

## Germ cell cluster formation and ovariole structure in *Puto albicans* and *Crypticerya morrilli* (Hemiptera: Coccinea). Phylogenetic implications

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**Abstract.** The organization and development of ovaries in representatives of two families (Putoidae and Monophlebidae) of scale insects are described. Developing ovaries of *Puto albicans* McKenzie, 1967 and *Crypticerya morrilli* (Cockerell, 1914) consist of numerous clusters of cystocytes that are arranged in the form of rosettes. At the end of the last nymphal instar these clusters start to protrude from the interior of the ovary into the body cavity and the ovarioles begin to be formed. The ovary of a young female is composed of about 200 spherical telotrophic ovarioles devoid of terminal filaments. The ovarioles of *C. morrilli* contain 8 germ cells (7 trophocytes and a single oocyte). From 25 to 45 germ cells (23–43 trophocytes and 2 or 3 oocytes) occur in the ovarioles of *P. albicans*. An ovariole of an adult female is subdivided into a trophic chamber (trophiarium), vitellarium and ovariolar stalk (pedicel). At each stage of development, the ovaries are accompanied by large cells (termed bacteriocytes) that contain endosymbiotic microorganisms. The organization of the ovary in *P. albicans* is more similar to that in archaeococcoid scale insects than in neococcoid taxa. In contrast, the number of germ cells per ovariole in *C. morrilli* is not typical of other archaeococcoids, but resembles the derived condition seen in other iceryine taxa. The classification and phylogeny of scale insects are discussed in the light of these results.

### INTRODUCTION

Scale insects are plant pests with a world-wide distribution. They are characterized by unusual sexual dimorphism. Adult females are larviform, apterous and usually immobile. Males have one pair of wings and nonfunctional mouthparts. The monophyletic origin of scale insects, which is based on the numerous synapomorphies in this group, is widely accepted by entomologists (for further details see e.g. Koteja, 1974, 1996; Miller & Kosztarab, 1979; Gullan & Cook, 2007). Scale insects are classified into two informal groups, the primitive archaeococcoids and advanced neococcoids (Borchsenius, 1956). The monophyly of neococcoids is well documented and recently supported by molecular analyses (Cook et al., 2002). The neococcoid scale insects are classified into 17 well defined families, including Pseudococcidae, Kermesidae, Eriococcidae, Coccidae, Asterolecaniidae, Diaspididae and several smaller families (Gullan & Cook, 2007). In contrast to the neococcoids, the phylogeny and classification of archaeococcoids is still being debated (Koteja, 1974, 1996; Gullan & Sjaarda, 2001; Cook et al., 2002; Gullan & Cook, 2007). Since this group is characterized by plesiomorphic characters only, its monophyly is uncertain (Koteja, 1996; Cook et al., 2002). Morrison (1928) distinguished two families of archaeococcoids, the Ortheziidae and Margarodidae. The latter contained five subfamilies: Coelostomidiinae, Margarodinae, Monophlebinae, Steingeliinae and Xylococcinae. Koteja (1974) on the basis of mouthpart morphology elevated these subfamilies and two tribes sensu Morrison (i.e. Kuwanini and Matsucoc-

cini) to family rank. Additionally, Koteja (1974) included the small family Phenacoleachiidae in the archaeococcoid group. It should be emphasized that recent morphological (Gullan & Sjaarda, 2001; Foldi, 2005; Szklarzewicz et al., 2005; Hodgson & Foldi, 2006) and molecular data (Cook et al., 2002) confirm Koteja's classification. For several years some entomologists placed the family Putoidae, containing the single genus *Puto* McKenzie, with the archaeococcoids (e.g. Foldi, 2005; Hodgson & Foldi, 2006; Gullan & Cook, 2007; Kondo et al., 2008). It should be noted that the taxonomic position of the genus *Puto* is still under discussion. For many years, due to similarity in female morphology, *Puto* was regarded as a member of the advanced family Pseudococcidae (e.g. Koteja, 1974; Miller & Miller, 1993). Beardsley (1969), based on the morphological characters of males, concluded that *Puto* is a primitive neococcoid and should be placed in its own family, the Putoidae. Koteja (1996; pers. commun.) considered *Puto* as the ancestor of all neococcoids on account of its primitive characters. Cook and co-workers (2002), on the basis of molecular analysis, suggested that *Puto* is not a neococcoid. Thus, the controversy surrounding this genus warrants further studies directed at clarifying its systematic position.

The ovaries of scale insects were extensively studied over the last three decades (Książkiewicz, 1980; Szklarzewicz & Biliński, 1995; Szklarzewicz, 1997, 1998a, b, c; Koteja et al., 2003; Szklarzewicz et al., 2005; Niżnik & Szklarzewicz, 2007). The ovaries consist of short telotrophic ovarioles devoid of terminal filaments. Each ovariole is composed of a trophic chamber (trophiarium),

vitellarium and ovariole stalk (pedicel). The vitellarium usually contains a single oocyte. The ovarioles of scale insects, on account of their atypical organization (lack of a terminal filament, a single oocyte in the vitellarium, a small number of trophocytes in the tropharium), were formerly regarded as polytrophic or dieroistic (Jura, 1958; Węglarska, 1961; Magakyan et al., 1975). Szklarzewicz and co-workers in a series of papers (Szklarzewicz & Biliński, 1995; Szklarzewicz, 1997, 1998a, b, c; Koteja et al., 2003; Szklarzewicz et al., 2005; Niżnik & Szklarzewicz, 2007) have shown that the ovarioles of archaeococcoid scale insects markedly differ in organization and functioning from those of neococcoid scale insects. First, the total number of germ cells (trophocytes + oocytes) constituting the ovariole in archaeococcoids is much larger than in neococcoids. Second, tropharia of archaeococcoids (except for members of the family Monophlebidae) contain trophocytes and early previtellogenic oocytes (termed arrested oocytes), whereas in neococcoids the tropharia contain only trophocytes (except for representatives of the family Kermesidae and some Eriococcidae). The arrested oocytes in archaeococcoid scale insects are capable of further development, while those in kermesids and eriococcids do not develop, but degenerate, even those in tropharia. Thus, the ovaries of archaeococcoids appear more similar in organization and functioning to the ovaries of aphids (for further details see Büning, 1985; Szklarzewicz et al., 2000) than to those of neococcoids. Apart from the above features that are atypical of insects, the ovaries of scale insects so far investigated are characterized by a unique mechanism of ovariole formation (Węglarska, 1961; Szklarzewicz, 1997; 1998a, b, c; Niżnik & Szklarzewicz, 2007). Although in most insects the ovarioles arise inside the ovary, in scale insects, the ovariole anlagen protrude from the interior of the ovary into the body cavity. However, there is no detailed description of this process in the literature.

Since comparative studies on the organization of the ovaries in scale insects (for further details see Szklarzewicz, 1998a; Koteja et al., 2003) and several other groups of insects (e.g. heteropterans, mecopterans, adaphagan beetles, dipterans, neuropterans) have shown that this character is very useful for phylogenetic studies (for fur-

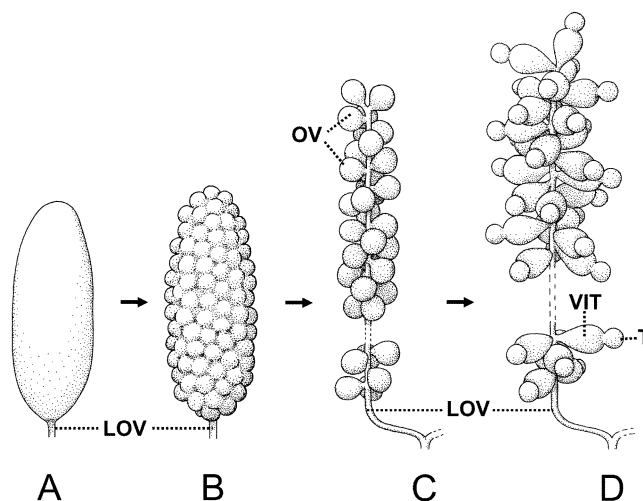


Fig. 1. Schematic representation of the development of an ovary of a scale insect. A – The spindle-shaped ovary in a last nymphal instar. B – The ovary at the end of the last nymphal instar. Clusters of cystocytes start to protrude from the interior of the ovary into the body cavity. C – The ovary of a young female. Ovarioles (OV) are radially arranged around the lateral oviduct (LOV). D – The ovary of an adult female. Ovarioles consist of tropharia (T) and vitellaria (VIT).

ther details see e.g. Biliński et al., 1998; Jaglarz, 1998; Kubrakiewicz et al., 1998a, b; Simiczjew et al., 1998), the aim of this work is to verify the taxonomic placement of *Puto* on the basis of the organization of its ovaries.

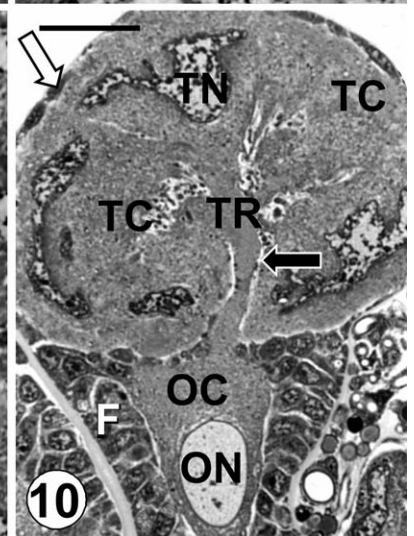
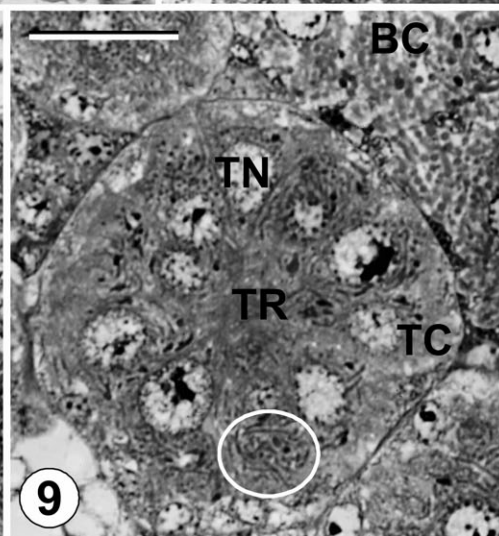
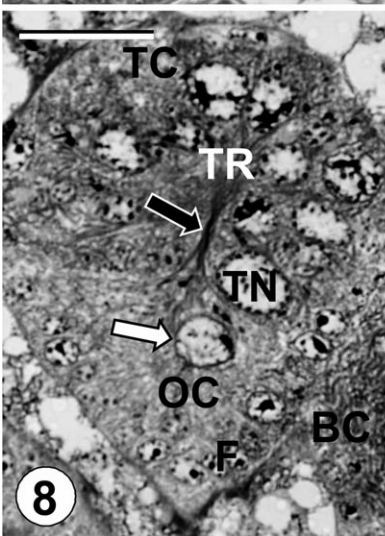
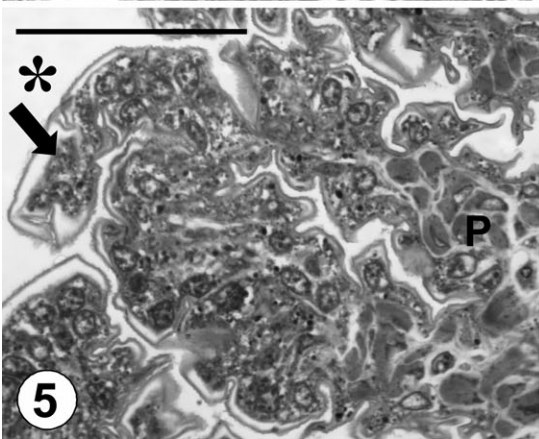
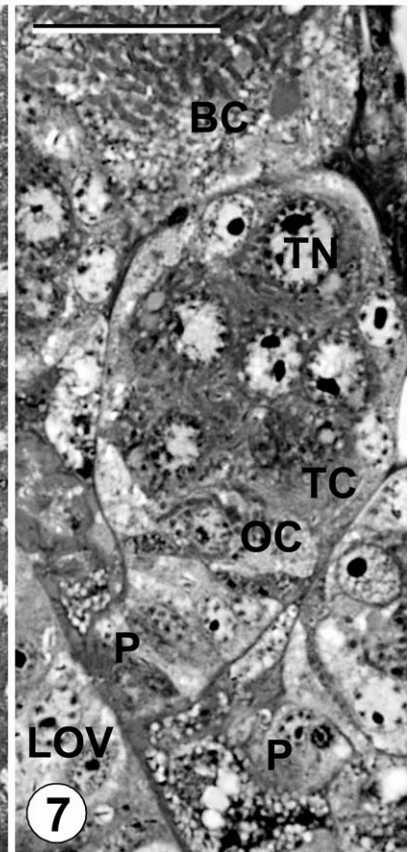
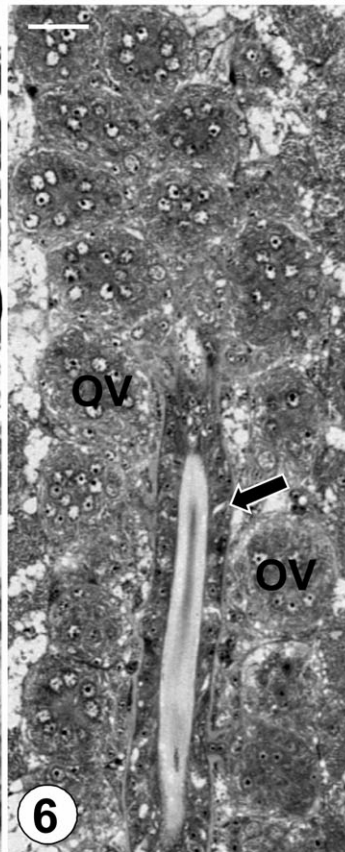
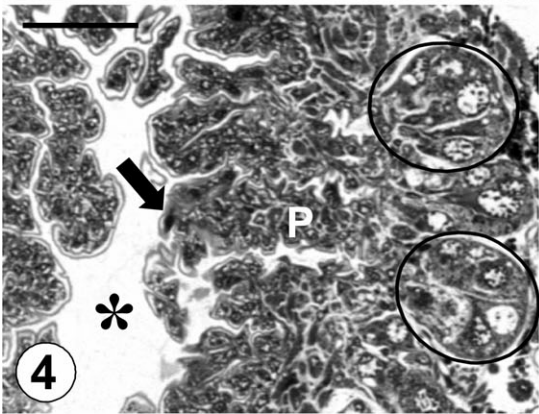
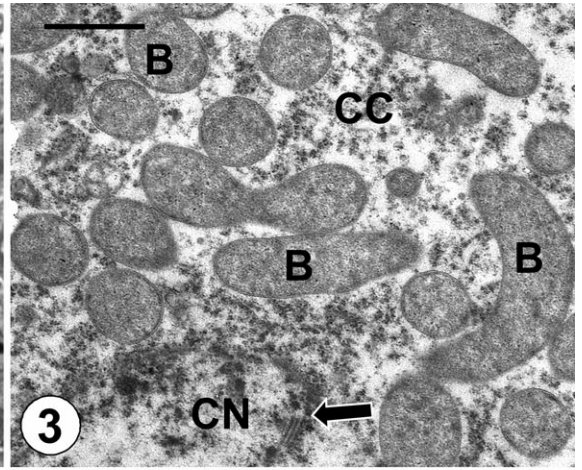
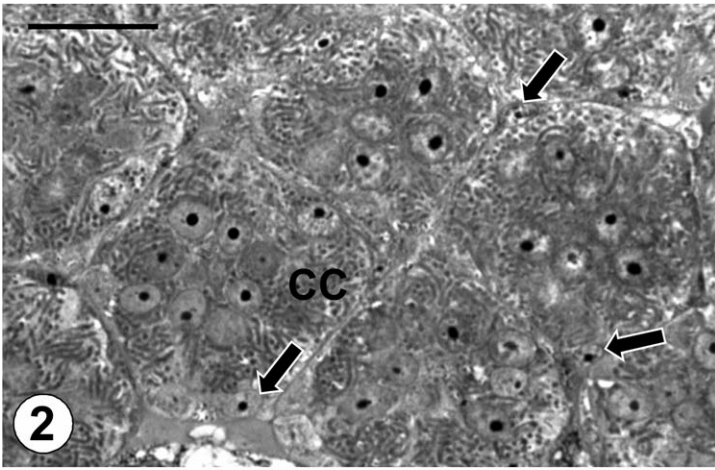
#### MATERIAL AND METHODS

The ovaries of *Puto albicans* and *Crypticeria morrilli* were studied using ultrastructural and histological methods.

McKenzie (1967) in his description of *P. albicans* stated that adult females do not produce ovisacs. The adult female of *C. morrilli*, which lays its eggs in a marsupium, was re-described by Unruh (2008).

Specimens of *P. albicans* were collected from twigs of *Arctostaphylos viscida* near Placerville (El Dorado County, California, USA) in May and specimens of *C. morrilli* (Cockerell, 1914) from twigs of *Acacia greggii* in Red Rock State Park (Yavapai County, Arizona, USA), also in May. The entire abdomens of individuals of each species were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 months. The

Figs 2–10. 2 – *P. albicans* (Putoidae). Fragment of the ovary in a last nymphal instar. The ovary is filled with clusters of cystocytes (CC). Arrows indicate somatic cells surrounding the clusters. Methylene blue, scale bar = 20 µm. 3 – *P. albicans* (Putoidae). Fragment of a cystocyte (CC) in the ovary of the last nymphal instar. The cystocyte cytoplasm is filled with numerous endosymbiotic bacteria (B). Note the synaptonemal complex (arrow) in the cystocyte nucleus (CN). TEM, scale bar = 2 µm. 4, 5 – *C. morrilli* (Monophlebidae). Fragment of the ovary (longitudinal section) at the end of the last nymphal instar. Clusters of cystocytes (encircled) start to protrude from the interior of the ovary into the body cavity. Note the long ovariole stalks (P) and deeply folded wall of the lateral oviduct (arrow). Asterisk – lumen of lateral oviduct. Methylene blue, scale bar = 20 µm. 6 – *P. albicans* (Putoidae). Fragment of the ovary (longitudinal section) of a young female. The spherical ovarioles (OV) are arranged in a radial pattern around the lateral oviduct (arrow). Methylene blue, scale bar = 20 µm. 7 – *P. albicans* (Putoidae). A longitudinal section of an ovariole of an adult female. The germ cells are differentiated into trophocytes (TC) and oocytes (OC). The ovary is accompanied by bacteriocytes (BC). LOV – lateral oviduct; P – ovariole stalk; TN – trophocyte nucleus. Methylene blue, scale bar = 20 µm. 8, 9 – *P. albicans* (Putoidae). An ovariole of an adult female. 8 – longitudinal section; 9 – cross section. In the centre of the tropharium is a trophic core (TR). Note the endosymbiotic bacteria (encircled) in the cytoplasm of the germ cells. Black arrow – nutritive cord; white arrow – oocyte nucleus; BC – bacteriocyte; F – follicular epithelium; OC – oocyte; TC – trophocyte; TN – trophocyte nucleus. Methylene blue, scale bar = 20 µm. 10 – *C. morrilli* (Monophlebidae). An ovariole of an adult female (longitudinal section). Note the large, lobate nuclei (TN) of trophocytes (TC). White arrow – flattened cells of the inner epithelial sheath; black arrow – nutritive cord; F – follicular epithelium; OC – oocyte; ON – oocyte nucleus; TR – trophic core. Methylene blue, scale bar = 20 µm.



material was next rinsed with a mixture of the buffer and sucrose (5.8 g / 100 ml) and postfixed for 1.5 h in 1% osmium tetroxide. After dehydration in a series of ethanol and acetone, material was embedded in the epoxy resin Epon 812 (Fullam, Latham, N.Y., USA). Semithin sections were stained with 1% methylene blue in 1% borax and photographed under a Jenalumar microscope. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a JEM 100× electron microscope at 80 kV.

## RESULTS

During the last nymphal instar the spindle-shaped ovaries (Fig. 1A) of *Puto albicans* and *Crypticerya morrilli* are filled with numerous clusters of undifferentiated germ cells termed cystocytes (Fig. 2). The cystocytes are integrated into rosettes (Fig. 2). Each cluster is surrounded by a single layer of flattened somatic cells (Fig. 2). Groups of somatic cells also occur among clusters (not shown). Analysis of serial semi-thin sections has shown that each cluster of *C. morrilli* consists of 8 cystocytes. The clusters in *P. albicans* consist of a greater number, even as many as 40 cystocytes. All of the cystocytes in a cluster are interconnected by intercellular bridges (not shown). Cystocytes are all morphologically similar. They are cone-shaped and have large spherical nuclei with a single nucleolus (Fig. 2). Synaptonemal complexes occur in the nuclei of all cystocytes (Fig. 3). In *P. albicans*, the cystocyte cytoplasm contains numerous endosymbiotic bacteria (Figs 2, 3). These endosymbionts are elongate and measure about 10 µm in length and 1 µm in diameter.

Long ovariole stalks (pedicles) are formed at the proximal pole of each spherical cluster at the end of the last nymphal instar (Fig. 4). Simultaneously, somatic cells start to form the distal part of the lateral oviduct (Figs 4, 5). At the beginning of this process the wall of the lateral oviduct is deeply folded (Figs 4, 5). At the stage when the formation of ovariole stalks is completed, the clusters of cystocytes start to protrude from the ovary into the body cavity (Figs 1B, 4). As this process progresses, the folds of the lateral oviduct wall stretch and the lateral oviduct takes on the form of an elongated tube (Fig. 6). Consequently the ovary of a young female (shortly after it emerges as an adult) is composed of numerous (about 200) spherical ovarioles, which are radially arranged around the lateral oviduct (Figs 1C, 6). The ovarioles are devoid of terminal filaments (Figs 1C, 6). As before, all of the cystocytes in a cluster are morphologically similar. In adult females, the ovarioles become elongated (Fig. 7). Simultaneously, the cystocytes differentiate into trophocytes and oocytes (Fig. 7). Analysis of serial sections of 10 ovarioles revealed a constant number of 7 trophocytes and a single oocyte in the ovarioles of *C. morrilli*. In those of *P. albicans* there are from 25 to 45 germ cells (23–43 trophocytes and 2 or 3 oocytes). In both these species the trophocytes occupy the distal part of an ovariole, whereas the oocyte or oocytes are localized in the proximal region (Fig. 7). In *P. albicans*, the oocyte closest to the lateral oviduct develops first (Figs 7, 8). Subsequently, the distal part of the ovariole transforms into a tropharium (trophic chamber) and proximal part

into the vitellarium (Figs 1D, 8). The latter contains a single oocyte (Fig. 8). In *P. albicans*, the remaining 1 or 2 oocytes (termed arrested oocytes) are localized in the proximal part of the tropharium (not shown). The somatic cells surrounding the ovariole differentiate into the flattened cells of the inner epithelial sheath and the follicular cells. The latter form a single-layered epithelium around the developing oocyte (Fig. 10). The inner epithelial sheath encompasses the tropharium (Fig. 10). The central area of the tropharium, termed the trophic core, is connected with both the trophocytes and oocytes (Figs 8–10). Trophocytes are joined to the trophic core by means of broad processes (Figs 8–10), whereas oocytes are connected via nutritive cords (Figs 8, 10). In the ovarioles of older females the trophocyte nuclei become large and lobate (Fig. 10). The trophocyte cytoplasm is filled mainly with ribosomes (not shown). Numerous elongate bacteria are present in the trophocytes and oocytes in *P. albicans* (Figs 8, 9).

Ovarioles develop synchronously in *P. albicans* and *C. morrilli*, i.e. they start to grow out of the spindle-shaped ovary into the body cavity at the same time (Figs 1B, 4). As a consequence, all the ovarioles in the ovary of an adult female are at the same developmental stage (Fig. 1D).

There are giant cells termed bacteriocytes associated with the ovaries in both of the species studied (Figs 7–9). The cytoplasm of these cells is tightly packed with endosymbiotic microorganisms (Figs 7–9).

## DISCUSSION

Results presented in this paper indicate that the ovaries of *Puto albicans* and *Crypticerya morrilli* develop in the same manner, i.e. the ovarioles protrude from the interior of the ovary into the body cavity. A comparison of these results with previous observations on the ovaries of archaeococcoids and neococcoids (Węglarska, 1961; Szklarzewicz, 1997, 1998a, b, c; Niżnik & Szklarzewicz, 2007) indicates that the ovary develops similarly in all scale insects. Thus, this character may be regarded as a synapomorphy for scale insects. Since the development of ovarioles in scale insects starts with the formation of ovariole stalks, it may be speculated that, as in other pterygote insects (for further details see Büning, 1994), the somatic cells of the ovaries play a crucial role in their development.

As a rule, ovarioles in scale insects develop asynchronously (Węglarska, 1961; Bielenin, 1962; Szklarzewicz & Biliński, 1995; Szklarzewicz, 1997, 1998a, b, c; Szklarzewicz et al., 2005; Niżnik & Szklarzewicz, 2007). Consequently, in the ovary of an adult female the neighbouring ovarioles are at different stages of oogenesis. Węglarska (1961) observed that in the oystershell scale, *Quadraspidiotus ostreaeformis* (Curtis) (Diaspididae), the ovarioles located in the distal region of an ovary develop earlier than those located in the proximal region. According to Węglarska (1961), young undifferentiated ovariole anlagen occur among well-developed ovarioles in the distal region. This study indicates that unlike the

situation in most scale insects, the ovarioles in *P. albicans* and *C. morrilli* develop synchronously. Koteja et al. (2003) postulated that synchronous development of ovarioles is associated with a shortening of the oviposition period.

The observations reported here indicate that the ovaries of both of the species investigated are accompanied by bacteriocytes (formerly termed mycetocytes), which contain endosymbiotic microorganisms. In *P. albicans*, endosymbiotic bacteria were observed both in the bacteriocytes and cytoplasm of germ cells. It is generally accepted that the presence of endosymbiotic microorganisms (bacteria or yeasts) in an insect's body is connected with its feeding on a diet deficient in some essential nutrients (for further details see Douglas, 1989; Baumann, 2005). In scale insects, as in other plant sap-sucking hemipterans, endosymbiotic microorganisms are responsible for the synthesis of essential amino acids, which are absent in the phloem sap. The endosymbionts are transovarially (vertically) transmitted from mother to offspring (Buchner, 1965; Szklarzewicz & Moskal, 2001; Szklarzewicz et al., 2006; Michalik et al., 2009). Variable modes of infection of germ cells by microorganisms have developed during the co-evolution of scale insects and their endosymbionts. The latter may invade young germ cells (oogonia or cystocytes) or older oocytes (vitellogenic or choriogenic). The latter process is more common among scale insects (Buchner, 1965; Szklarzewicz et al., 2006; Niżnik & Szklarzewicz, 2007). The occurrence of an enormous number of bacteria in all of the germ cells (cystocytes, trophocytes, oocytes) in *P. albicans* suggests that in this species the infection of germ cells occurs before their differentiation into oocytes and trophocytes.

### Phylogenetic conclusions

The results indicate that the ovarioles of *P. albicans* are composed of numerous germ cells (trophocytes + oocytes). The number of germ cells in the ovarioles analyzed varies between 25 and 44. Thus, it is similar or even larger than the number in ovarioles of other archaeococcoid families, such as the Ortheziidae (15–58 germ cells), Margarodidae s. str. (12–19 germ cells), Steingeliidae (21–41 germ cells) and Monophlebidae (8 germ cells) (Szklarzewicz & Biliński, 1995; Szklarzewicz, 1997, 1998c; Koteja et al., 2003; Szklarzewicz et al., 2005; Niżnik & Szklarzewicz, 2007). The neococcoid families are characterized by a smaller number of germ cells per ovariole. Among the neococcoid scale insects, the largest number of germ cells occurs in ovarioles of the Kerme-sidae (i.e. 8–20) (Szklarzewicz, 1998b). In the Pseudococcidae and some species of Eriococcidae and Coccidae, the number of germ cells is 8 or slightly more than 8 (e.g. 10 in some coccids) (Szklarzewicz, 1998b; Witek, 2002). The smallest number of germ cells (i.e. 4) is reported for the Diaspididae, Cryptococcidae and some species of Coccidae and Eriococcidae (Węglarska, 1961; Bielenin, 1962; Książkiewicz, 1980; Szklarzewicz, 1998b).

The considerations mentioned above clearly indicate that the ovarioles of *P. albicans* are more similar to those of archaeococcoids than neococcoids. This in turn,

strongly supports the conclusion based on molecular data that the genus *Puto* should be placed among the archaeococcoids. This assumption also corresponds well with the results of studies on chromosome behaviour in scale insects. For over sixty years it has been known that the genus *Puto* is characterized by a primitive chromosome system, termed a “XX–XO chromosome system” (Hughes-Scharader, 1944). Besides *Puto*, this sex determining system is found in aphids (Blackman, 1985) and most archaeococcoids (Hughes-Schrader, 1948). In neococcoids and a few archaeococcoids, there are several other systems (Miller & Kosztarab, 1979). It is noteworthy that Hughes-Schrader (1948) and Brown and Cleveland (1968), on the basis of chromosome behaviour, suggested that *Puto* is more distantly related to pseudococcids than usually supposed.

The family Monophlebidae, which includes *C. morrilli*, is regarded as one of the most primitive archaeococcoid families among scale insects (Koteja, 1974). The monophyly of this family was established on the basis of characters of female morphology (Koteja, 1974; Gullan & Sjaarda, 2001). The present study of the ovaries of *C. morrilli* (from the tribe Iceryini) and earlier examinations of ovaries of other members of the family Monophlebidae – *Icerya purchasi* Maskell (from the tribe Iceryini) and *Palaeococcus fuscipennis* (Burmeister) (from the tribe Monophlebini) (Hughes-Schrader, 1927; Szklarzewicz et al., 2005; Niżnik & Szklarzewicz, 2007), revealed that their ovarioles always contain 8 germ cells (7 trophocytes + single oocyte). Therefore, it is likely that the occurrence of 8 germ cells per ovariole is a characteristic feature of all monophlebidids. Thus, this character gives additional support for the monophyly of the family Monophlebidae. Szklarzewicz et al. (2005) observed that ovary organization in monophlebidids seems to be more similar to that in the neococcoid family Pseudococcidae than to that in other archaeococcoid families, such as Ortheziidae, Margarodidae s. str. and Steingeliidae. Assuming that the large number of germ cells per ovariole represents the ancestral condition within scale insects (Szklarzewicz, 1998a; Koteja et al., 2003), it may be speculated that the same number of germ cells in ovarioles in monophlebidids and pseudococcids may be the result of homoplasy, i.e. this character evolved independently in both families.

Previous (Szklarzewicz, 1998c; Koteja et al., 2003; Szklarzewicz et al., 2005) and the present study on the ovaries of representatives of families Margarodidae s. str., Steingeliidae and Monophlebidae (i.e. Margarodidae sensu Morrison) have revealed that they are characterized by distinct structure. They may contain large and variable numbers of germ cells per ovariole (Margarodidae s. str., Steingeliidae) or small and stable numbers (Monophlebidae). Several oocytes may develop in their vitellaria (Margarodidae s. str., Steingeliidae) or just a single oocyte (Monophlebidae). These observations strongly support the division of the Margarodidae sensu Morrison into several small families as suggested by Koteja (1974).

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