Sperm morphology of *Trichospilus diatraeae* and *Palmistichus elaeisis* (Hymenoptera: Chalcidoidea: Eulophidae)

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ABSTRACT

In this study, the sperm morphology of the parasitoids *Trichospilus diatraeae* and *Palmistichus elaeisis* (Eulophidae) was investigated using light and transmission electron microscopy. In the two species, the sperm are spiral along their entire length and measure about 130 μm and 195 μm in length, respectively. The head region consists of the acrosome and nucleus. The acrosome is composed of an acrosomal vesicle and, in *P. elaeisis*, a perforatorium. In both species, an extracellular layer in which several filaments are radiated covers the acrosome and the anterior nuclear region. The nuclei are filled with homogeneous and compact chromatin and measure about 50 μm in length in *P. elaeisis* and 20 μm in *T. diatraeae*. The flagellum consists of an axoneme with the 9+9+2 microtubule arrangement spiraled in a long helix, two mitochondrial derivatives coiling around the axoneme and, in *P. elaeisis*, two accessory bodies. In *T. diatraeae* were observed transverse striations throughout the central region of the axoneme, whereas the central pair of microtubules was rarely observed. In the final flagellar region in *T. diatraeae*, different from *P. elaeisis*, one mitochondrial derivative ends well before the other and both end before the axoneme. The sperm of these two species exhibit features that discriminate one species from each other, as well as characteristics suggest that Eulophidae is closely related to Trichogrammatidae and both of these families are more similar to Eurytomidae than Agaonidae.

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1. Introduction

The Chalcidoidea comprise up to a third of parasitic Hymenoptera species (LaSalle and Gauld, 1991), forming one of the most abundant groups of insects (Grissel and Schauff, 1997). Although some Chalcidoidea are phytophagous or hyperparasitoids, most are parasitoids of other arthropods, thus playing an important role in controlling the populations of other insects. Therefore, chalcidoids have been successfully used in most biological pest control programs (Greathead, 1986; Neumann et al., 2010; Polaszek et al., 2012).

Despite the economic and ecological importance of this group of insects, knowledge of evolutionary relationships among Chalcidoidea remains unclear, with is no consensus on the placement of several families (Heraty et al., 1997; Munro et al., 2011). Therefore, clear definitions of families based on characters that can be used in cladistic analyses are necessary (Grissel and Schauff, 1997).

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Authors like Heraty et al. (1997, 2012) drew attention to the use of new classification systems to clarify relationships between families and subfamilies of Chalcidoidea.

In many animal groups, including insects, sperm morphological data have been commonly used in phylogenetic analysis (Carcupino et al., 1995; Dallai and Afzelius, 1995; Dallai et al., 2011; Gottardo et al., 2012; Jamieson, 1987). In this regard, earlier studies have demonstrated that the structural diversity of spermatozoa in Chalcidoidea can provide a system of characters, which may be used in combination with others to study phylogenetic relationships of the group and resolve uncertainty at the family and genus levels.

In this paper we describe the sperm structure and ultrastructure of the parasitoids *Trichospilus diatraeae* and *Palmistichus elaeisis* (Chalcidoidea: Eulophidae) to provide spermatological data for future phylogenetic analyses of this insect group.

2. Materials and methods

Adult male *T. diatraeae* and *P. elaeisis* were purchased in the department of entomology at the Escola Superior de Agricultura Luiz Queiroz – ESALQ/USP.

2.1. Light microscopy

Seminal vesicles were dissected and squashed on clean glass microscope slides, followed by spreading, then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After drying at room temperature, the slides were observed with an Olympus BX41 photomicroscope equipped with a phase contrast lens to allow measurement of the sperm. For nuclei measurements, some slides were stained for 15 min with 0.2 µg/ml of 4,6-diamino-2-phenylidole (DAPI) and viewed with a epifluorescence Olympus BX60 microscope equipped with a BP360–370 nm excitation filter. The images were analyzed using the *ImagePro-Plus* program.

2.2. Transmission electron microscopy

Seminal vesicles were dissected in 0.1 M sodium cacodylate buffer, pH 7.2 and fixed in a 2.5% glutaraldehyde and 0.2% picric acid solution with the same buffer, for 24 h at 4 °C. The material was post-fixed in 1% osmium tetroxide solution in the same buffer. The material was dehydrated using acetone and embedded in Epon. Ultrathin sections were stained with 2% uranyl acetate in distilled water and 0.2% lead citrate in a 1 N sodium hydroxide solution and observed with a transmission electron microscope, Zeiss Leo 906.

For basic protein detection, the ethanolic phosphotungstic acid method (E-PTA) was applied. Seminal vesicles were fixed only in buffered glutaraldehyde solution for 24 h at 4 °C. The material was dehydrated in an alcoholic series and treated *en bloc* by a 2% PTA solution in absolute alcohol and embedded in Epon.

3. Results

The sperm of *P. elaeisis* (Fig. 1A) and *T. diatraeae* (Fig. 2A) are spiral, long and thin, approximately 195 µm and 130 µm in length, respectively. They are divided into head and flagellum regions (Fig. 1A and Fig. 2A). The head region consists of the acrosome and nucleus. In both species, the acrosome and the anterior nuclear region are covered by an extracellular layer in which several filaments are radiate (Figs. 1C–E and 2C–F). This extracellular layer is longer in the sperm of *T. diatraeae* (Fig. 2C) sperm than *P. elaeisis* (Fig. 1C), measuring around 2 µm and 0.3 µm, respectively. It in *P. elaeisis* contains two regions, with the inner region being more electron-dense (Fig. 1C). This layer and all the filaments arising from it are E-PTA positive, in both species (Fig. 2F).

The acrosome is very small in both species, measuring around 0.1 µm in length. It is composed of an acrosomal vesicle and, in *P. elaeisis*, a rod-shaped perforatorium. This latter has the base inserted into a small cavity at the nuclear tip and is covered by the acrosomal vesicle (Fig. 1C).

In both species the nucleus is filled with homogeneously compacted chromatin (Figs. 1F–H and 2G–J). In *P. elaeisis* it is long, measuring approximately 50 µm in length and 0.2 µm at the base, gradually tapering toward the tip (Fig. 1A and B). In *T. diatraeae* the nucleus measures 20 µm in length and 0.4 µm in diameter at the base. It tapers abruptly from base to near half (Fig. 2A and B).

In the transition region of the nucleus-flagellum in both species, the nucleus is connected to the flagellar structures by a centriolar adjunct (Figs. 1F, H, I and 2H–K). The centriolar adjunct overlaps both the base of the nucleus and the axoneme anterior region (centriole). In *P. elaeisis* the overlapped nuclear area is greater than in *T. diatraeae* (Figs. 1F and 2I, however, in *T. diatraeae* the centriolar adjunct surrounds almost the entire nuclear base (Figs. 1H and 2J).

The flagellum consists of an axoneme, two mitochondrial derivatives, and in *P. elaeisis*, two accessory bodies (Figs. 1J–M and 2H, I, L, N). The axoneme shows a microtubular arrangement of 9+9+2, nine accessory microtubules, nine

peripheral pairs and one central pair (Figs. 1N and 2N). However, in *T. diatraeae* the central pair was difficultly observed (Fig. 2L–O), while transverse striations were observed throughout the central region of the axoneme (Fig. 2 H, I, P and Q). These striations are compound by dense and pale lines with regular spaces between these ones, measuring among dense lines about 38 nm (Fig. 2I).

The axoneme is also coiled, so all pairs cannot simultaneously be sectioned at right angles (Figs. 1M–P and 2L–O). At the flagellar posterior end the axoneme is the last to finish, and the accessory microtubules are the first to disappear (Figs. 1O, P and 2L, M). In *T. diatraeae* sperm, when treated with ethanolic PTA, an EPTA-positive material is clearly observed around and inside the axoneme, but the microtubules are EPTA-negative (Fig. 2F).

The mitochondrial derivatives are arranged in a spiral along the entire axoneme. In cross-section they are oval shaped with equal areas and much smaller than the axoneme (Figs. 1L, M and 2H–P). In the final portion of the flagellum, the mitochondrial derivatives of *P. elaeisis* end approximately together and near the end of the axoneme, since flagella sectioned with only one mitochondrial derivative or just the axoneme rarely are observed (Fig. 1L). In *T. diatraeae* one derivative ends well before the other and both end before the axoneme, because Fig. 2L shows various flagella in cross section with only one mitochondrial derivative or only the axoneme.

In *P. elaeisis*, the accessories bodies are electron-dense, located between the mitochondrial derivatives and the axoneme, and in cross-sections are oval with a very small diameter (Fig. 1J and M). They were not observed in *T. diatraeae*.

4. Discussion

The sperm of *P. elaeisis* and *T. diatraeae* exhibit basic morphology similar to that of other Chalcidoidea, for example: (1) spiral sperm; (2) the presence of an extracellular layer coating the acrosome and part of the nucleus from which several filaments are irradiated; (3) the two mitochondrial derivatives have reduced and equal diameters; and (4) at the end of the axoneme, accessory microtubules finish first (Brito et al., 2009; Fiorillo et al., 2008; Lino-Neto et al., 2000; Quicke et al., 1992; Silva, 2010). Meanwhile, there are other features that discriminate these two species of others chalcidoids.

Although the presence of an acrosome is common in Chalcidoidea, its morphology can vary widely. It may be very small, e.g. *Mellitobia* (Brito et al., 2009), or absent, as in *Pegoscapus* (Fiorillo et al., 2008). Also, there are species in which the vesicle acrosomal is present but the perforatorium is not observed, as in *Trichogramma pretiosum* and *Trichogramma dendrolimi* (Lino-Neto and Dolder, 2001; Lino-Neto et al., 2000), *Mellitobia australica* and *Mellitobia hawaiiensis* (Brito et al., 2009), in *Idarnes* sp.1 and sp.2 (Silva, 2010) and in *T. diatraeae* studied here. Likewise, the extracellular layer was not observed in some chalcidoids as, for example, *Idarnes* sp.1 and sp.3 (Silva, 2010). However, this layer, with filaments radiating from it, is not unique to chalcidoids, as observed in Ichneumonoidea (Moreira et al., 2010; Quicke et al., 1992) and Cynipoidae (Newman and Quicke, 1998).

The centriolar adjunct, observed in these two eulophids, occurs in many insect orders including Hymenoptera. In chalcidoids, it can offer good diagnostic characters at the family and sometimes genus level. For example, in Eurytomidae (Lino-Neto et al., 1999), Trichogrammatidae (Lino-Neto et al., 2000; Lino-Neto and Dolder, 2001) and Eulophidae (Brito et al., 2009) this structure overlapping only the centriole region (about 0.5 µm). However, in Eurytomidae it overlaps the nucleus by a much larger distance (about 8 µm) than in Eulophidae and Trichogrammatidae (less than 0.5 µm). In the Agaonidae, *Pegoscapus* (Fiorillo et al., 2008) and *Idarnes* (Silva, 2010), the centriolar adjunct does not overlap the nucleus.

Fig. 1. Light (A and B) and transmission electron (C–Q) photomicrographs of spermatozoa from *P. elaeis* in longitudinal (C, G and K) and transversal (D, E, G–J and L–P) sections. (A) Sperm in phase contrast. The arrow indicates the limit between the head (h) and tail (t). (B) Nucleus (n) stained with DAPI. (C–E) Section of the head region showing the acrosomal vesicle (a), the perforatorium (p) inserted in a deep nuclear cavity and the filaments (f) radiating from the extracellular sheath (s). (F) Section of the nucleus–flagellum transition region showing centriolar adjunct (ca), centriole (c) and axoneme (ax). (G) Section of two nuclei. (H–J) Sections of the nucleus–flagellum transition region at different levels. Note in (J) the anterior tip of the mitochondrial derivatives (m). (K) A longitudinally sectioned flagellum showing the mitochondrial derivatives coiling around the axoneme. (L) Sperm at different levels. Note the presence of two mitochondrial derivatives in all flagella. (M) Middle region of the flagellum showing the axoneme formed of double external microtubules (curved arrow), a central pair (small arrows), and accessories tubules (arrowhead). The large arrows indicate the two accessory bodies. (N–P) Sections showing the flagella tips where one mitochondrial derivative (m) terminates before the other and both before the axoneme. In the latter, the accessory microtubules (arrowheads) are first to finish. Bars: A and B = 10 μm ; C–J and M–P = 0.1 μm ; K and L = 0.5 μm .

It overlaps only the centriole region in *Pegoscopus*, but in *Idarnes* it runs parallel to mitochondrial derivatives by at least 4 μm .

Although the axoneme with 9+9+2 microtubules is observed in many insects, changes in this pattern are commonly observed, which may represent important characters for systematics of these organisms. For example, transverse striations along the center of the axoneme, as occur in *T. diatraeae*, have never been observed in any other Hymenoptera including chalcidoids, this striations may constitute a unique characteristic for the species or genus. However, whether these striations are modifications of one existing flagellar components as the central sheath, and how they combine with other elements in the central region of the axoneme, are issues that need further investigation.

In most chalcidoids, including these two species, accessory microtubules are the first to finish, followed by the central pair

and the peripheral double. However, in *Pegoscopus* (Fiorillo et al., 2008), the central pair is the first to end. The last peripheral double ending differentiates chalcidoids as well as two ichneumonids species (Moreira et al., 2010), of the Aculeata, in which the last to finish are accessory microtubules (see Zama et al., 2005).

In these two eulophid species, the mitochondrial derivatives, in cross-section, are symmetrical, slightly oval and smaller than, and very closed to, axoneme. These same features are the same ones showed in Eurytomidae (Lino-Neto et al., 1999) and Trichogrammatidae (Lino-Neto et al., 2000; Lino-Neto and Dolder, 2001), indicating that these three families are closely related. However, mitochondrial derivatives of *M. hawaiiensis* and *M. australica* (Brito et al., 2009) are asymmetric, possibly representing a unique characteristic for this eulophid genus. In *Pegoscopus* (Fiorillo et al., 2008), derivatives differ from those of the three families mentioned

Fig. 2. Light (A and B) and transmission electron (C–Q) photomicrographs of spermatozoa from *T. diatraeae* in longitudinal (C, D, G–I, P and Q) and transversal (E, F and J–O) sections. (A) Sperm in phase contrast. The arrow indicates the limit between the head (h) and tail (t). (B) Nucleus (n) stained with DAPI. (C–F) Section of the head region showing the acrosomal vesicle (a) supported at the nuclear tip (arrow) and the filaments (f) radiating from the extracellular sheath (s). (G–K) Sections of a nuclear region (G) and nucleus–flagellum transition showing centriole (c), centriolar adjunct (ca) and mitochondrial derivatives (m) coiling around the axoneme (ax). Note the presence of transverse striations (arrows) throughout the central region of the axoneme. (L–Q) Sections of flagella at different levels. Note that it is common flagella with only one (large arrow) or no (arrowheads) mitochondrial derivative. Also note that the central pair of microtubules (arrows in O) is rarely observed and the accessory microtubules are first to finish (M). Bars: A and B = 10 μ m; C–F and I–O = 0.1 μ m; G, H, P and Q = 0.5 μ m.

above only by being slightly asymmetrical. In *Idarnes* (Silva, 2010), they are larger in diameter and completely surrounded by the axoneme, indicating that these two agaonid genera are distantly related.

Accessory bodies are very reduced (as in *P. elaeisis*) or possibly absent in some species (as in *T. diatraeae*), characteristics that differentiate chalcidoids from most Hymenoptera (Zama et al., 2005; Araújo et al., 2009; Moreira et al., 2012).

In conclusion, the morphological characteristics of chalcidoid sperm indicate that Eulophidae is closely related to Trichogrammatidae, as was observed by Heraty et al. (2012), and both of these families are closer to Eurytomidae than Agaonidae. In Agaonidae, *Pegoscopus* sperm are more similar to those three families above than *Idarnes*.

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