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. davi and Z. tuberculatus, the analyzed species had paracrystalline material in both mitochondrial derivatives. Moreover, the tests 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.

Introduction

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

Ultrastructural sperm analyses are important tools for study of the taxonomy and phylogeny of insects. 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.

Ultrastructural analyses of sperm in Zaprionus are restricted to the species Zaprionus indianus and Zaprionus sepsoides. 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. davi, Z. gabonicus, Z. megalorchis and Z. tuberculatus) and the species Drosophila willistoni (subgenus Sophophora). Scaptodrosophila latifasciaeformis (subgenus Scaptodrosophila) was...
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. 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.

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. 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. However, Z. vittiger was polymorphic for sheath color, which may be yellow or brownish purple.

The sperm of Pterygota (a primitive group of Insecta) has 2 mitochondrial derivatives that flank the axial filament. Mojica et al. used the size of these mitochondrial derivatives as an evolutionary tool to understand the radiation of the genus Drosophila. 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) 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

| 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  | vittiger| Zaprionus megalorchis| Congo                                 |
| Zaprionus   | vittiger  | davidi  | Zaprionus camerounensis| Amani (Tanzânia)                     |
| Zaprionus   | tuberculatus| tuberculatus| Zaprionus davidi | São Tomé (São Tomé)                     |
| Sophophora  | —         | willistoni| Drosophila willistoni| Ithala (South Africa)                  |
| Scaptodrosophila| —       | —       | Scaptodrosophila latifasciaeformis| São José do Rio Preto/SP (Brazil) |

**Table 1. Studied species and their geographical origin.**

![Figure 1](image1.png)  
**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.
material on both mitochondrial derivatives (Figs. 2A, 3A and C). This same characteristic has been observed in Z. indianus, Z. sepsoides and D. hydei.\textsuperscript{17,18} However, in most species of the genus Drosophila (Table 2), the paracrystalline material is present only in the larger mitochondrial derivative.\textsuperscript{17,29-32}

The structure of the sperm axoneme is of great importance for phylogenetic studies in insects.\textsuperscript{10,14,27}

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

| Species                | Granules | Mitochondrial derivatives | Paracrystalline material | Axoneme | 64 sperm per cyst |
|------------------------|----------|---------------------------|--------------------------|---------|------------------|
|                        | Same size | Different size | Largest derivative | Smallest derivative | 9 + 9 + 2 |
| *Zaprionus africanus*  | +        | -                         | +                        | +       | +                |
| *Zaprionus camerounensis*| +        | -                         | +                        | +       | +                |
| *Zaprionus davidii*    | -        | -                         | -                        | -       | -                |
| *Zaprionus gabonicus*  | +        | -                         | +                        | +       | +                |
| *Zaprionus megalorhisis*| +        | -                         | +                        | +       | +                |
| *Zaprionus tuberculatus*| +        | -                         | +                        | -       | +                |
| *Zaprionus indianus*\textsuperscript{1} | +        | -                         | +                        | +       | +                |
| *Zaprionus sepsoides*\textsuperscript{1} | +        | -                         | +                        | +       | +                |
| *Drosophila willistoni*  | +        | -                         | +                        | +       | +                |
| *Drosophila melanogaster*\textsuperscript{2,3} | na       | -                         | +                        | +       | +                |
| *Drosophila simulans*\textsuperscript{3} | na       | -                         | +                        | -       | +                |
| *Drosophila cardini*\textsuperscript{2} | na       | -                         | +                        | -       | +                |
| *Drosophila dunni*\textsuperscript{2} | na       | -                         | +                        | -       | +                |
| *Drosophila hydei*\textsuperscript{2,5} | na       | -                         | +                        | +       | +                |
| *Drosophila subobscura*\textsuperscript{6,7} | na       | -                         | +                        | -       | +                |
| *Scaptodrosophila latifasciaeformis* | +        | -                         | +                        | +       | +                |

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

**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. davidii* (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.
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, although some species have a peculiar number, such as 9 + 9 + 3 in Neuroptera, 9 + 9 + 1 in Culicidae (Diptera), 9 + 7 in Tricoptera, and 9 + 0 in Ephemeroptera. Moreover, as the majority of species of the suborder Brachycera, 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. So far, studies have indicated that all insects have cystic spermatogenesis. In Triatominae, the cysts develop independently; that is, a cyst does not influence the developmental stage of neighboring

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 mitochondrial 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.

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. In drosophilids, as described for *Platycentropus* (Trichoptera: Limnephilidae), 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. dunnii*, 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.

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. 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).

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.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr. Jean David (Center National de la Recherche Scientifique (CNRS) / GIF-SUR-Yvette / FRANCE) for providing some of the lines of *Zaprionus*, Rosana Silistino Souza (Academic Support Assistant II) for technical assistance in sample preparation and Luis Fernando Segala (Assistant Professor) for help with statistical analyses.

**Funding**

This paper was supported by Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP) (Process number 2010/01193-9) and Coordenadoria de Aperfeiçoamento do Pessoal do Ensino Superior (CAPES).

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