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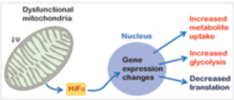

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Ultrastructural features of spermatozoa and their phylogenetic application in Zaprionus (Diptera, Drosophilidae)

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ABSTRACT

The genus *Zaprionus* consists of approximately 60 species of drosophilids that are native to the Afrotropical region. The phylogenetic position of *Zaprionus* within the Drosophilidae family is still unresolved. In the present study, ultrastructural features of spermatozoa of 6 species of *Zaprionus* as well as the species *Drosophila willistoni* and *Scaptodrosophila latifasciaeformis* were analyzed. The ultrastructure revealed that the species have the same flagellar ultrastructure. Two mitochondrial derivatives, one larger than the other, close to the axoneme were present, primarily in *D. willistoni* (subgenus Sophophora). Except for *Z. davidi* and *Z. tuberculatus*, the analyzed species had paracrystalline material in both mitochondrial derivatives. Moreover, the testes showed 64 spermatozoa per bundle in all of the species. In the cluster analysis, 6 *Zaprionus* species were grouped closely, but there were some incongruent positions in the cladogram. The results indicated that sperm ultrastructure is an important tool for elucidating the phylogeny and taxonomy of insects.

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Mitochondrial derivatives; spermatogenesis cystic; spermiogenesis

Introduction

Approximately 150,000 species of flies are described in the world and more than 24,000 species are described in the Neotropics.[1] The genus *Zaprionus* (Diptera, Drosophilidae) consists of approximately 60 species of which about 10 are grouped in the subgenus *Anaprionus* and 50 in the subgenus *Zaprionus*.[2,3] Phylogenetic relationships within groups and subgroups of drosophilids as well as the phylogenetic position of *Zaprionus* within the Drosophilidae family are still uncertain.[3-8] Although most phylogenetic analyses associate the genus *Zaprionus* to the subgenus *Drosophila*, new comparative analyses are needed to test the robustness of this association.[3,7,9]

Ultrastructural sperm analyses are important tools for study of the taxonomy and phylogeny of insects.[10-16] Mojica et al. characterized the primary evolutionary radiation that occurred in the *Drosophila* tripunctata group based on the ultrastructure of the mitochondrial derivatives and the number of sperm per cysts. The authors highlighted the need for new ultrastructural studies of the gametes of these insects to provide additional clarification of their evolutionary relationships.[17]

Ultrastructural analyses of sperm in *Zaprionus* are restricted to the species *Zaprionus indianus* and *Zaprionus sepsoides*.[18] The authors described important characteristics of these drosophilid gametes, such as the presence of granules in the peritoneal sheath, the presence of 2 mitochondrial derivatives of different sizes, the presence or absence of paracrystalline material in the derivatives, the arrangement of the axoneme, and the number of sperm per cyst.

This study aimed to characterize the ultrastructure of sperm of 6 other species of *Zaprionus* (*Z. africanus*, *Z. camerounensis*, *Z. davidi*, *Z. gabonicus*, *Z. megalorchis* and *Z. tuberculatus*) and the species *Drosophila willistoni* (subgenus *Sophophora*). *Scaptodrosophila latifasciaeformis* (subgenus *Scaptodrosophila*) was

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Table 1. Studied species and their geographical origin.

Subgenus	Group	Complex	Species	Geographic location of the collection
Zaprionus	vittiger	indianus	Zaprionus africanus	Kibale (Uganda)
Zaprionus	vittiger	indianus	Zaprionus gabonicus	Makokou (Gabão)
Zaprionus	vittiger	indianus	Zaprionus megalorchis	Congo
Zaprionus	vittiger	vittiger	Zaprionus camerounensis	Amani (Tanzânea)
Zaprionus	vittiger	davidi	Zaprionus davidi	São Tomé (São Tomé)
Zaprionus	tuberculatus	tuberculatus	Zaprionus tuberculatus	Ithala (South Africa)
Sophophora	—	willistoni	Drosophila willistoni	Matão/SP (Brazil)
Scaptodrosophila	—	—	Scaptodrosophila latifasciaeformis	São José do Rio Preto/SP (Brazil)

used as an outgroup (Table 1). In addition, data from the literature for other species of *Zaprionus* and *Drosophila* were used to help understand the relationships between *Zaprionus* and *Drosophila*.

Results and discussion

In this paper, intraspecific variation in spermatogenesis was not observed. All species showed globular granules in the cytoplasm of the coating layer of the testicular envelope, called the peritoneal sheath (Fig. 1A, Supplementary Figs. 1–6), except for *Z. davidi* (Fig. 1B, Supplementary Fig. 7) and *D. willistoni* (Supplementary Fig. 8). These pigmented granules are responsible for the color of the peritoneal coating sheath of the testes and for the formation of a physical barrier that can protect the testes and store nutrients.[19] Rego et al. detected the presence of glycogen in the composition of these granules. Cruz-Landim has also observed glycogen in the testicles of bees.[18,20]

The color of the peritoneal sheath of the testes is critically important for taxonomy. In the genus *Drosophila*, the color is diagnostic to the species level.[21] The peritoneal sheaths of the *Zaprionus* species analyzed in this study and those of *D. willistoni* and *S. latifasciaeformis* showed yellowing. Yellowing has also been observed in the sheaths of Z. *indianus, Z. sepsoides* and Z. *spinipilus* that were analyzed previously.[18,22,23] However, *Z. vittiger* was polymorphic for sheath color, which may be yellow or brownish purple.[23]

The sperm of Pterygota (a primitive group of Insecta) has 2 mitochondrial derivatives that flank the axial filament.[24] In drosophilids, the mitochondrial derivatives are of different sizes (Table 2).[10,17,25-28] Mojica et al. used the size of these mitochondrial derivatives as an evolutionary tool to understand the radiation of the genus *Drosophila*.[17] They observed that the 2 derivatives differed in size and that this size difference was greater in *Sophophora* than in *Drosophila*. In the present study, although measurements were not taken, the simple observation of prints showed that our results are consistent with those of Mojica et al.: the difference in the size of these mitochondrial derivatives was greater in *D. willistoni* (which belongs to the *Sophophora* subgenus) (Fig. 2B) than in the other species analyzed (Figs. 2A and 3A-C). The exception was *Z. davidi*, which was similar to *D. willistoni*. Mojica et al. suggested that the relative size of the mitochondrial derivatives may have changed as *Drosophila* species have evolved.

Except for Z. *davidi* and Z. *tuberculatus* (Figs. 2C and 3B), the analyzed species have paracrystalline

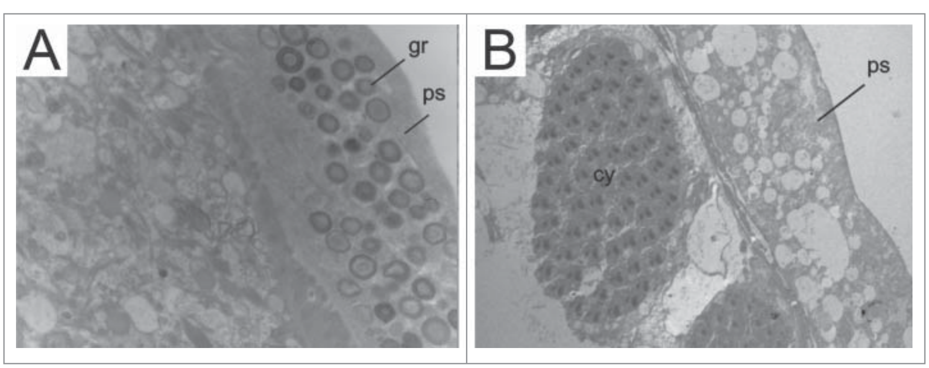

Figure 1. TEM micrographs of *Zaprionus* testes. A. *Z. africanus*; B. Z. *davidi*. Note peritoneal sheath (ps) filled with granules (gr) of different sizes and electron densities in Z. *africanus* and their absence in Z. *davidi*. Scale: Figure A: 11000 x; Figure B: 10000 x.

Table 2. Ultrastructural parameters of sperm used for comparisons of species.

Species	Granules	Mitochondrial derivatives		Paracrystalline material		Axoneme	64 sperm
		Same size	Different size	Largest derivative	Smallest derivative	9 + 9 + 2	per cyst
Zaprionus africanus	+	−	+	+	+	+	+
Zaprionus camerounensis	+	−	+	+	+	+	+
Zaprionus davidi	−	−	+	−	−	+	+
Zaprionus gabonicus	+	−	+	+	+	+	+
Zaprionus megalorchis	+	−	+	+	+	+	+
Zaprionus tuberculatus	+	−	+	−	−	+	+
*Zaprionus indianus**1	+	−	+	+	+	+	+
*Zaprionus sepsoides**1	+	−	+	+	+	+	+
Drosophila willistoni	−	−	+	+	+	+	+
*Drosophila melanogaster**2,3	na	−	+	+	−	+	+
*Drosophila simulans**4	na	−	+	+	−	+	+
*Drosophila cardini**2	na	−	+	+	−	+	−
*Drosophila dunni**2	na	−	+	+	−	+	−
*Drosophila hydei**2,5	na	−	+	+	+	+	−
*Drosophila subobscura**6,7	na	−	+	−	−	+	−
Scaptodrosophila latifasciaeformis	+	−	+	+	+	+	+

Note. *1Rego et al. (2013), *2Mojica et al. (2000), *3Noguchi and Miller (2003), *4Pasini et al. (1996), *5 Henning (1992), *6Ramamurthy et al. (1980), *7Hauschteck-Junguen and Maurer (1976), na: not analyzed.

material on both mitochondrial derivatives (Figs. 2A, 3A and C). This same characteristic has been observed in Z. *indianus*, Z. *sepsoides* and *D. hydei*.[17,18] However, in most species of the genus *Drosophila* (Table 2), the paracrystalline material is present only in the larger mitochondrial derivative.[17,29-32]

The structure of the sperm axoneme is of great importance for phylogenetic studies in insects.[10,14,27]

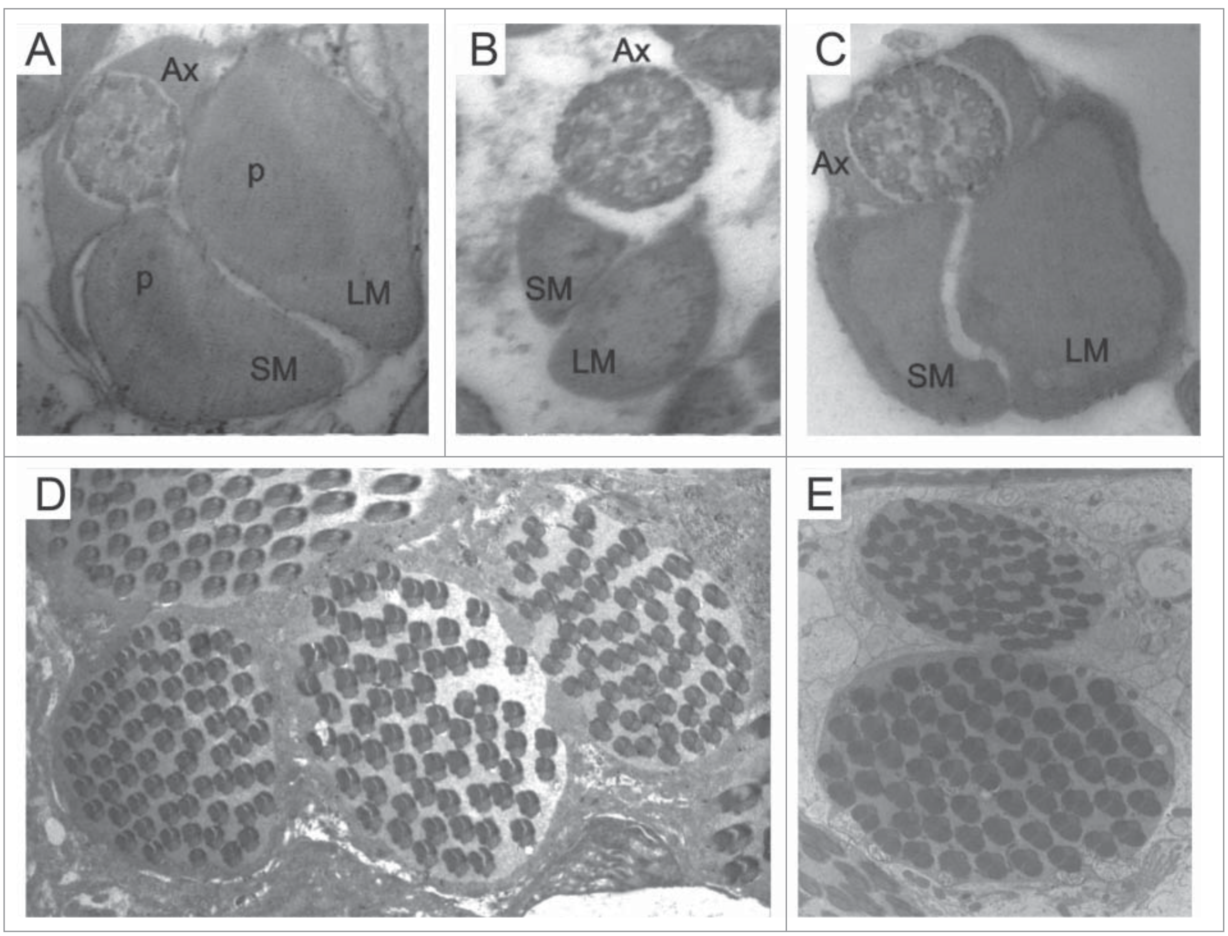

Figure 2. Ultrastructure of transverse sections of the spermatozoal tail of *Z. gabonicus* (A) showing the paracrystalline material (p) on both mitochondrial derivatives; the axonemes in *D. willistoni* (B) and *Z. davidi* (C) have the arrangement of 9 + 9 + 2 microtubules; the cysts containing 64 spermatozoa in *Z. camerounensis* (D) and *Z. tuberculatus* (E). Scale: Figures A, B, C: 84000 x; Figures D, E: 10000 x.

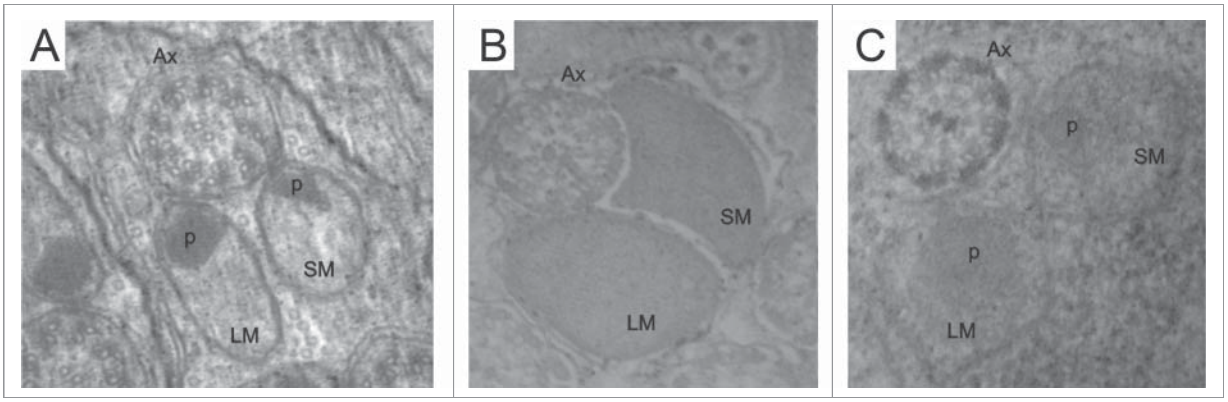

Figure 3. Ultrastructure of transverse section of the spermatozoal tail of *S. latifasciaeformis* (A), *Z. tuberculatus* (B) and Z. *megalorchis* (C) Note the presence of the axoneme (Ax) and 2 mitochondrial derivatives of different sizes: larger mitocondrial derivative (LM), smaller mitochondrial derivative (SM); the accumulation of paracrystalline material (p) is visible in *S. latifasciaeformis* and *Z megalorchis*. Scale: Figures A, B, C: 84000 x.

Most species of insects present the '9 + 9 + 2' arrangement, consisting of one pair of central microtubules and 9 double peripheral microtubules, surrounded by 9 additional accessory microtubules,[10,27] although some species have a peculiar number, such as 9 + 9 + 3 in Neuroptera,[33] 9 + 9 + 1 in Culicidae (Diptera),[34] 9 + 7 in Tricoptera,[25] and 9 + 0 in Ephemeroptera.[25] Moreover, as the majority of species of the suborder Brachyocera,[27] all drosophilids species analyzed had an axoneme structure of the 9 + 9 + 2 configuration, which is the typical arrangement of 9 + 2 internal microtubules surrounded by 9 additional accessories microtubules (Figs. 2A–C and 3A–C) (Table 2).

Spermiogenesis in all analyzed species occurs within cysts where the sperm are organized and exist at the same developmental stage (Fig. 2D). This phenomenon is referred to as cystic spermatogenesis and is characterized by synchronized cell division within a given cyst.[35] So far, studies have indicated that all insects have cystic spermatogenesis.[36] In Triatominae, the cysts develop independently; that is, a cyst does not influence the developmental stage of neighboring

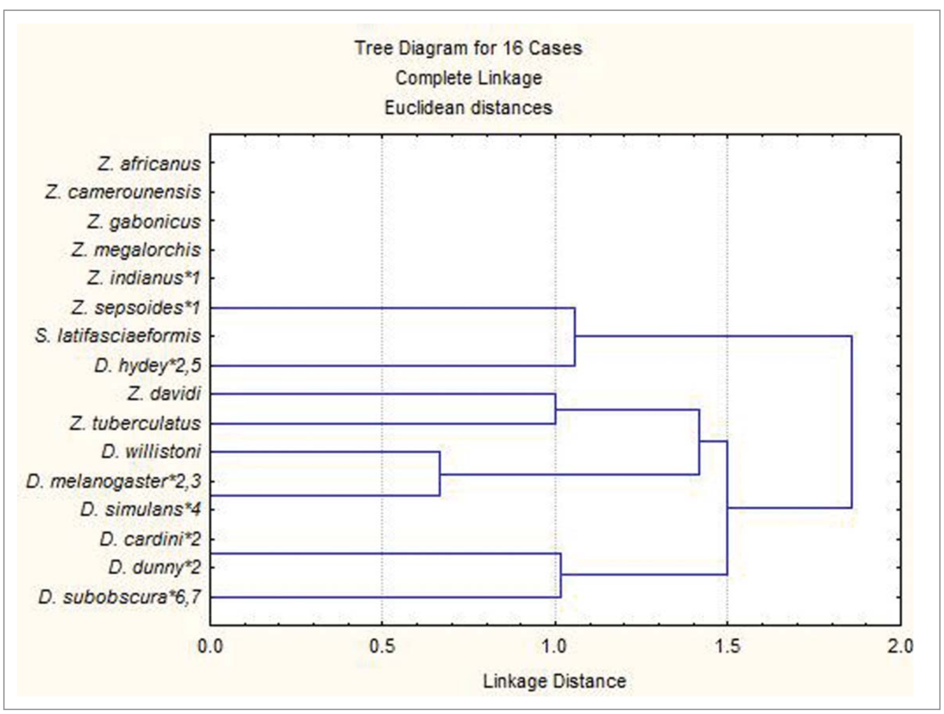

Figure 4. Cladogram obtained from cluster analysis using a presence-absence matrix for the characteristics analyzed in the present study and taken from literature.

cysts.[37] In drosophilids, as described for *Plalycentropus* (Trichoptera: Limnephilidae),[25] we suggest that neighboring cysts are also synchronized for cystic spermatogenesis (Fig. 2D and E).

For all of the species analyzed, we observed the presence of 64 cells inside a cyst (Fig. 2D and E) (Table 2). The number of cyst cells varies in some species of *Drosophila*. In *D. dunni*, this number varies from 44 to 56 sperm per cyst; in D. *cardini*, it varies from 36 to 40 sperm per cyst; in *D. melanogaster*, this fixed number is 64; and in *D. subobscura*, this number can reach up to 128 sperm per cyst.[17,26,28,29]

In the cluster analysis, on the basis of the analyzed characteristics, 5 species of *Zaprionus* as well as 6 species of *Drosophila* were grouped, suggesting their relatedness within each genus (Fig. 4). Although an increase in the number of characteristics and species is necessary to validate these results, they are already indicative that the ultrastructure of sperm is a promising tool for phylogenetic and taxonomic studies of insect groups.

Materials and methods

The species and strains of *Zaprionus* and other species used in this study and their geographic location are shown in Table 1. The testes of 24 3-day-old adults of each species were processed according to the methods of Cotta-Pereira et al. with modifications.[38] Ultrathin sections of 70 nm, contrasted with uranyl acetate and lead citrate, were examined with a transmission electron microscope.

The five ultrastructural parameters of sperm used by Rego et al. were used in this study for comparison between species (Table 2).[18]

A cluster analysis was conducted using the Euclidean distance and joining method (Statistica; Statsoft Inc.) with the data from Table 2 from which a presence-absence matrix of the characteristics was generated.[39]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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