carnoy's fluid. After rinsing and hydration, the slides were stained with crystal violet or safranin, according to the usual method (in Bourdon and Marchal, 1973).

Studied insects

See Table 1.

3. Results and Discussion

Non-symbiotic (= asymbiotic) species

In *Sitophilus linearis*, no bacteriocyte and no bacteriome were detected in the ovaries or gut squashes. Thus we concluded that there is an absence of "integrated" bacteria (Nardon and Grenier, 1993) such as Primary Endosymbionts (SOPE) (Heddi et al., 1999) in *Sitophilus oryzae*.

Symbiotic species

The cytological investigations of larvae have led to the discovery of bacteriocytes in *Diocalandra frumenti* (Figs. 3 and 4), *Trigonotarsus rugosus* (Figs. 6 and 9), *Dynamis borassi* (Figs. 11–13), *Sipalinus gigas* (Fig. 14) and *Rhynchophorus palmarum* (Fig. 15). The study of squashes of ovaries has revealed the presence of bacteria in *Sitophilus rugicollis* (Fig. 1) and *Sphenophorus striatopunctata* (Fig. 5). In *S. rugicollis* the bacteria are flexuous, 10 to 20 µm long and they seem to be thinner than SOPE (Fig. 1).

Concerning *S. vateriae*, we searched for bacteria in the anterior mesenteric ceca of adults only. The pictures obtained (Fig. 2) show the ceca invaded by cells of about 10 to 12 µm in diameter. They are organized in a manner that leaves 20 µm wide cytoplasmic spaces generated by cytoplasmic fusions. These cells present exactly the same aspect as those found in *S. oryzae* (unpublished data) with granules and threads which could be interpreted as being degenerative bacteria. Hence, *Sitophilus vateriae* could be symbiotic but this needs to be confirmed on the larvae.

Figs. 1–15. See following pages.

Figure 1. *Sitophilus rugicollis*. Squash of an ovary from an adult female. The nuclei are those of trophic cells. Crystal violet. Scale: 10 µm.

Figure 2. *Sitophilus vateriae*. Section in the anterior mesenteric cecum of an adult. Nuclei are disposed at the periphery. Fused cells form cytoplasmic area (p). RTF. Scale: 10 µm.

Figure 3. *Diocalandra frumenti*. Transverse section of the anterior part of a larva. Toluidine blue. at: adipose tissue, b: bacteriome, m: mesenteron, sn: nervous system, st: stomodeum. Scale: 50 µm.

Figure 4. *Diocalandra frumenti*. Larval bacteriome. RNase and toluidine blue. The bacteriocytes with their nuclei are quite visible. e: envelope of the bacteriome, t: trachea. Scale: 20 µm.

Figure 5. *Sphenophorus striatopunctata*. Squash of an ovary from an adult female. Gram. Scale: 10 µm.

Figure 6. *Trigonotarsus rugosus*. Bacteriome around the intestine. RTS. b: bacteriome, m: mesenteron. Scale: 10 µm.

Figure 7. *Trigonotarsus rugosus*. Larval bacteriome. Toluidine blue. Bacteriocytes with their nuclei showing several nucleoli. g: larval gut, i: interstitial tissue, t: trachea. Scale: 30 µm.

Figure 8. *Trigonotarsus rugosus*. Larval bacteriome. Bacteria in bacteriocytes. Rnase toluidine blue. We can appreciate the presence of a high content of RNA in the cytoplasm of bacteriocytes. Nucleoli are still clearly visible in some of them because the action of Rnase is not complete, to avoid the sections to be unsticked from the slides. i: interstitial cells, t: trachea. Scale: 30 µm.

Figure 9. *Trigonotarsus rugosus*. Contact of a huge bacteriocyte with the intestine. Bacteria in the cytoplasm. Presence of mucus at the bacteriome/gut contact. RTS. i: nucleus of interstitial tissue, t: trachea. Scale: 20 µm.

Figure 10. *Trigonotarsus rugosus*. Contact of a bacteriocyte with the intestine. Nucleoli and bacteria in the cytoplasm. RTS. Scale: 10 µm.

Figure 11. *Dynamis borassi*. Larval bacteriome. Pseudo-syncitium. RTF. i: interstitial tissue, p: cytoplasmic area with bacteria, t: trachea. Scale: 20 µm.

Figure 12. *Dynamis borassi*. Larval bacteriome. Pseudo-syncitium. Rnase, toluidine blue. e: envelope, i: interstitial tissue, p: cytoplasmic area with bacteria, t: trachea. Scale: 20 µm.

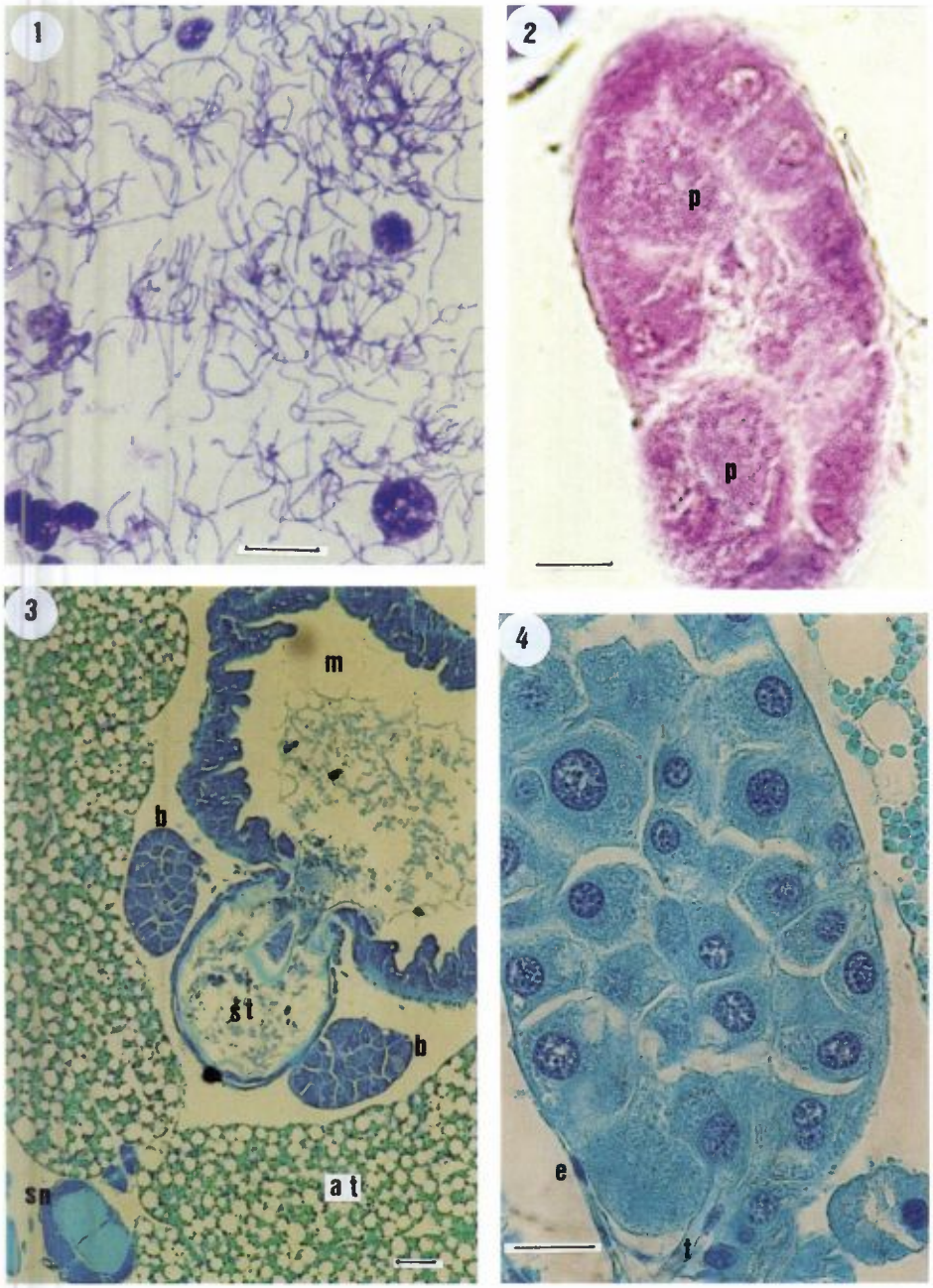
Figure 13. *Dynamis borassi*. Larval bacteriome. Cytoplasmic areas with intracellular symbionts. RTF. e: envelope, p: cytoplasmic area. Scale: 10 µm.

Figure 14. *Sipalinus gigas*. Larval bacteriome. String of bacteriocytes and contact with the mesenteron. Toluidine blue. b: bacteriome, m: mesenteron. Scale: 50 µm.

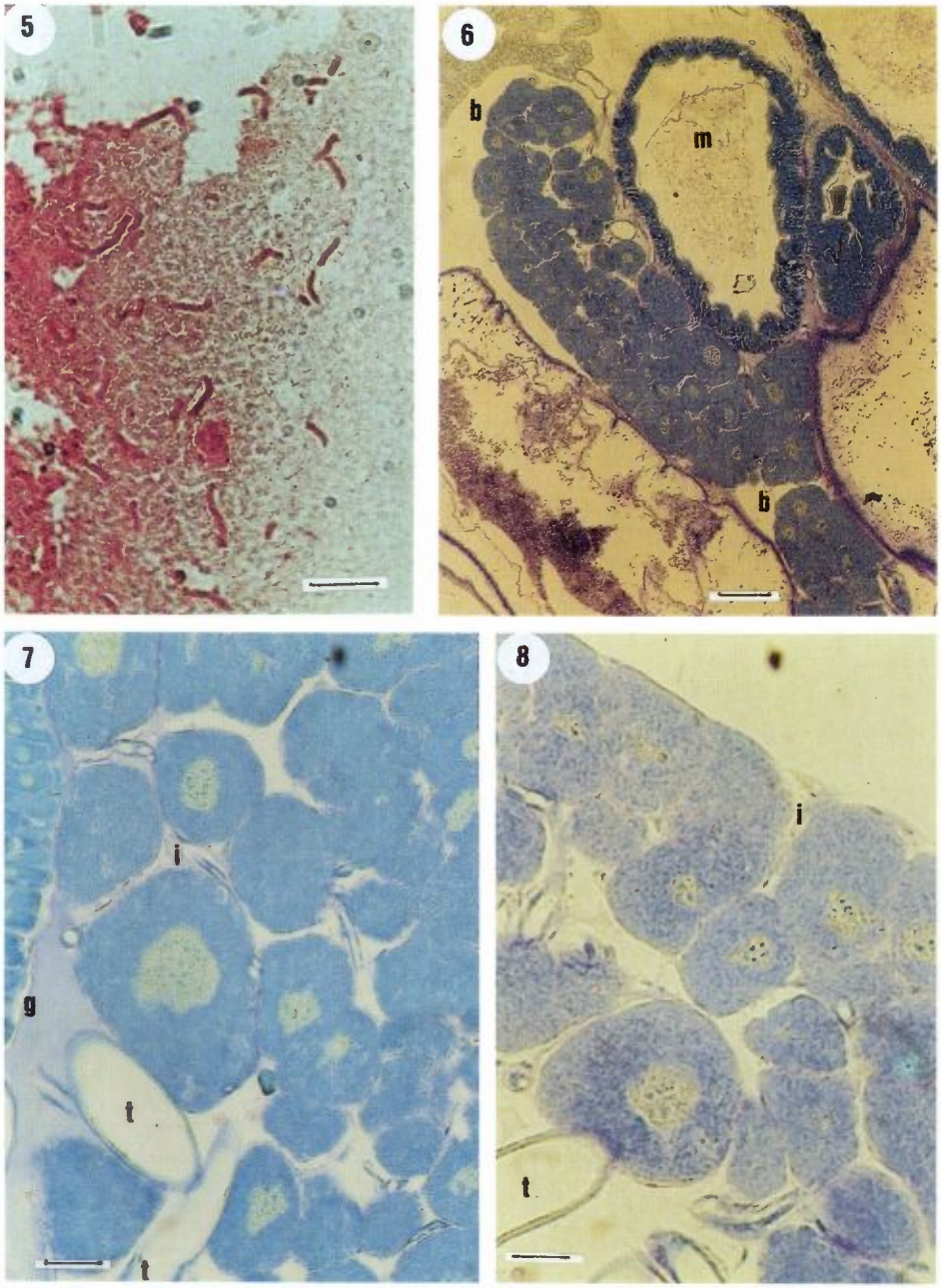
Figure 15. *Rhynchophorus palmarum*. Larval bacteriome with bacteria. Toluidine blue. e: envelope, i: interstitial tissue, t: trachea Scale: 10 µm.

Larval bacteriome characteristics

In Dryophthoridae, the bacteriome is a unique compact organ, always located at the anterior part of the larvae, between the fat body and the intestine (Figs. 3, 6, 14). It is always in intimate contact with the intestine at



Figures 1–4. See legend on pp. 232.



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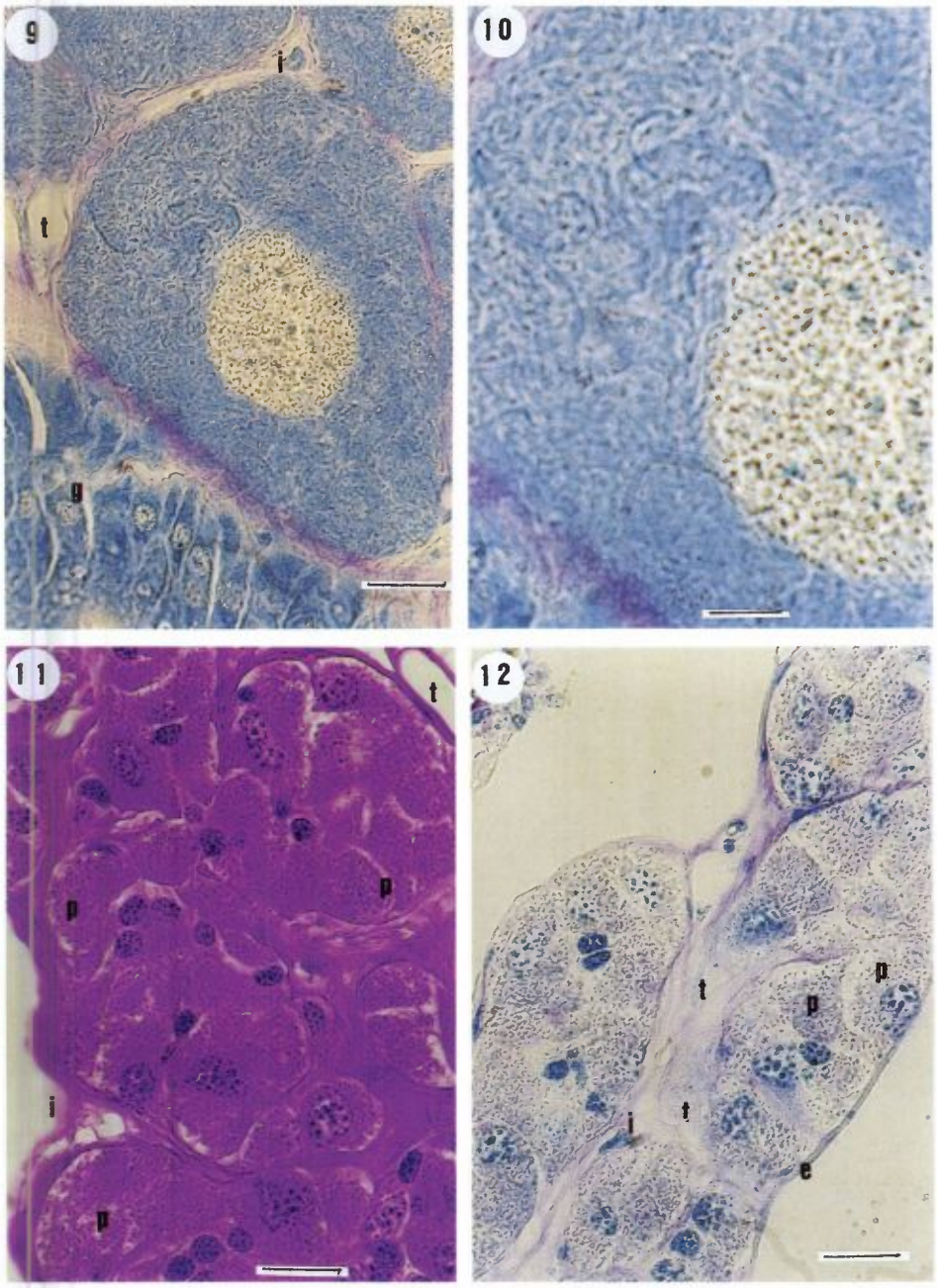

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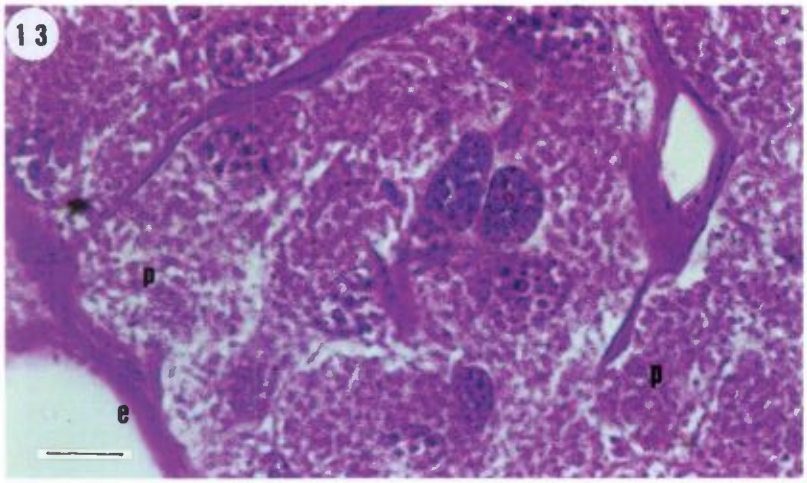

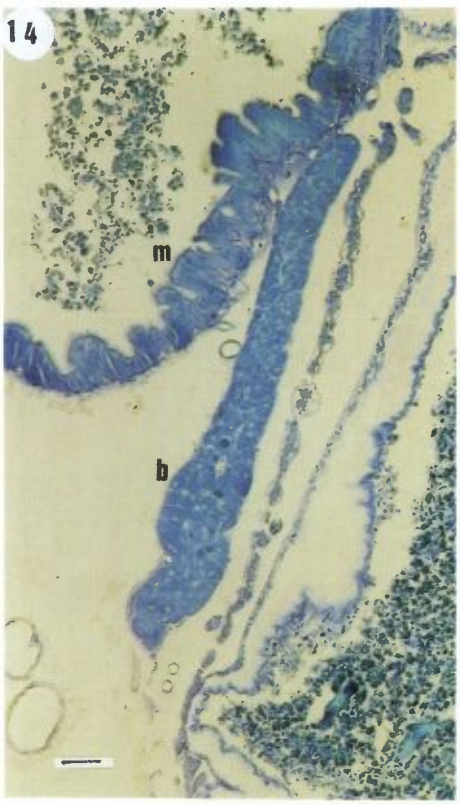

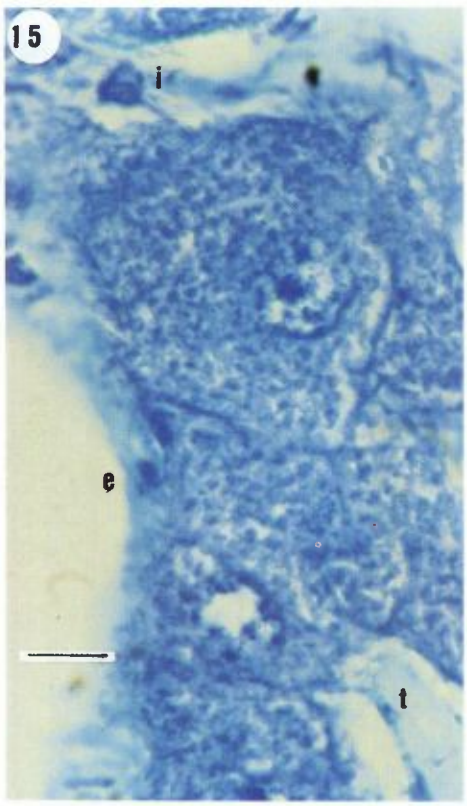

Figures 13–15. See legend on pp. 232.

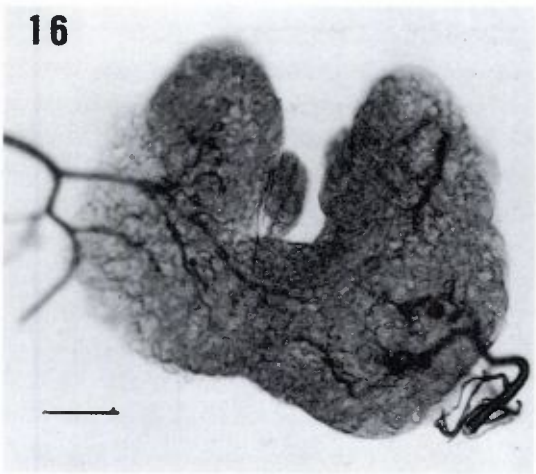

Figure 16. *Sitophilus oryzae*. Dissected larval bacteriome. Third instar. Numerous tracheae. Methylene blue staining. Scale: 20 µm.

the junction stomodeum/mesenteron, ventrally in *D. frumenti* (Fig. 3) and in *S. oryzae* (Nardon, 1973) or dorsally, as in *Cosmopolites sordidus* (Nardon et al., 1985). It is noteworthy that there is no direct connection between the gut and the bacteriome which can easily be separated. The fore-gut is short, so that the bacteriome is located just behind the head capsule.

The bacteriome presents a U shape (Fig. 16) in *S. oryzae*, *S. granarius*, *S. zeamais* and *D. frumenti* (Fig. 3). In other species, generally with bigger larvae, we observed the formation of strings of bacteriocytes which grow toward the posterior part of the insect, as in *Trigonotarsus rugosus* (strings 300 µm wide) (Fig. 6), *Dynamis borassi* (100 to 200 µm wide) (Fig. 12), *Sipalinus gigas* (Fig. 14) or *Rhynchophorus palmarum* (100 to 150 µm wide) (Fig. 15). Contacts with the gut are also observed along the strings (Figs. 6, 9, 14), but still without any direct connection between the bacteriome and the gut lumen. The contact area is especially rich in acid muco-polysaccharides, as identified by their metachromasia with toluidine blue (Fig. 9). Their role is currently unknown but it was demonstrated in the *Vibrio-Euprymna* symbiosis that the muco-polysaccharides take part in the process of the specific host-bacteria recognition and symbiosis establishment (Visick and McFall-Ngai, 2000).

The larval bacteriome is delimited by a thin cellular envelope with small flat nuclei (Figs. 4, 8, 12). It seems that the cells of this envelope can penetrate the bacteriome, between the bacteriocytes, and form the "interstitial tissue"

the importance of which depends on the species (Figs. 7–9). Their role is not determined yet but it seems to be important since all the described bacteriomes exhibit such a structure as those found in the Homoptera *Psylla pyricola* (Chang and Musgrave, 1969).

The bacteriome is mainly composed of bacteriocytes which are very similar in all the species studied. They are huge cells: 40 µm wide in *Diocalandra frumenti* (Fig. 4) or *Dynamis borassi* (Figs. 11 and 12) and even wider in bigger insects, like *Trigonotarsus rugosus* (Fig. 7), where they can reach 300 µm in diameter. In *Dynamis borassi*, the interstitial tissue is highly developed and delimits cytoplasmic areas composed of cells, the membranes of which are not or only poorly visible, resembling a sort of syncitium. In *Sipalinus gigas* the general characteristics are the same, except that in this species the cell membranes are more clearly visible. The presence of syncitia inside the bacteriome is not a particularity of Dryophthoridae. It has been described in other Coleoptera, like Lyctidae (*Lyctus linearis*) or Cucujidae (*Oryzaephilus surinamensis*) (in Nardon and Grenier, 1989). They are also present in numerous Homoptera, like *Psylla pyricola* (Chang and Musgrave, 1969) and in Hemiptera like *Arocatus continctus* (Carayon, 1974).

The nuclei of bacteriocytes are very large, 10 to 12 µm in diameter in *Diocalandra frumenti* (Fig. 4) and 50 to 60 µm in *Trigonotarsus rugosus* (Fig. 9). Chromatin either appears as aggregates as in *Dynamis borassi* (Figs. 11 and 12), *Diocalandra frumenti* or *Rhynchophorus palmarum*, or with a fibrillogranular aspect as in *Trigonotarsus rugosus* (Figs. 9 and 10), where 7 to 8 nucleoli are visible (Fig. 9). Such giant cells and nuclei suggest a ploidisation process. This has been confirmed in *Sitophilus oryzae* with the incorporation of ^{3}H thymidine into the bacteriocytes (Nardon, 1978). The phenomenon of polyploidisation is widespread in all the insects with endocellular symbionts. For instance, it has been reported in the Homoptera *Pseudococcus maritimus* (Louis, 1967), in the cockroaches (Richards and Brooks, 1958) and in the Coleoptera *Oryzaephilus surinamensis* (Nardon and Grenier, 1989). Nevertheless, the bacteriome growth is not the consequence of ploidisation only. An increase in nuclei number has also been demonstrated in *S. oryzae* from the second larval stage (308 nuclei) to the fourth stage (1659 nuclei) by a confocal microscope analysis.

Another characteristic of bacteriocytes is the very high amount of RNA, as exhibited by ribonuclease treatment experiments (Figs. 7 and 8). RNA degradation allows for a better view of the intracellular symbionts which fill the cytoplasm. On slides, it is often difficult to analyze the bacterial morphology, except in *Trigonotarsus rugosus* where the bacteria are long and flexuous bacilli (Figs. 9 and 10).

Finally, larval bacteriomes are characterized by the importance of the tracheal system reaching this organ (Fig. 16) and threading its way between

bacteriocytes (Figs. 4, 7, 8, 12, 14). Taken together, the abundance of RNA and the tracheal network suggest that bacteriome tissue is the site of intensive aerobic metabolism.

4. Conclusion

Laboratory breeding species have been repeatedly analyzed, which has facilitated precise and easily validated observations. However, conclusions can also be made regarding population symbiotic status for those wild species represented by even a small number, when the sample is well preserved. In this case, the examination of a single larva is relevant, since in insect integrated intracellular symbioses the whole population is symbiotic (Nardon and Grenier, 1993).

Among the Dryophthoridae family, five species only were known to host intracellular symbiotic bacteria. Buchner (1965) mentioned an additional one, *Rhynchophorus ferrugineus*, without any further characterization. The present work shows that 7 additional Dryophthoridae species harbor integrated intracellular bacteria. Hence, intracellular symbiosis in the Dryophthoridae family seems to be ubiquitous and more generalized throughout the species.

Furthermore, the structure of the Dryophthoridae bacteriome does not fundamentally differ from the structure described in other families and insect species. Symbiosis is probably very ancient, occurring in the ancestor of Dryophthoridae (unpublished data).

However, non-symbiotic species such as *S. linearis* also have indications that symbiosis could be lost in the course of evolution, probably as a consequence of diet switching. In line with this observation is the symbiotic status within the *Sitophilus* genus. Among the six species studied, *S. linearis*, *S. oryzae*, *S. zeamais*, *S. granarius*, and *S. rugicollis*, only the former is non-symbiotic. We should specify that this species feeds on the legume seed *Tamarindus indica*, which is known to be rich and well balanced nutritionally, in contrast to cereals.

Bacteriome shape examination in the Curculionidae (according to the former taxinomy) has allowed for the identification of as many as 9 different morphological types (Nardon and Grenier, 1989). But among the Dryophthoridae *sensus stricto*, only a single shape with few variations has been observed so far. The ontological differentiation of the bacteriome tissue is specifically induced by the intracellular symbionts (Nardon, 1973). This suggests the existence of molecular communication between prokaryotic and eukaryotic partners that leads to the establishment of similar bacteriome structures. So far, we know from the *Sitophilus* genus that integrated intracellular bacteria influence both the physiology and the metabolism,

thereby increasing the fitness of the insect (Nardon and Wicker, 1981; Nardon and Grenier, 1993; Heddi et al., 2001).

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