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# The sperm of Hylodinae species (Anura, Leptodactylidae): Ultrastructural characteristics and their relevance to interspecific taxonomic relationships

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Hylodinae leptodactylids (*sensu* Lynch 1971) form a group of diurnal frogs, which is hypothesized on the basis of morphological traits to be the closest relatives of the dendrobatid frogs. Our study describes ultrastructural characteristics of sperm from three hylodine species (*Hylodes phyllodes*, *Crossodactylus* sp. n. and *Megaelosia massarti*) to reassess the intergeneric relationships within the Hylodinae, as well as the supposed relationship between the Hylodinae and Dendrobatidae. The ultrastructure of the sperm is very similar among the three species and is indicative of its conserved nature within the Hylodinae. The structure of the acrosomal complex was very similar to that of other leptodactylid species, to most of the remaining species included in the Bufonoidea lineage, and also to that observed in the dendrobatid species examined so far. Since such a structure has been considered a plesiomorphic trait, it contributes little to our understanding of the relationships between the Hylodinae and Dendrobatidae. The flagellar apparatus of *Crossodactylus* sp. n. is very similar to that of most leptodactylids. The sperm of *Megaelosia massarti* and *Hylodes phyllodes* display a distinctive condition in their axial and juxtaxonemal fibers. This distinctive flagellar condition expands the already known variability in sperm structure within the Leptodactylidae.

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## 1. Introduction

Leptodactylid frogs are restricted to the Americas from the southern USA and the Antilles to southern South America, and more than 1,000 species are recognized within this neotropical family (Frost 2004). They represent about one quarter of the anuran species described so far. Within the Leptodactylidae, the subfamily Hylodinae (*sensu* Lynch 1971) encompasses three genera, namely *Hylodes*, *Crossodactylus*, and *Megaelosia*, and 34 species, distributed from northwestern to southern Brazil and northern Argentina (Frost 2004).

Monophyly of the Hylodinae frogs was proposed by Lynch (1971), who emphasized that the skull of *Megaelosia* is strikingly different from that of the other genera. The dietary habits, such as batrachophagy, and the absence of announcement calls are also peculiarities of *Megaelosia* not shared with the remaining hylodines (Giaretta *et al* 1993).

Based on morphological and karyotypical data, *Hylodes* and *Crossodactylus* have been tentatively considered as the closest relatives of the neotropical frogs belonging to the family Dendrobatidae (Noble 1931; Lynch 1971; Bogart 1991; reviewed by Ford 1993). Noble (1931), based on the presence of dermal scutes on the toes and the morphology

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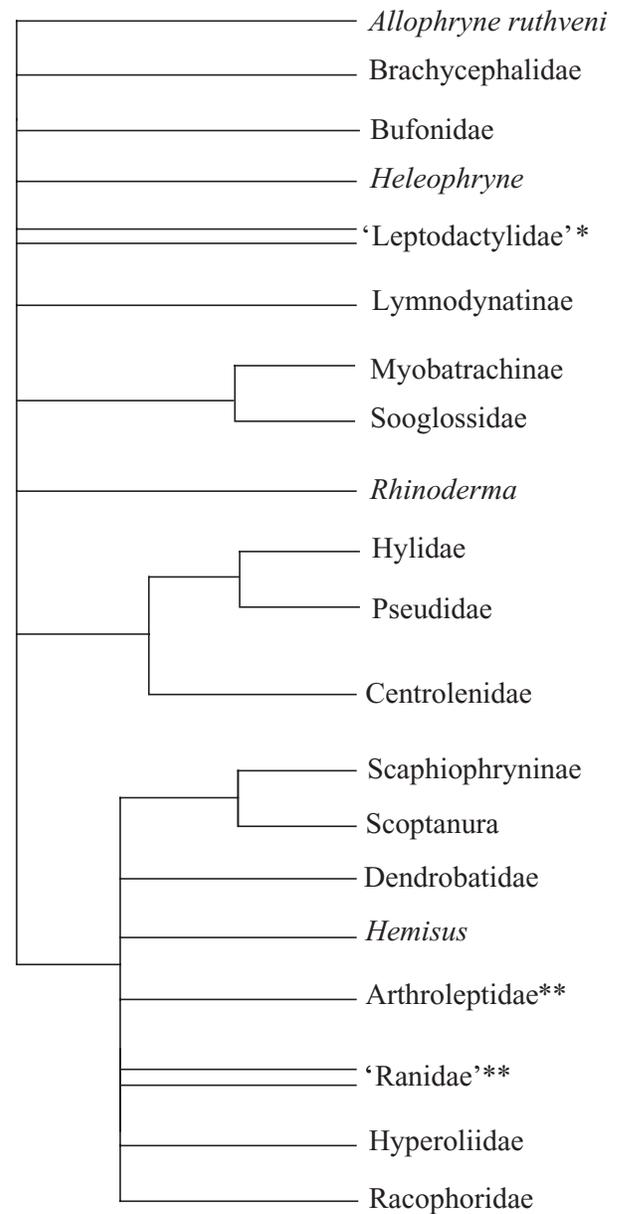
of the pectoral girdle, proposed *Crossodactylus* as the sister group of the dendrobatid frogs. Lynch (1971) found additional characters, such as a similar cranial morphology and the presence of toxic skin secretion, supporting hylodine-dendrobatids relationships. According to Bogart (1991), chromosome morphology data from *Hylodes* species also indicate such a relationship.

Alternatively, according to Griffiths (1959, *apud* Ford 1993) and Ford (1989, *apud* Ford 1993), the sister group of the Dendrobatidae could be an African ranoid frog belonging to the ranid subfamily Petropedetinae. This hypothesis offered by Griffiths (1959, *apud* Ford 1993) was based on the presence of a firmisternal pectoral girdle in both groups. Based on osteological characters, Ford (1989, *apud* Ford 1993) also pointed out a ranid-dendrobatid relationship. For an overview of such competing hypothesis, see figures 1 and 2.

Recently, Aguiar-Jr *et al* (2002), Rosa *et al* (2003), and Aguiar-Jr *et al* (2004a) have used a cytogenetic approach to examine the Hylodinae-Dendrobatidae relationships, describing the karyotypes of *Hylodes*, *Crossodactylus*, *Megaelosia* and *Epipedobates* species. These investigations have suggested that no unambiguous homeologies can be found among the karyotypes of these two groups, even with the use of banding techniques. In addition, according to Rosa *et al* (2003), the karyotypic peculiarities of *Megaelosia* indicate that this genus contributes little to the understanding of the relationships between the Hylodinae and Dendrobatidae in the context of cytogenetics.

A variety of alternative approaches have been introduced to provide new data to supplement the traditional morphological parameters for the evaluation of taxonomic and phylogenetic problems of Anura. For example, the analysis of sperm ultrastructure has been applied to many anuran groups belonging to both the Archaeo- and Neobatrachia and has been helpful in suggesting evolutionary trends and in elucidating taxonomic relationships (Pugin-Rios 1980; Jamieson *et al* 1993; Kwon and Lee 1995; Meyer *et al* 1997; Aguiar-Jr *et al* 2003, revision by Garda 2002). To further the phylogenetic reconstruction based on spermatological characteristics, however, a broader number of species is necessary.

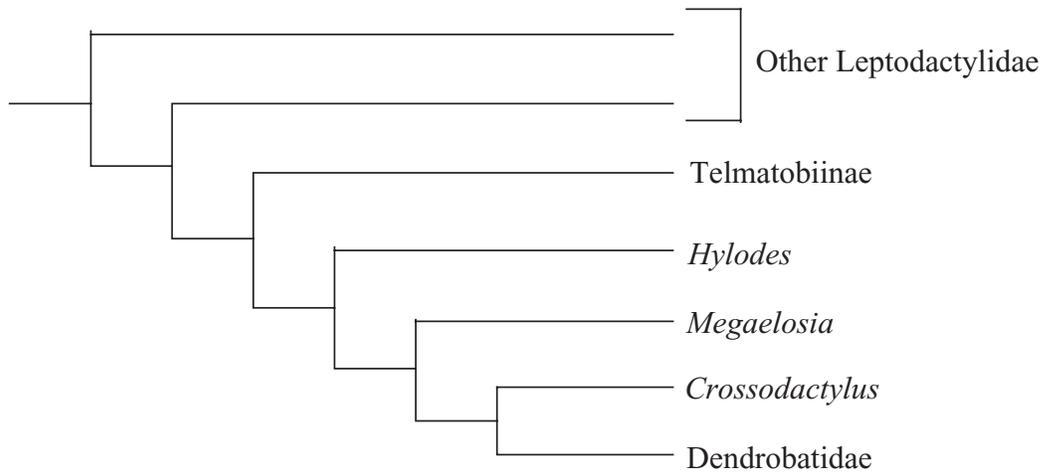
To date, the sperm ultrastructure has been studied in only 24 species belonging to three leptodactylid subfamilies, none of which include representatives of the Hylodinae (*sensu* Lynch 1971) (Pugin-Rios 1980; Pugin and Garrido 1981; Garrido *et al* 1989; B ao *et al* 1991; Amaral *et al* 1999, 2000; Scheltinga and Jamieson 2003). Thus, the main purpose of our study is to examine the sperm ultrastructure of hylodine frog species to contribute to the elucidation of the evolutionary trends in the Leptodactylidae and to assess their intergeneric relationships as well as the possible relationships between dendrobatid and leptodactylid species.



**Figure 1.** Phylogenetic relationships among neobatrachian Anura adapted from Ford and Cannatella (1993) and reproduced from Aguiar-Jr *et al* (2004a) (*Genetica* **121** 43–53, 2004, figure 1), with kind permission of Springer Science and Business Media. (\*) Indicates the group to which Hylodinae frog species belong, and (\*\*) indicates the other groups from which the Dendrobatidae hypothetically arose (Arthroleptidae was formerly the ranid subfamily Arthroleptinae). Within the Ranidae, the proposed dendrobatid sister group is the subfamily Petropedetinae. Names under quotation marks are not monophyletic groups.

## 2. Materials and methods

Adult male *Hylodes phyllodes* and *Megaelosia massarti* were collected at Paranapiacaba in the municipality of



**Figure 2.** Phylogeny illustrating the Hylodinae/Dendrobatidae relationships as postulated by Lynch (1971). Adapted from Ford (1993).



**Figure 3.** Map showing the localities of collection (▲, ■) of the species analysed in this study.

Santo André, São Paulo State. One adult specimen of *Crossodactylus* sp. n. was collected in the “Parque Florestal de Itapetinga” in the municipality of Atibaia, São Paulo State (figure 3). The specimens were collected under a permit from the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA) (20235/98-78 SP). Voucher

specimens were deposited in the Museu de História Natural “Prof. Adão José Cardoso” (ZUEC) of the Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil. The specimens were assigned the following accession numbers: *H. phyllodes* (ZUEC 11554), *M. massarti* (ZUEC 11396), and *Crossodactylus* sp. n. (ZUEC 11437).

The testes were removed by dissection, cut into small pieces, and fixed overnight at 4°C in 0.1 M sodium cacodylate buffer, pH 7.2, containing 2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose, and 5 mM CaCl<sub>2</sub>. Postfixation was done for 1 h in the same buffer containing 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM CaCl<sub>2</sub>. The tissue samples were subsequently rinsed in sodium cacodylate buffer and incubated for 2 h in 0.5% uranyl acetate. After rinsing in buffer, the samples were dehydrated in an increasing acetone series and embedded in Epon resin. Ultrathin sections, obtained with an ultramicrotome using a diamond knife, were stained with uranyl acetate and lead citrate and examined with a LEO 906 transmission electron microscope.

### 3. Results

The basic structure of the spermatozoa of the three hylodine species examined showed great uniformity, especially in structure of the acrosomal complex and midpiece. Differences were observed in the shape of the juxtaxonemal and axial fibers.

#### 3.1 Acrosomal complex

In all three species, a conical acrosomal vesicle covers the anterior portion of the nucleus. This vesicle is filled with a substance of variable electron density (figures 4A, 5A and 6A). In *Crossodactylus* sp. n., this vesicle is very thin (figure 5A). A subacrosomal cone is located behind the acrosomal vesicle. This cone consists of a substance of homogeneous electron density in all species (figures 4A, 5A and 6A) and extends posteriorly beyond the acrosome (seen only in figures 5A and 6A). In *M. massarti* and *H. phyllodes*, the subacrosomal cone has a granular appearance, in contrast to that in *Crossodactylus* sp. n., which appears solid. This characteristic is more apparent in transverse sections of the acrosomal complex (figures 4B-D, 5B, C, and 6B-D). A subacrosomal space is situated between the subacrosomal cone and the nucleus and is particularly large in *M. massarti* (figures 4A, 5A and 6A).

#### 3.2 Nucleus

An electron-lucent nuclear space is present at the apical tip of the nucleus in *H. phyllodes* and *Crossodactylus* sp. n., but not in *M. massarti*. The chromatin in the spermatozoa is not fully compacted in the three species, particularly in *M. massarti* (figures 4A, E, F, 5A, C, D-F and 6A, D-F). However, highly compacted chromatin was seen in transverse sections of the nucleus in *H. phyllodes* (figure 4D). The nucleus of the spermatids of *Crossodactylus* sp. n.

contains very loosely condensed chromatin, which borders a more condensed central region (figure 5D, E). Numerous mitochondria are associated with the posterior region of the nucleus in all species and surround the nucleus (figures 4E, 5D and 6F). This arrangement is not present in some of the sperm examined, as shown for *M. massarti* (figure 6E, F).

#### 3.3 Midpiece

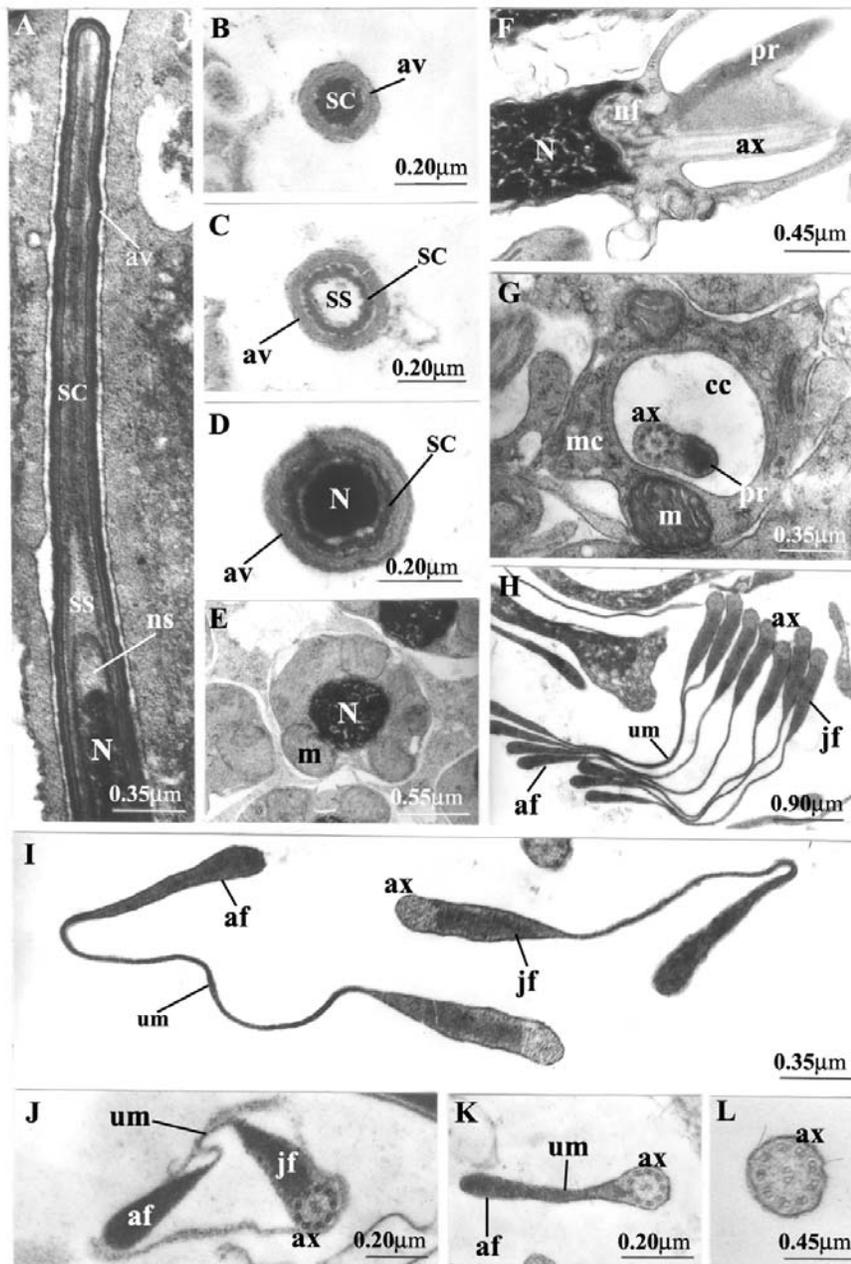
The midpiece is short and encloses the proximal and distal centrioles, which are arranged obliquely to each other (figures 4F, 5F and 6G). The distal centriole gives rise to the axoneme (figures 4F, 5F and 6G). Parallel to the distal centriole, a paraxonemal rod (*sensu* Jamieson *et al* 1993) is present in the spermatozoa of *H. phyllodes* and *Crossodactylus* sp. n. (figures 1F and 2E). A nuclear fossa is present at the base of the nucleus (figures 4F, 5F and 6G).

#### 3.4 Tail piece

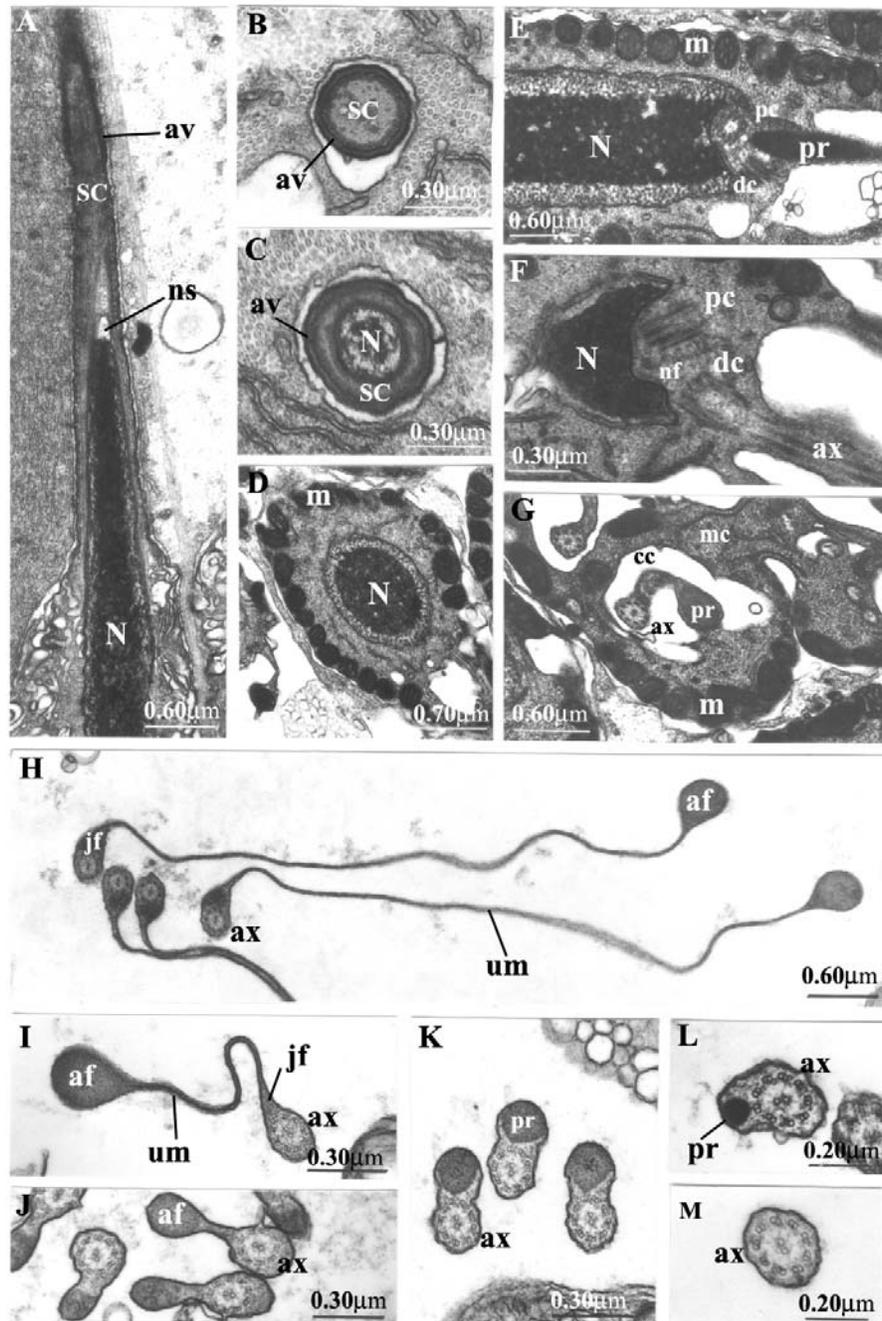
At the anteriormost portion of the tail, the flagellum is surrounded by the mitochondrial collar. This structure is poorly developed in the spermatozoa of *H. phyllodes*, which have only a few mitochondria or only a cytoplasmic sheath (figures 4F, G, 5G and 6H). The flagellum consists of an axoneme with the usual 9+2 pattern, a juxtaxonemal fiber (which is connected to the axoneme at doublet number 3), a very thin undulating membrane, and an axial fiber. The juxtaxonemal and axial fibers are connected by an electron-dense substance within the undulating membrane (figures 4H, I, 5H, I and 6I, J). At the anteriormost portion of the flagellum, the axial and juxtaxonemal fibers are not distinguishable and, hence, named paraxonemal rod (*sensu* Lee and Jamieson 1993) (figures 4G, 5G and 6H). *Hylodes phyllodes* and *M. massarti* have a very similar flagellar structure, in which the juxtaxonemal and axial fibers have an elongate conical shape, although both fiber types are also quite extensive in *H. phyllodes* (figures 4H, I and 6 I, J). This pattern is different from that of *Crossodactylus* sp. n., in which the juxtaxonemal fiber is very short, and the axial fiber has a globular shape (figure 5H, I). In more distal portions along the flagellum, the undulating membrane shrinks, and the accessory fibers move closer to each other. Posteriorly, the juxtaxonemal fiber narrows and progressively merges with the axial fiber (re-forming the paraxonemal rod) and, in the most distal portion, only the axoneme was observed (figures 4J-L, 5I-M and 6J-M).

### 4. Discussion

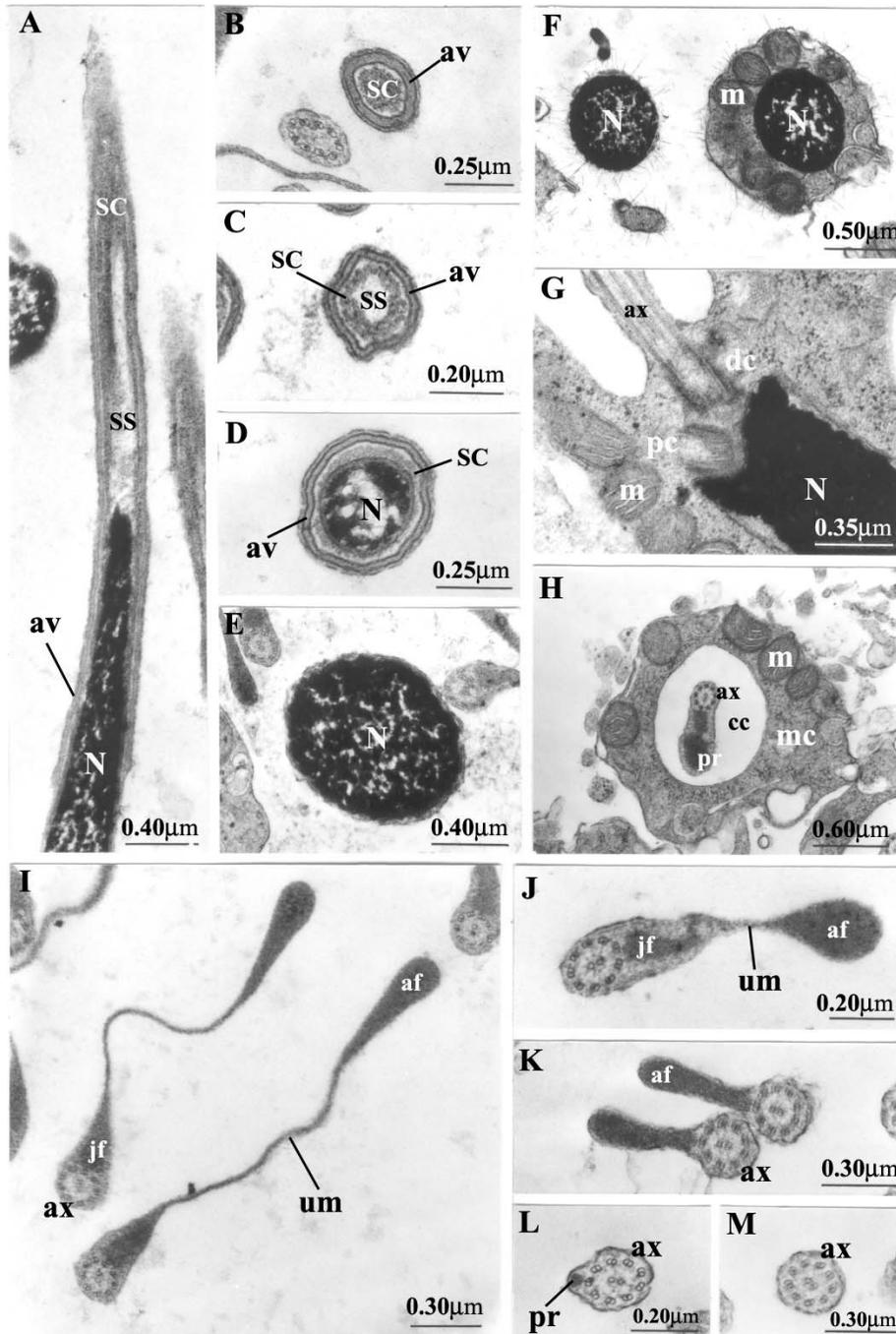
The similarity among the spermatozoa of the three Hylodinae species examined here indicates a conserved nature of the spermatological characteristics within the subfamily.



**Figure 4** Electron micrographs of *H. phyllodes* spermatozoa. (A) Longitudinal section through the anterior head region showing the acrosomal complex with the acrosomal vesicle (av) and subacrosomal cone (sc). Note the subacrosomal space (ss) behind the subacrosomal cone and the nuclear space (ns) at the tip of the nucleus (N). (B–E) Transverse sections through the head. In (D), note the high degree of chromatin condensation. In (E), note the mitochondria (m) surrounding the basal portion of the nucleus. (F) Longitudinal section through the posteriormost region of the nucleus, midpiece, and proximal portion of the flagellum, showing the nuclear fossa and the axoneme with an adjacent accessory fiber (i.e. paraxonemal rod, pr). Note the presence of a discrete cytoplasmic sheet without mitochondria. (G) Transverse section of the anterior portion of the flagellum showing a weakly developed mitochondrial collar (mc) separated from the flagellum by a cytoplasmic canal (cc). (H, I) Longitudinal section of the tail showing elongate conical axial (af) and juxtaxonemal fibers. Note the very thin undulating membrane (jf) connecting the accessory fibers and the axoneme. (J–L) Progressive shortening of the undulating membrane, and disappearance of the axial and juxtaxonemal fibers. In (K), note the remnant of the juxtaxonemal fiber (um) connect to the doublet 3. Note also that only the axoneme (ax) is seen at the tip of the tail (L).



**Figure 5** Electron micrographs of *Crossodactylus* sp. n. spermatozoa. (A) Longitudinal section of the anterior region of the head. Note the thin acrosomal vesicle (av), the subacrosomal cone (sc), and the subacrosomal (ss) and nuclear spaces (ns). (B–D) Transverse sections through different portions of the head. In (D), note the mitochondria surrounding the basal portion of the nucleus (N). (E, F) Longitudinal sections through an early spermatid. Note the oblique arrangement between the proximal (pc) and distal centrioles (dc), the paraxonemal rod (pr) adjacent to the distal centriole, and the low degree of chromatin compaction. (G) Transverse section of the anterior portion of the flagellum showing a well-developed mitochondrial (mc) collar separated from the flagellum by the cytoplasmic canal (cc). (H) Transverse sections of the tail. Note the globular shape of the axial fiber (af) and the short extension of the juxtaxonemal fiber (jf). (I–L) Progressive shortening of the undulating membrane (um) and approximation of the accessory fibers. In (I), note the narrowing of the juxtaxonemal fiber. (M) The tail lacks accessory fibers at its apical end, where only the axoneme (ax) is visible.



**Figure 6** Electron micrographs of *M. massarti* spermatozoa. (A) Longitudinal section through the anterior region of the head showing the poorly compacted chromatin, the acrosomal vesicle (av), the subacrosomal cone (sc), and a very large subacrosomal space (ss). (B–E) Transverse sections through the acrosomal complex and nucleus. Note the progressive enlargement of the nucleus (N). (F) Basal portion of the nucleus showing the facultative presence of mitochondria (m). (G) Longitudinal section through the centriolar region. Note the oblique arrangement of the proximal (pc) distal centrioles (dc). (H) Well-developed mitochondrial collar (mc) around the anteriormost portion of the flagellum. (I) Transverse section of the tail showing the elongate conical axial (af) and juxtaxonemal fibers (jf). Note the thin undulating membrane connecting the axoneme to accessory fibers. (J–L) Progressive shortening of the undulating membrane (um) and approximation of accessory fibers. (M) Only the axoneme (ax) was seen at the tip of the tail.

The acrosomal complex of the three species was similar to that of other leptodactylid species (Báo *et al* 1991; Amaral *et al* 2000), as well as to most of the other species included in the Bufonoidea lineage (Pugin-Rios 1980; Lee and Jamieson 1993; Kwon and Lee 1995; Báo *et al* 2001), as well as to that observed in the dendrobatid species examined so far (Garda *et al* 2002; Aguiar-Jr *et al* 2003, 2004b). In all of them, a conical acrosomal vesicle caps the anterior portion of the nucleus, having a subacrosomal cone behind it.

However, this similarity between the Hylodinae and Dendrobatidae does not contribute to support an evolutionary affinity between these two groups as it has been argued that the presence of a subacrosomal cone behind a conical acrosomal vesicle is possibly a plesiomorphic characteristic since it has been found in urodeles, gymnophionans, and the most primitive anurans (Scheltinga and Jamieson 2003).

Although Lee and Kwon (1996) have identified the uncompacted chromatin as an apomorphic trait (page 256, table 2) shared by the sperm cells of *Discoglossus* and *Bombina* genera, we consider that this character is a poor indicator of relationships since it is highly variable among species or even among sperm cells in the same tissue and individual. In all of the species studied here, the low degree of chromatin condensation seen in most of the cells examined probably reflects the different stages of maturation of the gametes rather than real affinities among them.

The number and distribution of mitochondria are very variable in lissamphibian sperm (Scheltinga *et al* 2001). The mitochondria observed around the base of the nucleus is probably a transient structure that is lost during spermiogenesis, as also seems to be the case of the mitochondrial collar in late testicular spermatozoa or in those within in the seminal vesicle (Pugin-Rios 1980; Garrido *et al* 1989). The well-developed mitochondrial collar seen in *Crossodactylus* sp. n. and *M. massarti* is common in bufonoid and also in dendrobatid species (Jamieson *et al* 1993; Kwon and Lee 1995; Garda *et al* 2002; Aguiar-Jr *et al* 2003, 2004b). However, in *H. phyllodes*, this structure is poorly developed and resembles the condition found in *Leiopelma hochstetteri* by Scheltinga *et al* (2001), who tentatively considered such a condition as a plesiomorphy of anurans since it was similar to that found in the lungfish *Neoceratodus forsteri*. At the moment, however, any tentative taxonomic inference based on this similarity among these two unrelated species would be speculative. Also, the well-developed mitochondrial collar was considered by Scheltinga *et al* (2001) as a possible plesiomorphy of anurans, as it resembled the condition found in acanthopterygian fishes, or a possible reversal to a pre-lissamphibian condition since a collar is absent from the sperm of urodeles, gymnophionans, and most of the basal Anura (see also Scheltinga and Jamieson 2003). So,

as a possible plesiomorphy, such a shared characteristic between hylodines (and the remaining bufonoid frogs) and dendrobatids is not indicative of phylogenetic relationships.

*Crossodactylus* sp. n. has a flagellar apparatus that is very similar to that of other leptodactylids, such as *Odontophrynus cultipes* (Báo *et al* 1991) and *Pseudopaludicola falcipes* (Amaral *et al* 2000), in which the axial fiber has a 'globular' shape, although the juxtaxonemal fiber of *Crossodactylus* is somewhat reduced. In contrast, the juxtaxonemal and axial fibers of *H. phyllodes* and *M. massarti* have a distinctive elongate conical shape. A juxtaxonemal fiber that is similar in shape to that of *Megaelosia* has been described from *Physalaemus* spp. (Amaral *et al* 1999), which surprisingly do not possess an axial fiber.

Despite some differences in skull morphology, Lynch (1971) retained *Megaelosia* in the Hylodinae due to similarities shared with *Hylodes*, including external morphology and the presence of paired vocal sacs, which could, however, be the result of convergence (see also Giaretta *et al* 1993). Maxson and Heyer (1982) determined a close relationship between these genera based on comparative albumin data. The spermatological features described here concerning the sperm flagellar apparatus may reinforce a possible relationship between these genera, supporting the retention of *Megaelosia* in the Hylodinae. However, no definite answer on this intergeneric relationship can be obtained until the phylogenetic significance of this trait is defined.

The ultrastructural features of the flagellar apparatus of the three hylodine species examined in our study differ somewhat from the pattern in dendrobatids (Garda *et al* 2002; Aguiar-Jr *et al* 2003, 2004b), in which a distinct juxtaxonemal fiber is absent and the undulating membrane is short and, in most cases, highly thickened. However, both groups retain a bufonoid-like configuration in which an axial fiber is connected to the axoneme through an axial sheath within an undulating membrane, in contrast to the condition found in ranids, in which no tail accessories are present (see Kwon and Lee 1995).

For the family Leptodactylidae, our data on the distinctive shape of the axial fiber in *H. phyllodes* and *M. massarti* increases the already known intrafamilial variability in sperm ultrastructure characteristics (for a detailed review see Scheltinga and Jamieson 2003). This variability includes some of the evolutionary trends proposed by Kwon and Lee (1995) and Lee and Jamieson (1993) within the 24 species already analysed (Pugin-Rios 1980; Pugin and Garrido 1981; Garrido *et al* 1989; Báo *et al* 1991; Amaral *et al* 1999, 2000; Scheltinga and Jamieson 2003).

Lee and Jamieson (1993) argued that the simplification of spermatozoa structures is an evolutionary trend in anurans. The simplifications include the absence of tail accessory filaments and the absence of the subacrosomal cone (or

conical perforatorium) in more highly apomorphic taxa. The Leptodactylidae, an apomorphic taxon (Neobatrachia), however, presents a mixture of characteristics. Auxiliary fibers are absent in *Caudiverbera caudiverbera* (subfamily Telmatobiinae) (Pugin and Garrido 1981), but well developed in *Odontophrynus cultripes* (Báo *et al* 1991), also a Telmatobiinae species. In addition, the leptodactylids of the genera *Odontophrynus*, *Pseudopaludicola* and *Telmatobius* have an acrosomal vesicle and a subacrosomal cone in the acrosome complex, in contrast to the genera *Caudiverbera*, *Leptodactylus*, and *Pleurodema*, in which the acrosome complex is composed of an acrosome vesicle only.

As previously emphasized by Amaral *et al* (2000), this variability supports the view that the Leptodactylidae may be a polyphyletic taxon, as suggested by Duellman and Trueb (1994).

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