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Ultrastructure and Transovarial Transmission of Endosymbiotic Microorganisms in *Conomelus anceps* and *Metcalfa pruinosa* (Insecta, Hemiptera, Fulgoromorpha)*

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Endosymbiotic microorganisms commonly occur in fulgoromorphans, as in other plant sap-sucking hemipterans. Large syncytial organs termed mycetomes are present in the body cavities of *Conomelus anceps* (Delphacidae) and *Metcalfa pruinosa* (Flatidae), in the close vicinity of the ovaries. The mycetomes are surrounded by a one-layered epithelium. The mycetome cytoplasm is filled with yeast-like symbiotic microorganisms (YLSs). The YLSs are transovarially transmitted to the next generation. The endosymbionts are released from the mycetomes and migrate towards the ovarioles containing vitellogenic oocytes. The YLSs pass through the cells of the ovariole stalk (pedicel) and enter the perivitelline space. Then, a deep depression is formed at the posterior pole of the oocyte. The YLSs accumulate in the oocyte depression and form a characteristic "symbiont ball". The mycetome cytoplasm of *Metcalfa pruinosa* as well as epithelial cells surrounding the mycetome contain small, rod-shaped bacteria.

Key words: Endosymbiotic microorganisms, transovarial transmission, ovariole, Fulgoromorpha, planthoppers.

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Fulgoromorphans, like other plant sap-sucking hemipterans, harbor obligate, intracellular symbiotic microorganisms (see BUCHNER 1965; HOUK & GRIFFITHS 1980; BAUMANN 2005, 2006, for further details). Endosymbiotic microorganisms are housed in large cells termed mycetocytes (=bacteriocytes). Mycetocytes are usually integrated into discrete organs termed mycetomes (=bacteriomes) that in some hemipterans may be surrounded by a one-layered epithelium. BUCHNER (1965) on the basis of comparative studies of all hemipteran groups distinguished two categories of endosymbiotic microorganisms: primary endosymbionts (currently called P-symbionts) and accessory endosymbionts (currently called facultative, secondary or S-symbionts). Primary endosymbionts are always present in all the specimens, whereas secondary endosymbionts occur in some populations only. More recent studies have shown that the occurrence of primary endosymbionts in

the insect body is related to a restricted diet, deficient in some essential nutrients (see BAUMANN 2005, 2006, for full review). Since hemipterans feed on phloem sap devoid of amino acids, their primary endosymbionts are responsible for amino acid synthesis and delivery to the host insect (e.g. SASAKI & ISHIKAWA 1995; WILKINSON & ISHIKAWA 2001). Thus, the occurrence of primary endosymbionts is necessary for the survival and reproduction of the host insects. In contrast to primary endosymbionts, the role of the secondary endosymbionts for their host insects remains unclear. Recent studies revealed that aphids harboring S-symbionts may better survive heat stress (MONTLOR *et al.* 2002) as well as attacks by parasitic hymenopterans (OLIVER *et al.* 2003) and fungal pathogens (SCARBOROUGH *et al.* 2005) than sterile specimens.

The symbiotic associations between primary endosymbionts and insects are the results of ancient in-

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fections with free living bacteria (BAUMANN 2006), whereas associations between secondary endosymbionts and insects are much younger and are due to multiple, independent infections (THAO *et al.* 2000).

Most hemipterans have prokaryotic endosymbionts, however, eukaryotic microorganisms historically termed “yeast-like symbionts” (YLSs) occur in some aphids, scale insects, leafhoppers and fulgoromorphans (see BUCHNER 1965; ISHIKAWA 2003, for further details).

Both bacterial and yeast-like endosymbionts are maternally inherited by transovarial transmission. Studies on transovarial transmission of bacterial endosymbionts have shown that variable modes of ovary infection by bacteria exist in insects (ŻELAZOWSKA & BILIŃSKI 1999; SZKLARZEWICZ & MOSKAL 2001; SZKLARZEWICZ *et al.* 2006). The endosymbiotic microorganisms may invade young germ cells (=cystocytes) or oocytes in the older stage of development (i.e. vitellogenic or choriogenic). The ovaries may be invaded by bacteria or by intact mycetocytes. The endosymbionts may migrate through the neighboring follicular cells (i.e. cells surrounding oocytes) or may enter the cytoplasm of follicular cells. In contrast to bacterial endosymbionts, the transovarial transmission of YLSs is poorly known, and there are only three published observations on this process (NODA 1977; CHENG & HOU 2001; SACCHI *et al.* 2008), two of which consider members of the family Delphacidae.

The present study was undertaken to provide information about the mode of transmission of YLSs in *Metcalfa pruinosa*, a member of the Flatidae family, and in another member of the family Delphacidae, *Conomelus anceps*.

Material and Methods

Adult specimens of *Conomelus anceps* (Germar, 1821) were collected in July near Nowy Targ (southern Poland). Adult specimens of *Metcalfa pruinosa* (Say, 1830) were collected in July in Grabels (southern France). The abdomens and dissected ovaries of individuals of each species were fixed in 2.5% glutaraldehyde in 0.1 phosphate buffer (pH 7.4) for three months. The material was then rinsed in 0.1 M phosphate buffer (pH 7.4) with addition of 5.8% sucrose, postfixed in 1% osmium tetroxide, dehydrated in a series of alcohol and acetone and embedded in epoxy resin Epox 812 (Fullam Inc., Latham, N.Y., USA). Semithin sections were stained with 1% methylene blue in 1% borax and photographed in a Jenalumar (Zeiss Jena) microscope. Ultrathin sections were stained with lead citrate and uranyl acetate and examined using a JEM 100 SX electron microscope at 80 kV.

Results

Gross morphology of the ovaries

The paired ovaries of *Metcalfa pruinosa* and *Conomelus anceps* consist of several ovarioles of telotrophic type (Fig. 1). The individual ovariole is subdivided into a terminal filament, tropharium (trophic chamber), vitellarium and ovariole stalk (pedicel) that joins the ovariole to the lateral oviduct (Fig. 1). The tropharium is composed of syncytial lobes containing 3-5 trophocyte nuclei (Fig. 1). Early pre-vitellogenic oocytes (termed arrested oocytes) and prefollicular cells are present at the base of the tropharium (Fig. 1). The vitellarium in mature females comprises 4-5 linearly arranged oocytes that are surrounded by a one-layered follicular epithelium (Fig. 1). The vitellarial oocytes develop through

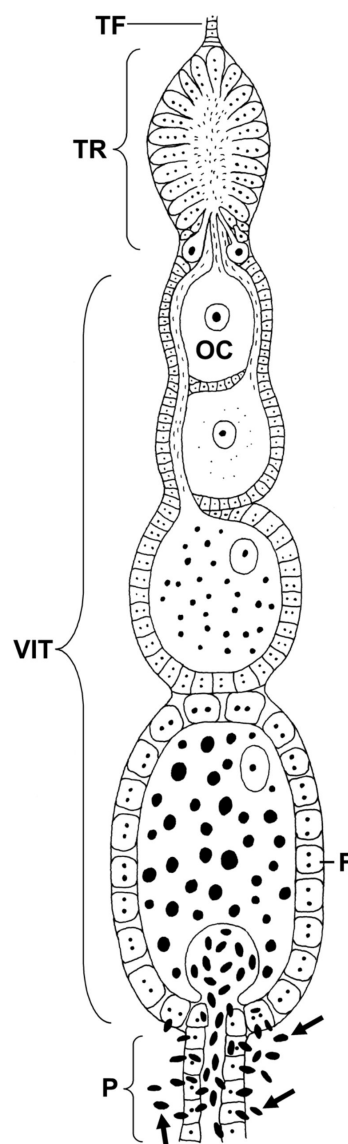
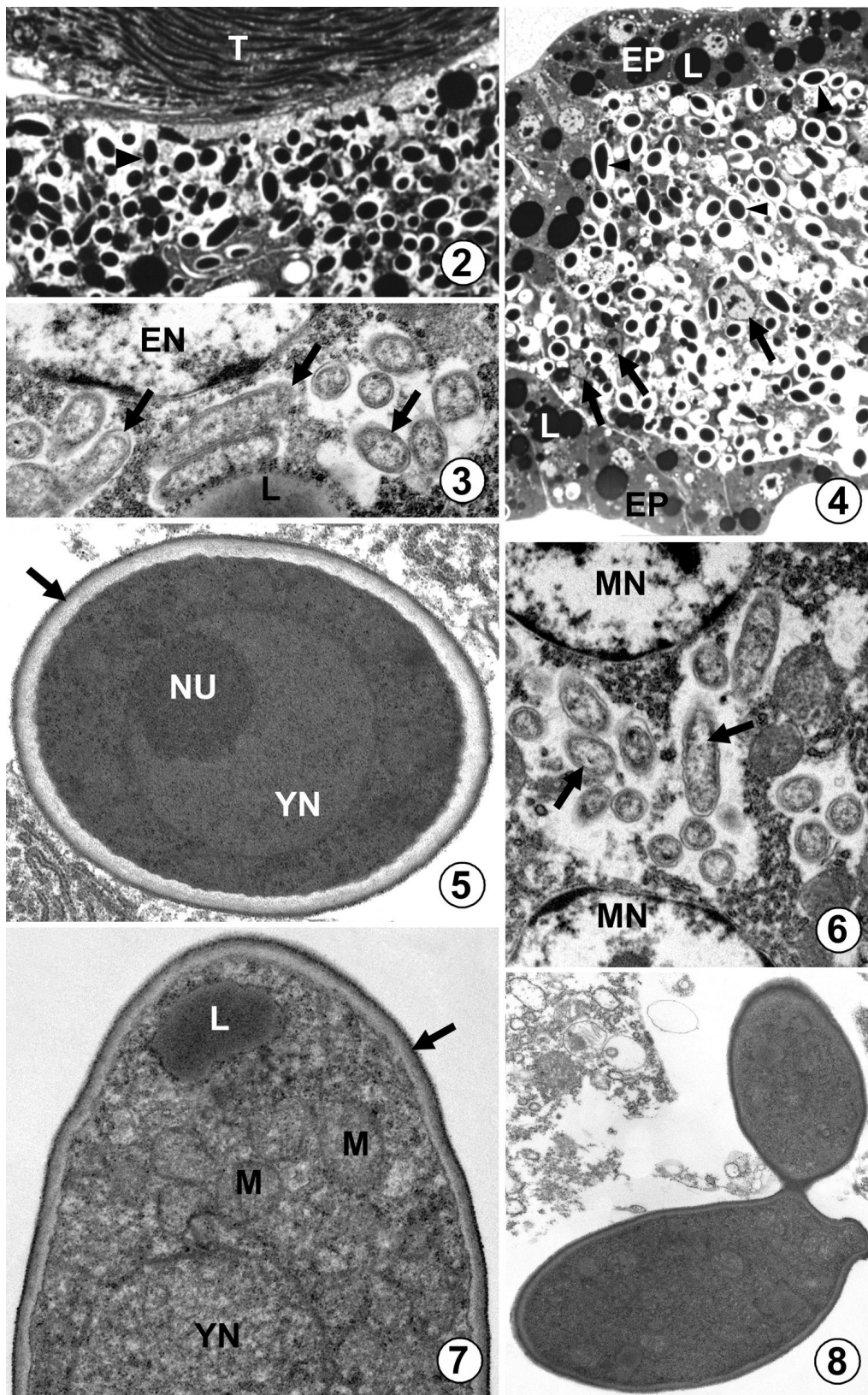


Fig. 1. Schematic representation of the ovariole of a planthopper. Arrows – YLSs; F – follicular epithelium; OC – oocyte; TF – terminal filament; TR – tropharium; VIT – vitellarium; P – pedicel.



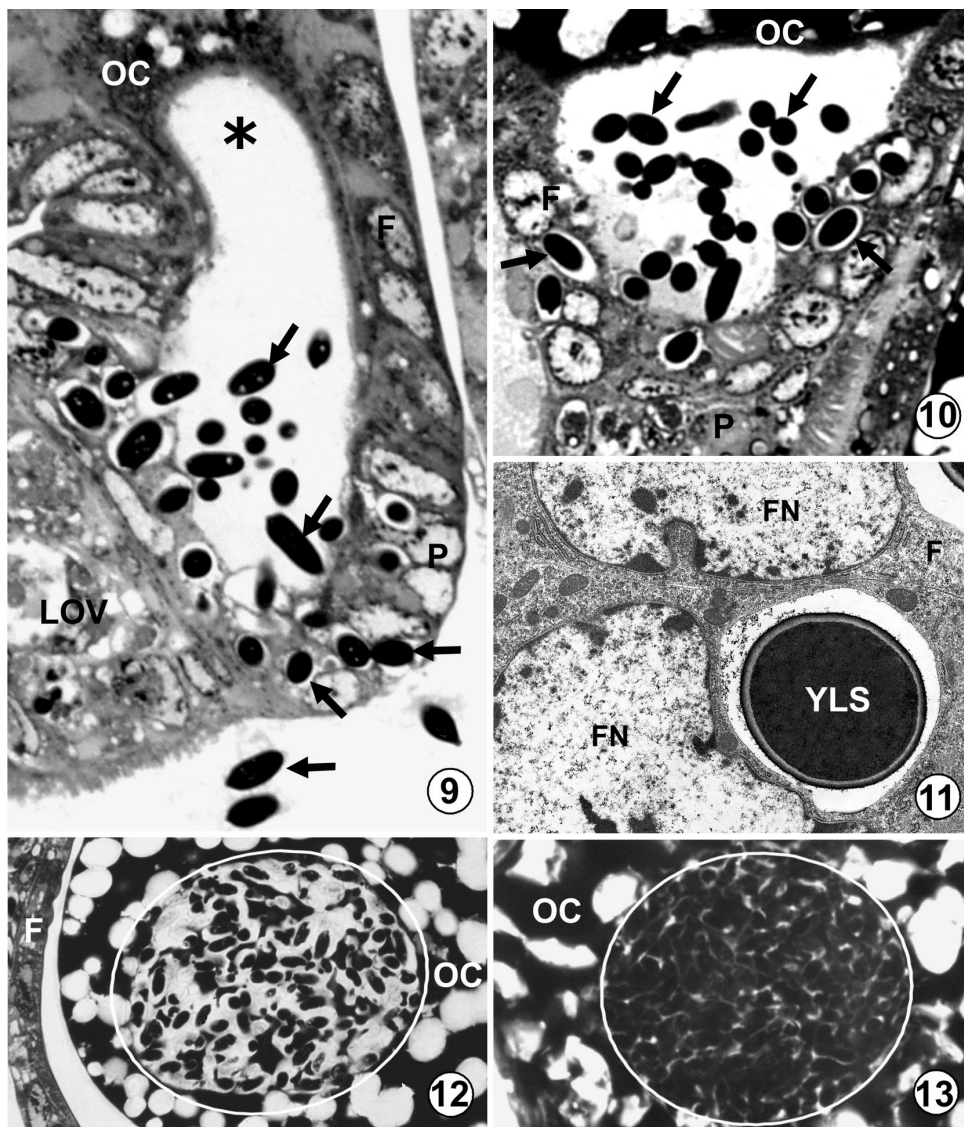
Figs 2-8. Fig. 2. *M. pruinosa*. The mycetome filled with YLSs (arrowhead) in the body of the male. T – testis. Methylene blue, $\times 570$. Fig. 3. *M. pruinosa*. Fragment of the epithelial cell surrounding the mycetome. Arrows – rod-shaped bacteria; EN – nucleus of the epithelial cell; L – lipid droplet. TEM, $\times 18\ 100$. Fig. 4. *C. anceps*. The mycetome filled with YLSs (arrowheads) in the body of the female. Arrows – mycetome nuclei embedded in the common cytoplasm; EP – epithelial cells surrounding the mycetome; L – lipid droplets. Methylene blue, $\times 530$. Fig. 5. *M. pruinosa*. Cross section through the cell of the YLS. Arrow – cell wall composed of two distinct layers; YN – nucleus; NU – nucleolus. TEM, $\times 16\ 500$. Fig. 6. *M. pruinosa*. Fragment of the mycetome cytoplasm. Arrows – rod-shaped bacteria; MN – mycetome nuclei embedded in the common cytoplasm. TEM, $\times 17\ 500$. Fig. 7. *M. pruinosa*. Fragment of the cell of the YLS. Arrow – cell wall; L – lipid droplet; M – mitochondria; YN – nucleus. TEM, $\times 15\ 100$. Fig. 8. *C. anceps*. The YLS reproducing by budding. TEM, $\times 9\ 300$.

three stages: previtellogenesis (i.e. synthesis and accumulation of RNA's), vitellogenesis (i.e. synthesis and accumulation of reserve substances) and choriogenesis (i.e. synthesis and secretion of precursors of eggshells and their deposition on the oocyte surface) (for a detailed description of ovaries of planthoppers see SZKLARZEWICZ *et al.* 2007).

Ultrastructure, distribution and transovarial transmission of yeast-like endosymbiotic microorganisms

Both in males and females of *C. anceps* and *M. pruinosa*, the spaces between internal organs are filled with large structures termed mycetomes (Figs 2, 4). The mycetomes have a syncytial character, i.e. they have numerous nuclei embedded in

a common cytoplasm (Figs 4, 6). The mycetomes are surrounded by a one-layered epithelium (Fig. 4). In young specimens epithelial cells are small, isodiametric and closely adhere to each other (not shown), while in the older specimens they become irregular and voluminous with large lipid droplets in the cytoplasm (Fig. 4). Numerous rod-shaped bacteria occur in epithelial cells (Fig. 3) as well as in mycetome cytoplasm (Fig. 6) of all examined specimens of *M. pruinosa*. The bacteria measure 1.2-1.6 μm in length and 0.3-0.5 μm in diameter. Both in *M. pruinosa* and *C. anceps*, the mycetome cytoplasm contains an enormous number of yeast-like symbionts (YLSs) (Figs 2, 4). The size of YLSs is 8-10 μm in length and 3,0-3,5 μm in diameter. They are surrounded by a thick cell wall composed of two distinct layers (Figs 5, 7, 8). The



Figs 9-13. Figs 9, 10. Longitudinal section through the posterior end of the ovariole, pedicel (P) and lateral oviduct (LOV) during infection by the YLSs (arrows). Methylene blue, $\times 1\ 000$. Fig. 9. *C. anceps*. Fig. 10. *M. pruinosa*. Asterisk – deep depression of the oolemma; F – follicular cells; OC – oocyte. Fig. 11. *C. anceps*. Fragment of the follicular epithelium (F) during migration of YLSs. FN – follicular cell nucleus. TEM, $\times 6\ 500$. Figs 12, 13. *C. anceps*. Cross section through the posterior pole of the ovariole containing a “symbiont ball” filled with YLSs (encircled). TEM, $\times 500$. Fig. 12. Oocyte during late vitellogenesis stage; Fig. 13. Full-grown oocyte. F – follicular epithelium; OC – oocyte.

outer layer is electron dense and has a thickness of 30 nm. The inner layer has lower electron density and is 140 nm thick. In the central part of the yeast cell a large, spherical nucleus with a single nucleolus is present (Fig. 5). The remaining cytoplasm is filled with ribosomes, mitochondria and large lipid droplets (Fig. 7). The YLSs reproduce by budding (Fig. 8). Sexual reproduction of YLSs was not observed. In older females (i.e. containing terminal oocytes in the stage of late vitellogenesis), the YLSs leave the mycetome cytoplasm. They become released into a haemolymph and migrate towards the terminal oocytes (Fig. 9). The endosymbionts pass through the cells of the ovariole stalk (pedicel) (Fig. 9) as well as follicular cells surrounding the posterior pole of the oocyte (Fig. 10, 11). Subsequently, they enter the perivitelline space (Figs 9, 10). At the same time a deep depression is formed at the posterior pole of the oocyte. The YLSs accumulate in the oocyte depression and form a characteristic “symbiont ball” (Fig. 12). At the end of oocyte growth, the “symbiont ball” is tightly packed with YLSs (Fig. 13). Until the end of oocyte growth the YLSs are isolated from the ooplasm by oolemma and do not enter the ooplasm. The endosymbionts gathered in the depression of the oocytes, like those harbored in the mycetome, undergo budding (not shown).

Discussion

Our observations revealed that both adult males and females of *M. pruinosa* (Flatidae) as well as *C. anceps* (Delphacidae) harbor a large number of intracellular YLSs. This observation shows that these endosymbionts are essential for both sexes of examined planthoppers. In recent years, the metabolic significance of YLSs for growth and reproduction of the host insects has been extensively studied using the rice brown planthopper, *Nilaparvata lugens* (SASAKI *et al.* 1996; HONGOI & ISHIKAWA 1997, 2000; WILKINSON & ISHIKAWA 2001). These studies revealed that planthopper YLSs are involved in nitrogen recycling using uric acid as a nitrogenous resource. Planthoppers, in addition to producing uric acid as a nitrogenous waste product, also synthesize it as a storage product during nitrogen deficiency. Uric acid is stored in mycetomes and converted by uricase, secreted by YLSs, into compounds of nutritional value. It should be noted that CHENG and HOU (2005) demonstrated that YLSs are also engaged in synthesis of yolk precursors in females of the rice brown planthopper, *Nilaparvata lugens*.

Our studies showed that YLSs in *M. pruinosa* and *C. anceps* are transmitted from the mother to the progeny by a similar route, i.e. via cytoplasm of cells of the pedicel as well as follicular cells sur-

rounding the posterior pole of the oocyte. Since the same situation has been observed in other members of the family Delphacidae, i.e. *Laodelphax striatellus* (NODA 1977) and *Nilaparvata lugens* (CHENG & HOU 2001) as well as in the leafhopper, *Scaphoideus titanus* (SACCHI *et al.* 2008), it seems probable that all hemipterans have developed the same mode of transmission of YLSs to the next generation. Thus, the transmission of YLSs in insects is more uniform than the transmission of bacteria (see Introduction). Moreover, molecular studies revealed that YLSs in hemipterans are not only similarly inherited but are also phylogenetically closely related to each other. The analysis of 18S ribosomal DNA sequences of YLSs in delphacids (NODA *et al.* 1995; XET-MULL *et al.* 2004), aphids (FUKATSU & ISHIKAWA 1996) and leafhoppers (SACCHI *et al.* 2008) indicated that they belong to the class Pyrenomycetes in the phylum Ascomyctina. The YLSs in the examined rice delphacids are characterized by a high degree of similarity of 18S ribosomal DNA sequences (NODA *et al.* 1995; XET-MULL *et al.* 2004). This finding strongly suggests that YLSs of delphacids are closely related (i.e. constitute a monophyletic group). This implies, in turn, that the symbiosis of YLSs and delphacids is the result of a single infection of the common ancestor of present delphacids. The YLSs harbored in members of the Flatidae family have so far not been examined by molecular methods, therefore their systematic position remains unknown. Since flatids and delphacids are phylogenetically distant within planthoppers (i.e. do not represent a monophyletic taxon) (BOURGOIN *et al.* 1997; URBAN & CRYAN 2007), it is unlikely that their endosymbionts have been acquired through a common ancestor. In this light, it may be assumed that YLSs were horizontally transferred between flatid and delphacid lineages. It is noteworthy that HONGOI and ISHIKAWA (2000), on the basis of an analysis of uricase gene sequences of YLSs in aphids and delphacids, provided evidence of a close relationship between YLSs in these phylogenetically distant groups of insects as a result of the horizontal transfer of microorganisms from the aphid to the planthopper lineage.

Both in *M. pruinosa* and *C. anceps*, the migration of YLSs is correlated with ovary development (the YLSs infect vitellogenic oocytes). Thus, this observation strongly supports the hypothesis that the movement of microorganisms is stimulated by an unknown factor released by ovaries (EBERLE & MCLEAN 1982; ŻELAZOWSKA & BILIŃSKI 1999; SZKLARZEWICZ & MOSKAL 2001; SZKLARZEWICZ *et al.* 2006).

We observed that apart from YLSs, rod-shaped bacteria are present in the body of all specimens of *M. pruinosa*. In contrast to YLSs, bacteria have never been found in the ovaries of *M. pruinosa*.

The absence of these bacteria in the ovaries indicates that they may be horizontally transmitted between specimens. The large number of bacteria both in the mycetome cytoplasm as well as in its epithelium suggests that they may have a significant (positive or negative) influence on the host insect. These microorganisms may represent S-symbionts of *M. pruinosa*, but may also prove to be pathogenic. It should be noted that NODA and SAITO (1979) detected rod-shaped bacteria in mycetomes of the planthopper *Laodelphax striatellus* (Delphacidae) but did not suggest a possible role for them. It may be also speculated that these bacteria belong to the widespread within arthropods rickettsia-like genus *Wolbachia pipientis*. The last assumption is supported by PCR detection of *Wolbachia* in two delphacids, *Laodelphax striatellus* and *Sogatella furcifera* (NODA *et al.* 2001), as well as by the observation that specimens of *Laodelphax striatellus* may be horizontally infected by *Wolbachia* (KANG *et al.* 2003). To verify these hypotheses, further studies of specimens of *M. pruinosa* taken from different populations are needed.

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