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Redescription of sperm structure and ultrastructure of *Trichogramma dendrolimi* (Hymenoptera: Chalcidoidea: Trichogrammatidae)

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Abstract

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To verify the questionable description of sperm structure of *Trichogramma dendrolimi*, in relation to Chalcidoids, a reinvestigation was undertaken. The spermatozoa appear wavy along their entire length. A small acrosome, together with the anterior nuclear region, is surrounded by an extracellular sheath, from which filaments radiate. The nucleus is helicoidal and attached to the flagellum by a centriolar adjunct. The axoneme has the 9 + 9 + 2 microtubule arrangement pitched in a long helix, with the spiralling mitochondrial derivatives coiling around it. Therefore, the spermatozoa of *T. dendrolimi* are very similar to those of other chalcidoids and different from the first description. It is now possible to affirm that the helical sperm structures are an apomorphic homology for Trichogrammatidae as well as Eulophidae, Pteromalidae, Eurytomidae, Torymidae, Mymaridae and for some other chalcidoids, if not all.

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Introduction

The Chalcidoidea is the second most species-rich superfamily of Hymenoptera and biologically it is one of the most diverse (Hanson and LaSalle 1995). It accounts for roughly one-third of the world's parasitic species of Hymenoptera (LaSalle and Gauld 1991). Most Chalcidoidea are parasites of other insects and they have been used extensively in the biological control of insect pests (Grissell and Schauff 1997). According to Greathead (1986), the majority of the successful biological control projects of insect pests have used chalcidoids to achieve substantial or complete control.

In spite of the considerable importance to applied entomology, the knowledge of evolutionary relationships among chalcidoids is in many respects the same today as it was in the early 1900s (Grissell and Schauff 1997), perhaps because this group includes some of the smallest insects, such as Trichogrammatidae at 0.2–1.5 mm. There is still disagreement as to the placement of several subfamilies and genera. According to Heraty *et al.* (1997) new character systems are needed to resolve the relationships among families and subfamilies of Chalcidoidea. However, very little has so far been done and the pattern of relationships is still vague for most groups (Grissell and Schauff 1997).

The ultrastructure of the spermatozoa has been extensively used in taxonomic and phylogenetic studies of various animal groups, including the insects (see Baccetti 1972; Dallai 1979; Dallai and Afzelius 1990, 1995; Carcupino *et al.* 1995; Jamieson *et al.* 1999). In Chalcidoidea, the spermatozoa present sufficient ultrastructural diversity to furnish a character system (Quicke *et al.* 1992; Lino-Neto *et al.* 1999, 2000a). This system, associated with other character systems, may be used as a basis for phylogeny, as well as resolving some uncertainty about the relationships among families and genera. However, to apply these characters to phylogenetic studies, the structures must be positively identified so that homology can be correctly established.

The typical hymenopteran sperm, as in most insects (Phillips 1970), is long, ranging from approximately 40 μm to 250 μm in length (Quicke 1997). It is comprises an anterior region, called the head, and a posterior region, the flagellum. The

head includes an anterior acrosomal complex, followed by the nucleus. The flagellum, in most hymenopterans, is formed by an axoneme with a 9 + 9 + 2 microtubule arrangement, two mitochondrial derivatives that are alike or of unequal diameter and two accessory bodies. The basic structure of the spermatozoa known for chalcidoids is similar to that of the rest of the hymenopterans, but can be distinguished by the mitochondrial derivatives following a spiral course around a similarly twisted axoneme. The nucleus is often also spirally twisted (Lee and Wilkes 1965; Wilkes and Lee 1965; Hogge and King 1975; Quicke *et al.* 1992; Lino-Neto *et al.* 1999, 2000a).

In the present work we describe the structure and ultrastructure of the spermatozoa of *Trichogramma dendrolimi*, which contrast with the previous description of Lingmei and Dunsu (1987). Here we show that the sperm ultrastructure of this species is basically the same as other Chalcidoidea and widely different from the ultrastructure presented by those authors.

Materials and Methods

Adult virgin males of *Trichogramma dendrolimi* Matsumura, 1925 were obtained from colonies maintained in the Laboratoire de Biologie Appliquée, INSA, Institut National de la Recherche Agronomique, Lyon, France. Voucher specimens, mounted on glass microscope slides, have been deposited in the entomological collection of the Entomology Department of the 'Luiz de Queiroz' Agronomic School (ESALQ/USP), Piracicaba, SP, Brazil.

Light microscopy

Seminal vesicles were dissected and broken open on clean glass microscope slides, where spermatozoa were spread and fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. After drying at room temperature, the preparations were observed with a photomicroscope (Olympus, BX60) equipped with phase contrast.

To measure the nucleus, some of these preparations were stained for 15 min with 0.2 µg/mL 4,6-diamino-2-phenylindole (DAPI) in phosphate-buffered saline, washed and mounted with Vectashield. They were examined with an epifluorescence microscope (Olympus, BX60), equipped with a BP360–370 nm excitation filter.

Scanning electron microscopy

Spermatozoa from the seminal vesicle were spread on a coverslip, fixed in 2.5% glutaraldehyde, dehydrated in acetone, critical-point-dried and sputter-coated with gold. They were observed with a scanning electron microscope, JEOL JSM5800LV.

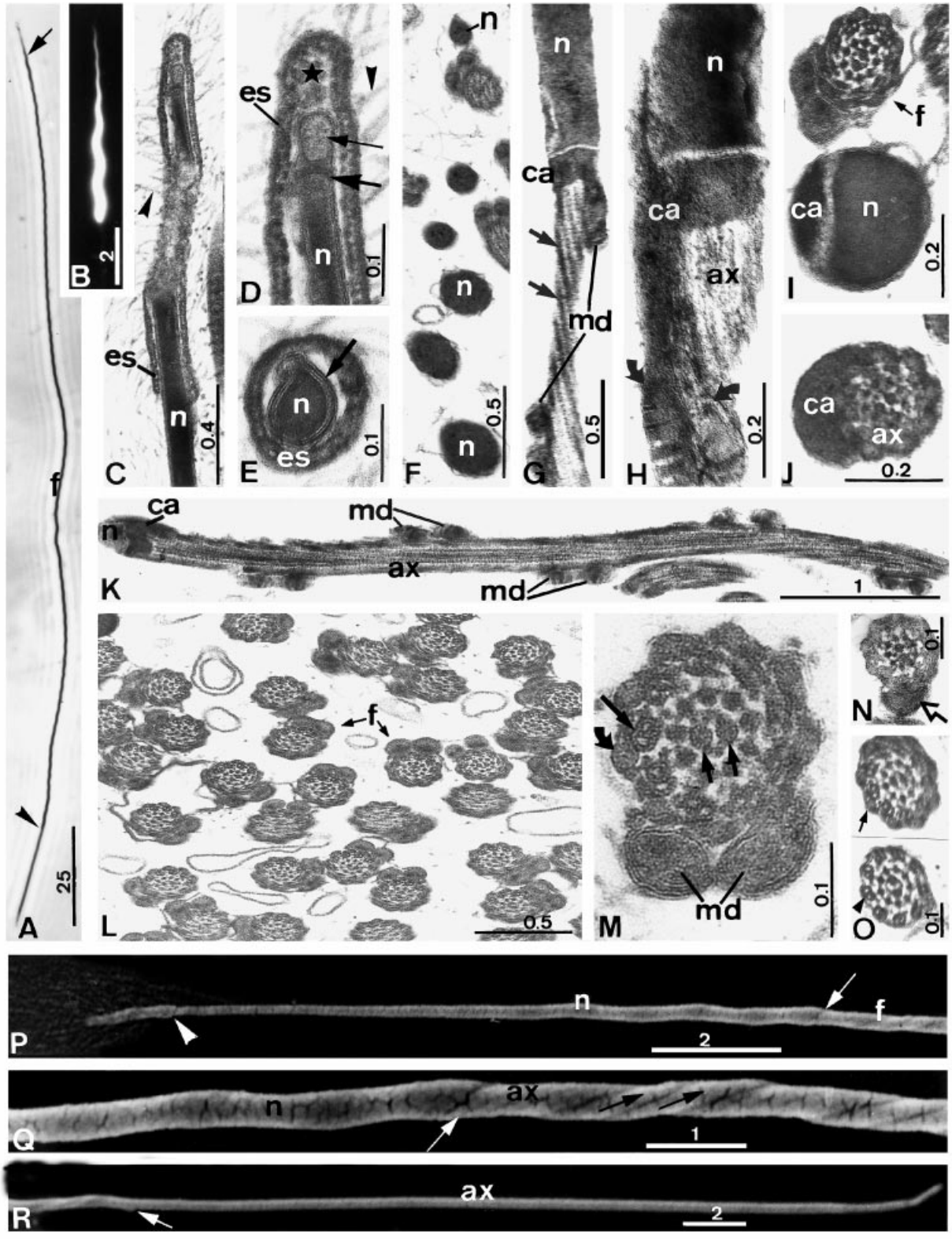
Transmission electron microscopy

Seminal vesicles were dissected and fixed in a mixture of 2.5% glutaraldehyde, 1% tannic acid, 1.8% sucrose in 0.1 M phosphate buffer followed by block staining in 1% uranyl acetate in distilled water (Afzelius 1988). After dehydrating in acetone and embedding in Epon 812 resin, the specimens were sectioned, the sections were stained with uranyl acetate and lead citrate and were examined in a transmission electron microscope (Zeiss, Leo 906), operating at 40 or 80 kV.

Results

The spermatozoa of *Trichogramma dendrolimi* are long and slender, measuring about 0.32 µm by 238 µm and under phase contrast microscopy they appear wavy along their entire length (Fig. 1A). The acrosomal complex is very small, measuring about 1.5 µm in length (Fig. 1C,P). It is formed by a small structure with a diameter equal to that of the nucleus at their junction (smaller arrow in Fig. 1D), where it fits with a concave base onto the rounded nuclear tip (larger arrow in Fig. 1D). In the sections we obtained of this region, it was not possible to clearly identify this structure as being a perforatorium or an acrosomal vesicle. This structure,

Fig. 1—**A**, Light micrographs of spermatozoa. The arrow indicates the head and the arrowhead, the posterior end of the mitochondrial derivatives. —**B**, DAPI stained fluorescence of the helicoidal nucleus. —**C**, Longitudinal sections of the acrosomal complex showing the filaments irradiating (arrowhead) from the extracellular sheath(es). —**D**, High power image of a longitudinal section through of the anterior acrosomal region. The larger arrow indicates the junction of nucleus (n) and acrosomal vesicle (or perforatorium) (smaller arrow). The arrowhead indicates the filaments and a star, the inside region of the extracellular sheath tip. —**E**, Cross-section of the acrosomal complex region. The arrow shows the cellular and nuclear membranes. —**F**, Cross-section of nuclei at different levels. —**G**, **H**, Longitudinal sections of the nucleus–flagellum transition region. Arrows indicate the axonemal microtubules and curved arrows, the anterior tip of the mitochondrial derivatives. —**I**, Cross-section of the nuclear base. —**J**, Cross-section of the axonemal anterior extremity at the level of the centriolar adjunct. —**K**, Anterior tip of a longitudinally sectioned flagellum. —**L**, Various cross-sectioned flagella. —**M**, Cross-section of a flagellum with high magnification showing the axoneme made up of nine doublets (longer arrow), a central pair (shorter arrows) and accessory tubules (curved arrow). —**N**, **O**, Cross-sections of the posterior flagellar region. Observe that one mitochondrial derivative is longer (open arrow) and the accessory microtubules (arrow) finish before the doublets (arrowhead). —**P**, **R**, Scanning electron micrographs of the head, of the nucleus–flagellum transition and of the flagellar extremity, respectively. Arrowhead indicates the acrosomal inferior limit, the white arrows indicate the nucleus–flagellum transition, the black arrows, the mitochondrial derivatives and the shorter arrow, the posterior end of the mitochondrial derivatives. Abbreviations: ax = axoneme; c = centriolar adjunct; es = extracellular sheath; f = flagellum; md = mitochondrial derivatives; n = nucleus. Scale bars in µm.



as well as the anterior nuclear region (about 1.3 μm), is surrounded by an extracellular sheath, from which numerous filaments arise (Fig. 1C,D). The extracellular sheath extends anteriorly producing a small space, which is filled by a roughly granular material (star in Fig. 1D). The nucleus measures about 16 μm in length, it is twisted in a helix (Fig. 1B,P) and completely filled with homogeneous, compact chromatin (Fig. 1C–I). It is circular in cross-section (Fig. 1F), but in the anterior region, where it is covered by the extracellular sheath, it has a pear-shaped cross-section (Fig. 1E). The nucleus tapers more strongly from the mid-region towards the apex (Fig. 1B). Its posterior, truncated extremity, with a diameter of approximately 270 nm, is flattened on one side where an anterior projection of the centriolar adjunct fits tightly (Fig. 1G–I). The nucleus is attached to the flagellum by a centriolar adjunct that is electron dense, forming a 160-nm thick disk between nucleus and axoneme with an anterior projection extending 400 nm, juxtaposed basolaterally to the nucleus. A posterior expansion of this adjunct extends approximately 320 nm and is intimately associated with the axoneme's anterior tip (Fig. 1G–K). The flagellum consists of an axoneme and a pair of mitochondrial derivatives (Fig. 1K–M). The axoneme follows the 9 + 9 + 2 microtubule arrangement, including nine outer accessory tubules, nine doublets and two central single microtubules (Fig. 1M). The microtubules are twisted, as can be clearly observed in cross-sections, since not all of the doublets can be sectioned at perfect right angles (Fig. 1L–O), and in longitudinal sections (Fig. 1G,K). Anteriorly, the axoneme begins just below the nuclear base with the microtubules inserted in the centriolar adjunct (Fig. 1G–H,K). Posteriorly, the accessory microtubules terminate before the other microtubules (Fig. 1O). The mitochondrial derivatives are alike in cross-section, oval, measuring on average 100 nm in the longer diameter and are placed very close to the axoneme (Fig. 1K–N,Q). In longitudinal sections as well as in scanning electron micrographs, they can be seen coiling regularly around the axoneme (Fig. 1G,K,Q). Anteriorly, the mitochondrial derivatives begin together in contact with the posterior extremity of the centriolar adjunct, approximately 480 nm from the nuclear base (Fig. 1H). In the final flagellar region, one mitochondrial derivative terminates shortly before the other (Fig. 1N), about 28 μm above the axoneme tip (Fig. 1O,R). Typical accessory bodies, as found in most Hymenoptera, do not occur (Fig. 1L,M).

Discussion

The ultrastructure of the spermatozoon of *Trichogramma dendrolimi* is basically the same as that of the majority of the Hymenoptera (Quicke *et al.* 1992; Newman and Quicke 1998; 1999a,b). In all species of chalcidoids in which the sperm ultrastructure has already been described (Lee and Wilkes 1965; Wilkes and Lee 1965; Hogge and King 1975; Quicke *et al.* 1992; Lino-Neto *et al.* 1999, 2000a), the nucleus is twisted into a helix and the mitochondrial derivatives coil around the twisted axoneme.

The presence of an extracellular sheath surrounding the true acrosome and an anterior region of the nucleus has also been described for the chalcidoids, *Nasonia vitripennis* (Hogge and King 1975), *Bephratelloides pomorum* (Lino-Neto *et al.* 1999), *Trichogramma atopovirilia* and *T. pretiosum* (Lino-Neto *et al.* 2000a) and many other wasp groups (Quicke *et al.* 1992; Newman and Quicke 1998; 1999b). To date, however, only in chalcidoids has the presence of numerous filaments arising from the extracellular sheath been clearly demonstrated (Lino-Neto *et al.* 1999, 2000a).

In most hymenopterans the acrosomal complex is basically formed by a cone-shaped acrosomal vesicle surrounding a cavity which holds the perforatorium. This structure, as a rule, is inserted in a cavity of the nuclear tip (Quicke 1997). However, in this species, as in *B. pomorum* (Lino-Neto *et al.* 1999), besides the extracellular sheath, only one other small structure has been found, which has a concave base fitting onto the rounded nuclear tip. In *B. pomorum* (Lino-Neto *et al.* 1999), it was identified as perforatorium. In spite of the fact that it is still not possible to define this structure as being a true perforatorium or an acrosomal vesicle, we now believe that it probably corresponds to the latter. In any case, one of the two structures is, apparently, absent and this may be another characteristic that differentiates chalcidoids from most hymenopterans, although Newman and Quicke (1999a) indicate 'the apparent absence of the acrosomal rod' (perforatorium) in the xyeloid, *Xyela julli* (Symphyta).

The mitochondrial derivatives in *T. dendrolimi*, as in the rest of the chalcidoids (Quicke *et al.* 1992; Lino-Neto *et al.* 1999; 2000a), are oval, alike in diameter, lie very close to the axoneme and coil regularly around the axoneme. This characteristic differs from other Hymenoptera. In the latter, the mitochondrial derivatives are straight, more or less circular or, when oval, extend outward from the axoneme (Cruz-Höfling *et al.* 1970; Cruz-Landim and Silva de Moraes 1980; Wheeler *et al.* 1990; Quicke *et al.* 1992; Newman and Quicke 1998; 1999a,b; Lino-Neto *et al.* 2000b). The mitochondrial derivatives beginning together at a small distance from the nucleus and in contact with the posterior base of the centriolar adjunct have been observed in chalcidoids (Lino-Neto *et al.* 1999, 2000a), including this species, as well as in the siricoid, *Tremex* sp. (Newman and Quicke 1999a) and, apparently, in ants (Wheeler *et al.* 1990). In the rest of the hymenopterans where this characteristic has been observed the mitochondrial derivatives began one after the other, that is, one in contact with the centriolar adjunct and the other, more anteriorly, in contact with the nuclear base (e.g. the pamphiliid, *Cephalcia arvensis*, Newman and Quicke 1999a) or above the nuclear base (e.g. *Leptopilina heterotoma*, Newman and Quicke 1999b).

The axoneme begins in all the hymenopterans examined to date directly below the nuclear base, either juxtaposed to the nucleus, as described in chalcidoids (Lino-Neto *et al.*

1999, 2000a) including the species studied here, or the initial portion of the axonemal microtubules surrounds the strongly tapered nuclear base, as occurs in *Apis mellifera* (Hoage and Kessel 1968; Lino-Neto *et al.* 2000b). No description has been found in which the axoneme begins laterally in relation to the nucleus, as in Lingmei and Dunsu (1987).

According to Jamieson *et al.* (1999), the centriolar adjunct usually surrounds asymmetrically the base of the mitochondrial derivatives and the axoneme where all of these structures attach to the posterior end of the nucleus. In the other hymenopterans where this structure has been observed (Wheeler *et al.* 1990; Newman and Quicke 1998; 1999a,b; Lino-Neto *et al.* 2000b), it is located only between the nuclear base and one or both mitochondrial derivatives. Still, in these hymenopterans, the anterior extremities of the axonemal microtubules are parallel to the adjunct and not inserted into it, as in chalcidoids (Lino-Neto *et al.* 1999; 2000a).

Lingmei and Dunsu (1987) observed the following structural characteristics in the spermatozoa which they described as *T. dendrolimi*. A total length of approximately 110 μm, of which the nucleus occupied about 7 μm and the flagellum, 100 μm. A well-developed acrosome, lying laterally in relation to the nucleus. The mitochondrial derivatives and axoneme also lie parallel to the nucleus and the first extend as far as the acrosome, although no mitochondrial derivative can be observed in the micrographs of the basal nuclear region viewed in cross section (Fig. 2b,c in Lingmei and Dunsu 1987). Also, micrographs of flagella in cross-sections show the mitochondrial derivatives as being approximately triangular, not compressed to the axoneme. The accessory bodies are clearly visible and no helicoidally twisted structure was found (see Figs 3, 4 in Lingmei and Dunsu 1987).

Therefore, as we suggested previously (Lino-Neto *et al.* 2000a), the first description appears to be mistaken, since spermatozoa with such structural characteristics cannot belong to *T. dendrolimi*, which we describe in this study. It is possible that the specimens used by these authors did not even belong to the order Hymenoptera, since the ultrastructural characteristics observed by the authors are completely different from those observed in all other known hymenopteran species. This is true even for Symphyta (Newman and Quicke 1999a), which is considered the most primitive hymenopteran taxon.

Quicke *et al.* (1992), considering the article of Lingmei and Dunsu (1987), described the absence of a spiralling structure in Trichogrammatidae as either plesiomorphous or as a character reversal. However, based on these results, as well as those presented by Lino-Neto *et al.* (2000a), we believe that the spiralling of the nucleus and of the mitochondrial derivatives as well as the twisting of the axonemal microtubules are synapomorphies for the Trichogrammatidae as well as for the Eulophidae (Lee and Wilkes 1965; Wilkes and Lee 1965), Pteromalidae (Hogge and King 1975), Eurytomidae (Quicke *et al.* 1992; Lino-Neto *et al.* 1999) and Aphelinidae (Quicke 1997). Besides these five chalcidoid families, this spiralling is probably also a synapomorphy for species of the Chalcididae, Encyrtidae, Agaonidae, Torymidae and Mymaridae families, in which we have observed, with the light microscope, typical twisted spermatozoa.

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