UNIVERSIDADE ESTADUAL DE CAMPINAS

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A ULTRA-ESTRUTURA DO ESPERMATOZÓIDE DE ANUROS DAS FAMÍLIAS DENDROBATIDAE, MICROHYLIDAE E PSEUDIDAE

Tese apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural na área de Biologia Celular.

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**FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP**

G166u

Garda, Adrian Antonio
A ultra-estrutura do espermatozóide de anuros das famílias Dendrobatidae, Microhylidae e Pseudidae/Adrian Antonio Garda. --
Campinas, SP:[s.n.], 2002

Orientadora: Sônia Nair Báo
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Dissertação (mestrado) – Universidade Estadual de Campinas. Instituto de Biologia

1. Amphibia. 2. Ultra-estrutura. 3. Sistemática. I. Báo, Sônia Nair. II. Colli, Guarino Rinaldi. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Data da Defesa: 27/02/2002

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O presente trabalho foi realizado no Laboratório de Microscopia Eletrônica da Universidade de Brasília (UnB), sob a orientação da Prof. Dra. Sônia Nair Báo (Depto. de Biologia Celular, UnB) com a co-orientação do Prof. Dr. Guarino Rinaldi Colli (Departamento de Zoologia, UnB).

- Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq);

- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES);

- Universidade de Brasília (UnB);

- Universidade Estadual de Campinas (UNICAMP).

Ao grande amigo BG

AGRADECIMENTOS MAIS QUE SINCEROS

Aos meus pais e minha irmã, pelo amor e pelo apoio psicológico, filosófico e também financeiro durante os últimos 25 anos.

À minha orientadora, Sônia Nair Báo, e a meu orientador, Guarino R. Colli, por todo tempo gentilmente dedicado a essa presente tese, e por conseguinte, à minha formação.

Ao Professor, amigo e colega de mesa de bar Antônio Sebben, que despertou em mim a paixão pelos anfíbios, sem a qual esse trabalho não existiria.

Aos meus orientadores Guarino Colli, Heloísa Miranda e Sônia Báo, pelo biólogo que sou e venha a ser.

Aos meus grandes e eternos amigos: Fernanda Werneck, Reuber Brandão, Saulão, Santos Balbino, Daniel Martinelli, Núbia Shelli, Frederico França, Ayrton Péres, Marcelo BG, Gustavo Vieira, Gabriel Costa, Helga Wiederhecker, Mari Zatz, Daniéis Diniz e Mesquita, Eni Braga, Leonora Bastos e Ruscaia Teixeira.

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RESUMO

As relações filogenéticas entre anuros permanecem mal resolvidas, qualquer que seja o nível taxonômico em questão. Reconstruções baseadas em dados morfológicos tradicionais não foram capazes de fornecer soluções consistentes para uma compreensão da história evolutiva do grupo. Recentemente, a ultra-estrutura do espermatozóide tem sido utilizada para avaliar as relações entre as famílias de muitos grupos de vertebrados, inclusive anfíbios. Todavia, a ausência de dados para muitas famílias impede uma reconstrução filogenética com base nesses novos caracteres. A presente tese descreve pela primeira vez a ultra-estrutura de anuros de três famílias neotropicais (Dendrobatidae, Microhylidae e Pseudidae) e as implicações evolutivas derivadas dessas observações. Ainda que o número de famílias descritas não possibilite a utilização desse rol de dados para uma análise de parcimônia das famílias de anuros, um grupo de 22 caracteres pôde ser proposto para a classe com base nos presentes dados e na literatura.

ABSTRACT

Phylogenetic relationships among anurans remain poorly resolved, regardless of the taxonomic level under study. Reconstructions based on traditional morphological data did not result in well corroborated solutions. Recently, sperm ultrastructure has been used to evaluate relationships among several vertebrate families, including anurans. Nevertheless, the lack of descriptions for several families hinders the possibility of using such data in phylogenetic reconstruction. This thesis describes, for the first time, the ultrastructure of three neotropical anuran families (Dendrobatidae, Microhylidae, and Pseudidae) and evaluates the evolutionary implications for these families. Even though the number of families described is still small for a good phyloganetic analysis, we present 22 characters that can be used in phylogenetic reconstruction.

1. INTRODUÇÃO

1.1 A Importância da História Evolutiva dos Anuros

Desde a sua divergência a partir dos ancestrais que primeiro colonizaram os continentes, os anfíbios passaram por uma radiação adaptativa surpreendente que resultou na enorme variedade de histórias de vida observada hoje (Duellman & Trueb, 1986). A complexidade de sua ecologia, comportamento e fisiologia, bem como a ampla distribuição geográfica e a ocupação de praticamente todos os ambientes terrestres atestam o sucesso do grupo até os dias de hoje. Ainda que esse *status* de importância ecológica e evolutiva seja hoje bem aceito, nosso conhecimento a respeito dos anfíbios deixa muito a desejar. O número total de espécies conhecidas de anfíbios recentemente superou o de mamíferos e a taxa de descrição de espécies novas é a maior entre os vertebrados terrestres (Glaw & Köhler, 1998). Isso é um indício inegável da condição primária de conhecimento do grupo, visto que ainda estamos no passo inicial que é o de delimitar morfológica e geograficamente os táxons. As relações filogenéticas, de maneira análoga, são pouco conhecidas e mesmo em níveis taxonômicos elevados o problema é crítico. A questão da monofilia dos anfíbios e da relação entre três seus três clados (Gymnophiona, Caudata e Anura) é debatida até os dias de hoje (Hedges *et al.*, 1990; Feller & Hedges, 1998; Sá, 1999) e as incertezas a respeito das relações entre as famílias de anuros exemplificam a precariedade em que se encontra o conhecimento da história evolutiva dos anfíbios.

As salamandras pertencem à ordem sobre a qual há mais informação, enquanto as enigmáticas e furtivas cecílias são sem dúvida o grupo que menos conhecemos. A ordem

anura pode ser considerada especialmente problemática por possuir ao mesmo tempo a maior diversidade e relações filogenéticas pouco conhecidas. Como a maioria das espécies encontra-se nos trópicos, onde o volume de estudos e o conhecimento do ambiente são menores que nas regiões temperadas, a compreensão das relações filogenéticas entre espécies, gêneros e famílias é precária. Muitas famílias, como Leptodactylidae e Microhylidae, provavelmente não são monofiléticas (Duellman, 1975; Ford & Cannatella, 1993), enquanto a posição de outras, como Dendrobatidae, permanece obscura (Ford, 1993).

A razão para essa dificuldade em se estabelecer uma história do grupo advém tanto da falta de conhecimentos a respeito de espécies tropicais como de certas características intrínsecas dos próprios anfíbios. O plano corporal uniforme dos anuros, por exemplo, torna difícil uma boa separação das famílias (Inger, 1967). A ausência de escamas ou pêlos e o esqueleto altamente reduzido são exemplos dessa incômoda simplicidade morfológica. Assim, os caracteres tradicionais de osteologia e morfologia externa, largamente usados em outros grupos, acabam por não ser suficientes e caracteres alternativos passam a ser necessários para uma reconstrução fidedigna da filogenia da ordem (Wake, 1993).

Essa ausência de reconstruções filogenéticas consistentes pode causar graves problemas. O estudo de diversos aspectos da ecologia dos seres vivos (como interações) só pode ser feito com base em uma filogenia bem estabelecida, de modo que possamos avaliar a importância das relações filogenéticas na determinação das características observadas. Uma hipótese de relação entre os táxons é a única forma de podermos discernir características resultantes da história recente de outras meramente herdadas, resultantes de pressões evolutivas muito mais antigas e sem importância ecológica nos dias atuais. Essa

dificuldade imposta ao estudo da ecologia dos anfíbios é agravada ante a atual condição do grupo, que sabidamente está declinando em todo mundo (Stebbins & Cohen, 1995; Pough *et al.*, 1999). Desse modo, faz-se necessário compreender as diferenças de abundância entre diferentes espécies, bem como o grau e a natureza das flutuações naturais intra-específicas. Esse conhecimento da ecologia do grupo depende, pois, de uma boa compreensão das relações históricas entre os táxons em estudo.

Por conseguinte, a elucidação da história evolutiva dos anfíbios ultrapassa a barreira de uma questão sistemática e torna-se de suma importância, tanto para a compreensão da dinâmica das oscilações das populações como para a compreensão de toda a ecologia da ordem.

1.2 As relações entre as famílias de anuros

As primeiras tentativas de se estabelecer uma hipótese de relações entre as famílias de anuros remontam a meados do século 19 (Dumèril & Bibron, 1841, em Griffiths, 1963). Esses primeiros trabalhos valeram-se de caracteres individuais, como língua, discos adesivos dos dedos e dentes para separar as principais subordens de anuros. Cope (1865) (em Griffiths, 1963) foi o primeiro a reconhecer a importância da cintura escapular para a classificação dos anuros, dividindo a ordem em Arcífera e Firmisterna. Em seguida, utilizando a base fornecida por Nichols (1916) (em Griffiths, 1963). Noble (1931) concentrou-se em redefinir as 5 subordens e propor uma hipótese para a relação entre as famílias que as compunham. Seu trabalho consolidou agrupamentos substancialmente importantes, como as famílias Pipidae, Discoglossidae e Ascaphidae. Famílias mais recentes e com muitos representantes neotropicais (como Bufonidae e Hylidae), todavia,

foram pobremente definidas pela escassez de dados para suas espécies (Bufonidae continha, por exemplo, Myobatrachidae, Pseudidae, Bufonidae, Leptodactylidae e Rhinophrynidae).

A hipótese de Noble (1931) foi durante muito tempo a mais aceita. Cerca de 30 anos mais tarde, Griffiths (1963) apresentou uma excelente compilação a respeito da estrutura morfológica dos anuros. Nesse trabalho ele fez uma avaliação dos caracteres passíveis de serem usados na reconstrução da história evolutiva dos anuros, além de rever as famílias e suas composições, utilizando principalmente o número de vértebras pré-sacrais e a estrutura do esterno na cintura escapular (Firmisterna ou Arcífera). Com base na sua nova definição para as famílias, Griffiths (1963) sugeriu uma hipótese para a relação entre essas. Ele reconheceu agrupamentos de famílias ainda hoje bem aceitos, como Ranidae+Rhacophoridae+Microhylidae, e Bufonidae+Hylidae+Leptodactylidae, que mais tarde viriam a ser chamadas Ranoidea e Bufonoidea (=Hyloidea), respectivamente.

Inger (1967) reacendeu a discussão ao alertar para a importância dos caracteres larvais na reconstrução da história dos adultos. Pioneiramente, ele utilizou um programa computacional para avaliar as relações filogenéticas entre as famílias de anuros. Com o avanço na parte computacional para a análise filogenética, novas metodologias e programas surgiram. A filética quantitativa foi usada pela primeira vez em um trabalho onde as relações filogenéticas entre as famílias de anuros foram analisadas (Kluge & Farris, 1969). Os autores comparam os seus novos métodos com as classificações artificiais anteriores, bem como com os resultados obtidos por Inger (1967). Ainda que o objetivo central do trabalho tenha sido a introdução da nova técnica, os resultados produziram um dendrograma onde os grupos Archaeobatrachia e Neobatrachia são prontamente distinguidos. Em Neobatrachia, Bufonoidea é prontamente separado de

Ranidae+Rhacophoridae e Microhylidae.

Em seguida, um livro onde diversos aspectos da biologia evolutiva dos anuros são debatidos fez um apanhado geral a respeito dos conhecimentos e problemas a respeito da biologia evolutiva de anuros (Vial, 1973). Nesse livro, Lynch (1973) propõe um dendrograma de relações, juntamente com uma redefinição das famílias de anuros, reconhecendo, por exemplo, a família Pseudidae, inicialmente proposta por Savage & Carvalho (1953) e interpretada como uma subfamília de Hylidae por Griffiths (1963). Lynch (1973) ainda define superfamílias: Pipoidea, Ascaphoidea, Pelobatoidea, Bufonoidea e Ranoidea. Todavia, seu trabalho não utiliza metodologias cladísticas para avaliar as relações entre esses clados. Duellman & Trueb (1986) apresentaram uma nova proposta baseada em 16 caracteres morfológicos. Essa reconstrução apresenta diversas politomias não resolvidas. Contudo, a relação entre superfamílias aparece razoavelmente bem delineada. Assim como no dendrograma de Kluge & Farris (1969), Archaeobatrachia e Neobatrachia são prontamente distinguidos. Analogamente, em Neobatrachia, Bufonoidea é bem separado de Ranoidea e Microhylidae. Entretanto, além de apresentar sugestões diferentes com respeito à posição de certas famílias (como o agrupamento de Dendrobatidae com Ranidae), as relações entre as famílias, notoriamente em Bufonoidea, apresentam-se com pouca ou nenhuma resolução.

Com a subseqüente utilização de dados moleculares o problema foi novamente revisto. Hedges *et al.* (1990), ao apresentar uma proposta para as relações em Tetrapoda baseada em seqüências dos fragmentos 18S e 28S do RNA ribossomal, deram especial atenção para os anfíbios, visto que as únicas diferenças entre suas 12 árvores mais parcimoniosas eram todas nas relações entre grupos de Lissamphibia. A análise de máxima

parcimônia foi incapaz de resolver as relações entre as famílias de anfíbios. Ao utilizar um método de análise de distâncias (Neighbor-joining), a árvore resultante apresentou diferenças substanciais com respeito às hipóteses morfológicas, formando clados com a incômoda situação de conter ao mesmo tempo representantes das três ordens, opondo-se desse modo à monofilia das ordens de anfíbios.

Hedges & Maxson (1993) avaliaram a utilização de dados moleculares para resolver as relações em Lissamphibia. Eles introduziram seqüências de RNA ribossomal mitocondrial 12S, o qual seria mais adequado devido à sua taxa de evolução mais rápida que o 18S. O cladograma resultante prontamente separa Archaeobatrachia e Neobatrachia, bem como Bufonoidea e Ranoidea dentro do último. Estranhamente, uma relação próxima entre Hylidae e Rhacophoridae foi encontrada, o que seria mais tarde desconsiderado, visto que a espécie seqüenciada tratava-se, na verdade, do hilídeo *Smilisca phaeota*, e não de um membro da família Rhacophoridae (Hay *et al.*, 1995). Esses autores também reanalizaram, utilizando métodos de máxima parcimônia, os dados de Duellman & Trueb (1986). A nova avaliação desses caracteres morfológicos produziu dezoito árvores, com um consenso onde praticamente nada pode ser dito a respeito das relações entre as famílias de Neobatrachia. Isso acabou por estimular o uso de técnicas moleculares, visto que em pouco tempo de estudo os resultados foram bastante promissores.

Hillis *et al.* (1993) apresentaram, por sua vez, uma análise com dados de RNA ribossomal 28S, além de uma combinação com dados morfológicos. As relações suprafamiliais, onde a formação dos grupos Bufonoidea, Pipanura (Mesobatrachia, formado por Pelobatoidea e Pipoidea, associado a Neobatrachia) e Neobatrachia foi bem corroborada. Dendrobatidae foi tida como mais associado a Bufonoidea, ao contrário de

Ranoidea, como sugerido pelos dados morfológicos (Duellman & Trueb, 1986). Apesar dessa diferença, a concordância dos dados morfológicos com os moleculares aparentemente sugere uma estabilização da volátil classificação das famílias de anuros (ao menos das subordens).

Em seguida, valendo-se dos dados já dispostos na literatura para RNA 12S e introduzindo seqüências de 16S, Hay *et al.* (1995) analisaram as relações entre 28 famílias de anfíbios. Dessa vez, a monofilia das três ordens foi confirmada. Neobatrachia e Archaeobatrachia foram bem substanciadas como subordens. Em Archaeobatrachia os seguintes pares foram definidos: Pelobatidae+Pelodytidae, Pipidae+Rhinophrynidae, *Ascaphus*+Leiopelmatidae e *Bombina*+Discoglossidae. Em Neobatrachia três linhagens foram definidas: Bufonoidea (contendo Dendrobatidae), Ranoidea (contendo Microhylidae) e Sooglossidae. Apesar dessa consistência para as relações suprafamiliares, o grau de suporte para as relações entre as famílias foi baixo.

Feller & Hedges (1998) voltaram a debater as relações entre as ordens e forneceram novas e fortes evidências para uma relação mais próxima entre salamandras e cecílias, ao contrário da hipótese até então mais aceita, entre anuros e salamandras. Seus dados basearam-se nas seqüências de quatro genes de RNA mitocondrial. Em sua discussão, os autores repassam caracteres morfológicos que poderiam sustentar as duas hipóteses em conflito. Os dados do trabalho corroboram a relação cecílias+salamandras com base na análise de três espécies de cada ordem, bem como pela ambigüidade dos dados morfológicos, passíveis de sustentar tanto uma como outra hipótese. Com esse cenário, os autores especularam a respeito dos eventos biogeográficos que teriam levado à distribuição atual. Vences *et al.* (2000), utilizando seqüências de RNA mitocondrial 12S e 16S,

apresentam os primeiros resultados para as relações dentro da família Dendrobatidae. Os autores endossaram, novamente, a colocação de Dendrobatidae em Bufonoidea, ao contrário de Ranidae (Duellman & Trueb, 1986).

Diversos trabalhos apresentam, por meio do uso de técnicas moleculares, resultados que vêm esclarecendo cada vez mais as relações filogenéticas entre e dentro de gêneros de várias famílias de anfíbios (Graybeal, 1997; Pramuk *et al.*, 2001). Todavia, as relações entre as famílias, nas três ordens, permanecem sem uma hipótese convincente. Alguns autores julgam que com o acúmulo contínuo de seqüências de diferentes genes esse problema será, cedo ou tarde, sanado (Hedges *et al.*, 1990; Feller & Hedges, 1998). Ainda que exista um aparente avanço em curto espaço de tempo com a utilização de caracteres moleculares, não se pode esquecer que essas reconstruções só foram reconhecidas como significativas por terem corroborado muitos dados morfológicos acumulados ao longo de quase dois séculos. Uma congruência significativa em diversos níveis entre esses dois diferentes grupos de dados (Hillis *et al.*, 1993) é o que realmente acaba por nos convencer que estamos nos aproximando de soluções convincentes para as relações filogenéticas da classe. Ao contrário do proposto em alguns trabalhos (Hedges *et al.*, 1990; Hedges, 1996), não há razão, *a priori*, para julgar um grupo de dados melhor que outro (Sá & Hillis, 1993).

Assim, a introdução de um novo grupo de caracteres (moleculares) consolidou conclusões previamente obtidas por meio da morfologia e também forneceu novas e excitantes hipóteses a respeito da origem, das relações e da distribuição geográfica dos anfíbios. A introdução de um novo grupo de dados, e não qualquer característica superior intrínseca dos dados moleculares, pode ser responsabilizada por esses recentes avanços.

A utilização de um número maior de grupos de dados, aliada ao estudo contínuo dos

grupos de caracteres anteriores, parece uma abordagem mais promissora para a resolução da filogenia dos anfíbios do que o investimento em um único rol de dados, seja ele morfológico ou molecular. Além disso, novos conjunto de caracteres influenciam de forma substancial o conhecimento do grupo em estudo, de modo que a descrição detalhada de outros caracteres morfológicos e novas seqüências fornecerão bons resultados não só para a sistemática dos anfíbios, como também para a anatomia comparada, fisiologia, ecologia, biologia molecular e tantos outros campos de conhecimento desses animais.

Nos últimos anos a ultra-estrutura do espermatozóide tem sido usada como um grupo alternativo de caracteres para vários grupos animais, como para peixes (Jamieson, 1991; Buckland-Nicks, 1995), répteis (Jamieson, 1995; Teixeira *et al.*, 1999a,b) e invertebrados (Jamieson, 1987). Para anuros, a estrutura do espermatozóide foi uma das razões iniciais que levou à criação do gênero *Ololygon* (= *Scinax*) (Fouquette & Delahoussaye, 1977). Entretanto, a reconstrução filogenética por meio de técnicas cladísticas ainda não foi possível devido à escassez de dados para muitas famílias de anuros. Não obstante, alguns autores já propuseram diversas sinapormorfias e autapormofias para caracteres de ultra-estrutura do espermatozóide de anuros (Lee & Jamieson, 1992; Jamieson *et al.*, 1993; Jamieson, 1999; Scheltinga *et al.*, 2001). Com o intuito de confirmar essas sugestões bem como analisar a consistência dessa base de dados, urge descrever a ultra-estrutura do espermatozóide das famílias sobre as quais não há informação, além de criar um grupo de caracteres para os quais as espécies devem ser analisadas nesses estudos.

1.3 A ULTRA-ESTRUTURA DO ESPERMATOZÓIDE EM ANUROS

A morfologia dos espermatozóides de anfíbios é bastante diversa. O comprimento dos espermatozóides varia normalmente entre 40 e 110μm, mas pode chegar a 2300μm, como é o caso de *Discoglossus pictus* (Furieri, 1975). Células com o peculiar formato de um saca-rolhas, por exemplo, são encontradas na família Rhacophoridae (Mainoya, 1981; Mizuhira *et al.*, 1986; Jamieson, 1999). Em anuros da família Discoglossidae, um longo e espesso perforatório pode ser observado entrando no núcleo (Furieri, 1975; Pugin-Rios, 1980). Em outras famílias como Ranidae, Pipidae e Leptodactylidae (alguns membros da subfamília Telmatobiinae) o flagelo é formado apenas pelo axonema. Outras, tais como Bufonidae, Leptodactylidae (diversos), Hylidae e Rhinodermatidae apresentam uma cauda onde o axonema está ligado a uma membrana ondulante por meio de uma lâmina axial, que pode ser dilatada na extremidade de contato com os microtúbulos, formando a fibra justa-axonemal, e ao final da membrana ondulante, formando a fibra axial. A região da cabeça é composta pelo núcleo, vesícula acrossomal e estruturas associadas (perforatório, cone subacrossomal e espaço epinuclear). Essas estruturas são encontradas em combinações variadas nas espécies. Por uma questão de praticidade, o espermatozóide de anuros será dividido em três partes: cabeça, peça intermediária e cauda.

1.3.1 CABEÇA

Em geral, a cabeça é composta por diversas estruturas: uma vesícula acrossomal cônica que recobre o terço superior do longo e cilíndrico núcleo, um espaço epinuclear, um

cone subacrossomal, um perforatório endonuclear e núcleo. Combinações variadas dessas estruturas são observadas nas famílias de anuros. Em algumas espécies, tais como *Odontophrynus cultripes* (Báo *et al.*, 1991), *Hyla japonica* (Kwon & Lee, 1995), *Bufo* spp. (Burgos & Fawcett, 1956; Swan *et al.*, 1980; Lee & Jamieson, 1993), *Ascaphus truei* (James, 1970; Jamieson *et al.*, 1993) e *Leiopelma hochstetteri* (Scheltinga *et al.*, 2001), pode-se observar, abaixo do acrossoma, um cone subacrossomal que pode ser maciço (*A. truei* e *L hochstetteri*) ou formado por feixes individualizados (*Bufo* spp., e *H. japonica*). Feixes individualizados porém maiores e mais densos formam uma condição intermediária entre as formas descritas anteriormente (*O. cultripes*). O espaço epinuclear pode ser caracterizado como a região elétron lúcida abaixo do cone subacrossomal e acima do núcleo (*A. truei* e *L. hochstetteri*). Em certas espécies um perforatório endonuclear em forma de bastão pode ser observado (*A. truei* e *L. hochstetteri*). Essa estrutura é observada desde o início do espaço epinuclear até uma certa porção núcleo adentro, onde insere-se em um canal endonuclear.

Outras espécies, por sua vez, apresentam a região do acrossoma sem cone subacrossomal ou perforatório, de modo que a vesícula acrossomal fica bem próxima da membrana nuclear. Esse é o caso de *Rana* spp. (Poirier & Spink, 1971; Pugin-Rios, 1980), *Xenopus laevis* (Reed & Stanley, 1972; Bernardini *et al.*, 1986) e *Rhacophorus* spp. (Mizuhira *et al.*, 1986). Além disso, em *Rana* spp. a vesícula acrossomal possui a forma de um pequeno botão na região apical da célula, sem formar um cone recobrindo o núcleo.

Grau e forma de compactação da cromatina, de maneira semelhante, variam muito entre as espécies, podendo apresentar uma forma globular relaxada, onde diversos espaços nucleares podem ser vistos, como em *Batrachyla* spp. (Garrido *et al.*, 1989), ou uma

densidade maior, com espaços ausentes, como em *Rhacophorus* spp (Mizuhira *et al.*, 1986).

1.3.2 Peça Intermediária

A região da peça intermediária compreende a porção da fossa nuclear, onde insere-se o flagelo, os centríolos proximal e distal e a porção que contém as mitocôndrias. Alguns autores atentam para as diferenças dos ângulos entre os centríolos proximal e distal (Kwon & Lee, 1995). A disposição das mitocôndrias é uma das características mais lábeis entre diferentes espécies. Um grande colar de mitocôndrias é observado em *Bufo* spp. (Burgos & Fawcett, 1956; Swan *et al.*, 1980; Lee & Jamieson, 1993), *Melanophryniscus cambaraensis* (Báo *et al.*, 2001) e *Ciclorana* spp. (Meyer *et al.*, 1997). *Ascaphus truei* (Jamieson *et al.*, 1993) apresenta as mitocôndrias em contato direto com os componentes auxiliares da cauda, no meio da membrana ondulante. Um pequeno colar é observado em *Leiopelma hochstetteri* (Scheltinga *et al.*, 2001), ao passo que na família Myobatrachidae (Lee & Jamieson, 1992) as mitocôndrias podem ser observadas ao redor da fibra axial. A fossa nuclear possui um material de moderada elétron densidade, o material pericentriolar. Nas Famílias que possuem fibra axial, essa pode acompanhar o axonema até o centríolo distal na região de inserção, como em Bufonidae (Lee & Jamieson, 1993) ou entrar na fossa nuclear e posicionar-se ao lado do centríolo proximal, como em Myobatrachidae (Lee & Jamieson, 1992). Outras estruturas são ocasionalmente observadas, como as fibras periféricas do axonema. Pequenos adensamentos podem ser observados ao lado de cada par externo de microtúbulos na região próxima à inserção do flagelo (Pugin-Rios, 1980).

1.3.3 CAUDA

Assim como a região acrossomal, a cauda também varia muito em anuros. Pode-se observar, como foi mencionado, flagelos formados unicamente pelo axonema, como é o caso de Pipidae (Reed & Stanley, 1972; Bernardini *et al.*, 1986), Ranidae (Poirier & Spink, 1971; Pugin-Rios, 1980), Microhylidae (Jamieson, 1999), alguns membros da família Leptodactylidae (Pisanó & Adler, 1968; Pugin-Rios, 1980; Pugin & Garrido, 1981) e alguns membros da família Pelobatidae (*Scaphiopus holbrookii*) (James, 1970). Outras espécies apresentam, além do axonema, uma membrana ondulante. Essa possui em uma das extremidades uma fibra axial, geralmente mais espessa que o axonema. Na extremidade oposta a membrana ondulante liga-se ao axonema no 3° par de microtúbulos. A região da membrana ondulante aloja um material fino e de elétron densidade semelhante às fibras, a lâmina axial. Esse é o caso, por exemplo, de Bufonidae (Burgos & Fawcett, 1956; Lee & Jamieson, 1993; Báo *et al.*, 2001), Hylidae (Rastogi *et al.*, 1988; Meyer *et al.*, 1997), Ascaphidae (Jamieson *et al.*, 1993), Leiopelmatidae (Scheltinga *et al.*, 2001), Discoglossidae e Rhinodermatidae (Pugin-Rios, 1980). Ainda, uma terceira fibra no oitavo par de microtúbulos, à semelhança de urodelos, é observada em *Leiopelma hochstetteri* (Scheltinga *et al.*, 2001). O número de axonemas também pode variar: Pelobatidae (James, 1970), Rhacophoridae (Mainoya, 1981; Mizuhira *et al.*, 1986) e alguns leptodactilídeos (*Telmatobufo australis*, em Pugin-Ríos, 1980) possuem dois axonemas formando a cauda. Essa biaxonemalidade, todavia, é bem distinta entre essas espécies: a família Rhacophoridae possui dois axonemas na mesma cauda embebidos numa matriz de centenas de microtúbulos (Jamieson, 1999); o pelobatídeo *Scaphiopus holbrookii* possui dois

axonemas dentro da mesma cauda, enquanto uma verdadeira biflagelaridade pode ser observada em *Telmatobufo australis*, onde os dois axonemas estão fisicamente isolados pela membrana plasmática, formado dois flagelos individualizados.

Condições intermediárias entre a presença e ausência de elementos auxiliares caudais (membrana ondulante e fibras axial e justa-axonemal) são observadas em algumas espécies. *Hyla meridionalis* (Pugin-Rios, 1980) e *H. japonica* (Lee & Kwon, 1992) apresentam uma única fibra ao lado do axonema e sua membrana ondulante está ausente. O gênero *Physalaemus* da família Leptodactylidae, por sua vez, não possui a fibra axial, mas a membrana ondulante e uma fibra justa-axonemal bem desenvolvidas estão presentes (Amaral *et al.*, 1999).

1.3.4 Famílias Estudadas

As espécies utilizadas na revisão bibliográfica desta tese estão listadas, segundo as suas famílias, na Tabela 1. Podemos notar que algumas famílias apresentam uma grande quantidade de espécies descritas (Leptodactylidae e Hylidae), enquanto outras possuem apenas um indivíduo estudado (Pipidae).

Outro problema com a presente base de dados reside na imprecisão da descrição do espermatozóide de diversas espécies. Muitos trabalhos foram feitos com um cunho puramente morfológico e, desse modo, diversas estruturas importantes e passíveis de serem utilizadas em comparações filogenéticas não foram contempladas.

É notória a ausência de diversas famílias da região Neotropical: Brachycephalidae, Dendrobatidae, Pseudidae, Centrolenidae e Rhinophrynidae. A família Microhylidae apresenta uma pequena menção em Jamieson (1999) e sua ultra-estrutura detalhada ainda

não foi publicada. Além dessas famílias, Hyperoliidae, Heleophrynidae (África do Sul) e Sooglossidae (Ilhas Seychelles) ainda carecem de descrições de qualquer um de seus membros.

Tabela 1: Relação das espécies de anuros sobre as quais existem informações sobre a ultra-estrutura do espermatozóide e os trabalhos utilizados na presente tese.

Famílias e Subfamílias	Espécie	Trabalhos
ASCAPHIDAE		
	Ascaphus truei	James (1970), van der Horst (1979), Jamieson *et al.* (1993), Lee & Kwon (1996)
DISCOGLOSSIDAE		
	Alytes obstetricans	Pugin-Ríos (1980)
	Bombina orientalis	Kwon & Lee (1995), Lee & Kwon (1996)
	B. variegata	Furieri (1975), Pugin-Ríos (1980)
	Discoglossus pictus	Favard (1955), Pugin-Ríos (1980), Lee & Kwon (1996)
LEIOPELMATIDAE		
	Leiopelma hochstetteri	Scheltinga *et al.* (2001)
PELOBATIDAE		
	Megophrys montana	Asa & Phillips (1988)
	Scaphiopus holbrookii	James (1970)
PELODYTIDAE		
	Pelodytes punctatus	Pugin-Ríos (1980)
PIPIDAE		
	Xenopus laevis	James (1970), Reed & Stanley (1972), Bernardini *et al.* (1986), Yoshizaki (1987)
BUFONIDAE		
	Bufo arenarum	Burgos & Fawcett (1956), Lopez *et al.* (1983), Lemos *et al.* (1983)
	B. bufo	Pugin-Ríos (1980), Mo (1985), Kwon & Lee (1995)
	B. calamita	Pugin-Ríos (1980)
	B. chilensis	Pugin & Garrido(1981)
	B. marinus	Swan *et al.* (1980), Lee & Jamieson (1993)
	B. rangeri	van der Horst (1979)
	B. variegatus	Pugin-Ríos (1980), Pungin & Garrido (1981)
	B. vulgaris	Nicander (1970)
	Melanophryniscus cambaraensis	Báo *et al.* (2001)
	Nimbaphrynoides occidentalis	Pugin-Ríos (1980)

Tabela 1 (continuação)

Famílias e Subfamílias	Espécie	Trabalhos
HYLIDAE		
	Ciclorana brevipes	Meyer *et al.* (1997)
	C. cryptotis	Meyer *et al.* (1997)
	C. novaehollandiae	Meyer *et al.* (1997)
	Hyla japonica	Kwon & Lee (1995)
	H. meridionalis	Pugin-Ríos (1980)
	Litoria albogutatta	Meyer *et al.* (1997)
	L. aurea	Meyer *et al.* (1997)
	L. caerulea	Lee & Jamieson (1993)
	L. fallax	Lee & Jamieson (1993)
	L. gracilenta	Lee & Jamieson (1993)
	L. lesueuri	Lee & Jamieson (1993)
	L. moorei	Meyer *et al.* (1997)
	L. peronii	Lee & Jamieson (1993)
	L. rubella	Lee & Jamieson (1993)
	Pachymedusa dacnicolor	Rastogi *et al.* (1988)
	Scinax ranki	Taboga & Dolder (1993), Taboga & Dolder (1994), Taboga & Dolder (1998)
LEPTODACTYLIDAE		
subf. Telmatobiinae	*Alsodes vittatus*	Pugin-Ríos (1980)
	Batrachyla antartandica	Pugin-Ríos (1980), Pugin & Garrido (1981), Garrido *et al.* (1989)
	B. leptopus	Pugin-Ríos (1980), Pugin & Garrido (1981), Garrido *et al.* (1989)
	B. taeniata	Pugin-Ríos (1980), Pugin & Garrido (1981), Garrido *et al.* (1989)
	Caudiverbera caudiverbera	Pugin-Ríos (1980), Pugin & Garrido (1981)
	Eupsophus roseus	Pugin-Ríos (1980), Pugin & Garrido (1981)
	Hylorina sylvatica	Pugin-Ríos (1980), Pugin & Garrido (1981)
	Telmatobius hauthali	Pisanó & Adler (1968)
	Telmatobufo australis	Pugin-Ríos (1980), Pugin & Garrido (1981)
	Odontophrynus cultripes	Báo *et al.* (1991), Fernandes & Báo (1998)
subf. Ceratophryinae	*Lepidobatrachus laevis*	Waggener & Carroll (1998)
subf. Leptodactylinae	*Physalaemus biligonigerus*	Amaral *et al.* (1999)
	P. fuscomaculatus	Amaral *et al.* (1999)
	P. gracilis	Amaral *et al.* (1999)

Tabela 1: (Continuação)

Famílias e Subfamílias	Espécie	Trabalhos
subf. Leptodactylinae	*Pleurodema thaul*	Pugin-Ríos (1980), Pugin & Garrido (1981)
	Pseudopaludicola falcipes	Amaral *et al.* (2000)
MICROHYLIDAE		
	Cophixalus ornatus	Jamieson (1999)
MYOBATRACHIDAE		
	Adelotus brevis	Lee & Jamieson (1993)
	Limnodynastes peronii	Lee & Jamieson (1992)
	Mixophyes fasciolatus	Lee & Jamieson (1992)
	Neobatrachus pelobatoides	Lee & Jamieson (1992)
RANIDAE		
	Rana clamitans	Poirier & Spink (1971)
	R. dalmatina	Pugin-Ríos (1980)
	R. dybowskii	Kwon & Lee (1995)
	R. esculenta	Pugin-Ríos (1980)
	R. japonica	Yoshizaki (1987)
	R. nicromaculata	Mo (1985)
	R. pipiens	Poirier & Spink (1971)
	R. ridbunda	Pugin-Ríos (1980)
	R. rugosa	Kwon & Lee (1995)
	R. temporaria	Pugin-Ríos (1980)
RHACOPHORIDAE		
	Chiromantis xerampelina	Mainoya (1981), Wilson *et al.* (1991) , Jamieson (1999)
	Rhacophorus arboreus	Mizuhira *et al.* (1986)
	R. schlegelii	Mizuhira *et al.* (1986)
RHINODERMATIDAE		
	Rhinoderma darwinii	Pugin-Ríos (1980), Pugin & Garrido (1981)
	R. rufum	Pugin-Ríos (1980), Pugin & Garrido (1981)

ARCHAEOBATRACHIA

ASCAPHIDAE

A ultra-estrutura do espermatozóide de *Ascaphus truei* foi analisada em dois trabalhos (James, 1970; Jamieson *et al.*, 1993). A região acrossomal é formada pela vesícula acrossomal cônica, por um cone subacrossomal maciço e bem desenvolvido e por um perforatório em bastão alojado dentro de um canal nuclear, considerado uma plesiomorfia para Anura. A membrana ondulante é reduzida e possivelmente pedomórfica (Jamieson *et al.*, 1993). As fibras auxiliares são condensadas, formando uma estrutura com um sulco central que contém as mitocôndrias. Essa fibra única e espessa observada em *A. truei* é chamada bastão paraxonemal (sensu Jamieson *et al.*, 1993), o qual é análogo ao conjunto formado pelas fibra axial, justa-axonemal e lâmina axial.

DISCOGLOSSIDAE

A família Discoglossidae teve a ultra-estrutura de quatro representantes analisada. Em *Bombina variegata* (Furieri, 1975; Pugin-Rios, 1980) o espermatozóide é extremamente peculiar. A célula não possui a forma característica de cabeça, peça intermediária e cauda. Ao invés disso, a fossa nuclear está no terço superior do núcleo. O acrossoma termina bruscamente com uma forma cilíndrica onde um grande perforatório é observado. De maneira semelhante, *B. orientalis* (Kwon & Lee, 1995; Lee & Kwon, 1996) também possui a inserção do flagelo no ápice da célula e a estrutura da região apical é a mesma. *Discoglossus pictus* (Sandoz, 1973; Pugin-Rios, 1980) e *Alytes obstetricans* (Pugin-Rios, 1980) não apresentam a forma peculiar de *Bombina*, mas compartilham com

essa diversas características, tais como a presença de um espesso perforatório endonuclear, a terminação abrupta e a forma cilíndrica da região apical, a presença de membrana ondulante, fibras auxiliares e mitocôndrias adjacentes à fibra axial.

LEIOPELMATIDAE

A ultra-estrutura de *Leiopelma hochstetteri* foi recentemente descrita (Scheltinga *et al.*, 2001). Seu espermatozóide é plesiomórfico e apresenta várias características não observadas em outros anuros. A região acrossomal apresenta um cone subacrossomal bem distinto; em secções transversais a região acrossomal é angulada (circular na região apical e variando de quadrado até um heptágono na a base da vesícula acrossomal). Um pequeno colar de mitocôndrias é observável. A cauda possui uma fibra axial, membrana ondulante e fibra justa-axonemal associada ao terceiro par de microtúbulos. Ainda, uma terceira fibra auxiliar associada ao oitavo par de microtúbulos pode ser observada (de maneira semelhante ao encontrado em salamandras).

PELOBATIDAE

Scaphiopus holbrookii (James, 1970) e *Megophrys montana* (Asa & Phillips, 1988) são os membros da família Pelobatidae aqui considerados [*Scaphiopus. couchi* foi analisado na tese de Morriset (1974). Todavia, a mesma não pode ser recuperada na revisão bibliográfica]. Ambas espécies possuem dois axonemas formando a cauda. *S. holbrookii* possui um espermatozóide helicoidal, assim como *M. montana*, que difere de *S. holbrookii* devido à presença de uma fibra auxiliar adjacente aos dois axonemas.

PELODYTIDAE

A única espécie descrita na família foi *Pelodytes punctatus* (Pugin-Rios, 1980). A célula apresenta membrana ondulante e cone subacrossomal maciço. O colar de mitocôndrias não é observável e as mesmas aparentemente se encontram na região terminal do núcleo.

PIPIDAE

Provavelmente em função da sua ampla utilização em laboratório, o pipídeo *Xenopus laevis* foi estudado diversas vezes (James, 1970; Reed & Stanley, 1972; van der Horst, 1979; Pugin-Rios, 1980; Bernardini *et al.*, 1986; Yoshizaki, 1987). As características particulares dessa espécie são o espermatozóide em S, o grupo de membranas multilaminares na região acrossômica e a cauda sem membrana ondulante ou fibras auxiliares. Ainda, fibras periaxiais são observadas ao lado de cada um dos pares de microtúbulos externos na região da peça intermediária.

NEOBATRACHIA

BUFONIDAE

A família Bufonidae possui um gênero bem estudado (*Bufo*). O primeiro trabalho é o de Burgos & Fawcett (1956), onde a ultra-estrutura do espermatozóide de *Bufo arenarum* foi descrita. As características fundamentais da família foram descritas nesse trabalho que mesmo sendo um dos mais antigos é um dos mais completos. Dentre as principais observações feitas, a presença de membrana ondulante e fibras axial e justa-axonemal na cauda, a presença de colar de mitocôndrias e o cone subacrossomal dividido em fibras

individualizadas são as mais importantes. Essa forma do espermatozóide repete-se em *B. marinus* (Swan *et al.*, 1980; Lee & Jamieson, 1993), *B. bufo* (Pugin-Rios, 1980; Kwon & Lee, 1995), *B. calamita* (Pugin-Rios, 1980), *B. chilensis* (Pugin & Garrido, 1981) e *B. variegatus* (Pugin-Rios, 1980).

Apenas dois outros gêneros foram estudados: *Nimbaphrynoides occidentalis* (=*Nectophrynoides occidentalis*) (Pugin-Rios, 1980) e *Melanophryniscus cambaraensis* (Báo *et al.*, 2001). *Nimbaphrynoides occidentalis* possui um cone subacrossomal maciço, sem fibras individualizadas. Além disso, as mitocôndrias não se encontram em um anel, estando na verdade adjacentes ao axonema, onde a fibra justa-axonemal está ausente. Membrana ondulante e fibra axial são observadas e a última insere-se na fossa nuclear. *Melanophryniscus cambaraensis* apresenta a cauda muito semelhante a *Bufo* spp., com um colar de mitocôndrias, membrana ondulante e fibras auxiliares presentes. O cone subacrossomal é mais espesso que *Bufo* spp. e o bastão paraxonemal não entra na fossa nuclear, ligando-se à mesma por estrias transversais.

HYLIDAE

A família Hylidae tem 15 espécies descritas em 4 gêneros. *Hyla meridionalis* (Pugin-Rios, 1980) e *H. japonica* (Lee & Kwon, 1992; Kwon & Lee, 1995) apresentam a membrana ondulante reduzida, com apenas um bastão paraxonemal presente. As demais espécies, *Pachymedusa dacnicolor* (Rastogi *et al.*, 1988), *Ciclorana* spp. e *Litoria* spp. (Lee & Jamieson, 1993; Meyer *et al.*, 1997) possuem a membrana ondulante e diferem em estruturas pormenores, como espessura da lâmina axial e fibra justa-axonemal. Todas as espécies possuem cone subacrossomal dividido em fibras.

LEPTODACTYLIDAE

Uma das famílias mais estudadas, teve 16 espécies descritas até o presente, compreendendo três subfamílias e 12 gêneros. A variação morfológica do espermatozóide é grande. Membros da subfamília Telmatobiinae, *Telmatobius hauthali* (Pisanó & Adler, 1968), *Caudiverbera caudiverbera* e *Telmatobufo australis* (Pugin-Rios, 1980), não possuem elementos auxiliares na cauda, enquanto outras espécies da mesma subfamília, como *Odontophrynus cultripes* (Báo *et al.*, 1991), *Batrachyla* spp., *Eupsophus roseus* e *Alsodes vittatus* (Pugin-Rios, 1980) possuem.

Três gêneros da subfamília Leptodactylinae foram analisados: *Pleurodema* (Pugin-Rios, 1980), que não apresenta cone subacrossomal e o colar de mitocôndrias, *Physalaemus* (Amaral *et al.*, 1999), onde as três espécies descritas curiosamente não apresentam a fibra axial e *Pseudopaludicola* (Amaral *et al.*, 2000), onde todas as fibras caudais são observadas. Os dois últimos gêneros possuem cone subacrossomal em fibras e colar de mitocôndrias.

Lepidobatrachus laevis foi descrito para a subfamília Ceratophryinae (Waggener & Carroll, 1998). A espécie foi considerada biflagelar pelos autores, mas essa conclusão foi feita com base em uma interpretação errônea das micrografias. O cone subacrossomal está presente e bem desenvolvido, ao passo que a cauda é formada por membrana ondulante e fibras auxiliares. Um colar de mitocôndrias não pode ser observado.

MICROHYLIDAE

A família nunca teve a ultra-estrutura de qualquer membro completamente descrita, mas algumas considerações sobre *Cophixalus ornatus* foram feitas por Jamieson (1999). Segundo o autor, o espermatozóide seria apomórfico devido à perda da membrana ondulante e das fibras auxiliares, estando o axonema sozinho na cauda.

MYOBATRACHIDAE

Três gêneros foram analisados, contendo as espécies *Limnodynastines peronii*, *Mixophyes fasciolatus* e *Neobatrachus pelobatoides* (Lee & Jamieson, 1992; Jamieson, 1999). O cone subacrossomal apresenta-se em feixes de fibras (*N. pelobatoides* e *L. peronii*) e maciço (*M. fasciolatus*). As mitocôndrias apresentam-se aderidas à fibra axial e essa insere-se profundamente na fossa nuclear. Membrana ondulante e fibras auxiliares são observadas nos três gêneros.

RANIDAE

Ainda que 9 espécies tenham sido abordadas na família, todas pertencem ao gênero *Rana*, os outros 46 ainda não tendo sido estudados. Em todas as espécies o flagelo é composto apenas do axonema. O cone subacrossomal é ausente e a vesícula acrossomal não tem a forma cônica, apresentando-se na verdade como um botão apical ou lateralmente disposto.

RHACOPHORIDAE

A família Rhacophoridae teve três espécies descritas:. *Chiromantis xerampelina*

(Mainoya, 1981; Jamieson, 1999), *Rhacophorus arboreus* e *R. schlegelii* (Mizuhira *et al.*, 1986). O espermatozóide é bastante peculiar, apresentando um formato de saca-rolhas, com dois axonemas embebidos em uma matriz de centenas de microtúbulos. Cone subacrossomal, membrana ondulante e fibras auxiliares estão ausentes. A vesícula acrossomal é lateralmente disposta, acompanhando a espiral condensada da célula.

RHINODERMATIDAE

Rhinoderma darwinii e *R. rufum* foram analisadas por Pungin-Ríos (1980) e Pungin & Garrido (1981). O espermatozóide das duas espécies é bastante similar e apresenta características típicas dos Bufonoidea, com cone o subacrossomal desenvolvido, membrana ondulante e fibras auxiliares.

1.4 ULTRA-ESTRUTURA DO ESPERMATOZÓIDE E FILOGENIA DE ANUROS

Desde as primeiras publicações onde a ultra-estrutura do espermatozóide de um anuro foi analisada (Burgos & Fawcett, 1956), os trabalhos apresentaram um cunho descritivo, sem inferências ou comparações devido, obviamente, ao pequeno número de espécies estudadas. O crescente acúmulo de novas espécies descritas começou a ampliar as possibilidades de uso desses caracteres. Duas teses apresentaram contribuições importantes. James (1970) incluiu a descrição de três famílias (Ascaphidae, Pelobatidae e Pipidae). Uma das contribuições mais importantes, a tese de Pungin-Ríos (1980) acrescentou a descrição de 25 espécies em 8 famílias.

O primeiro trabalho comparativo foi justamente a tese de Pungin-Ríos (1980). Aqui,

o autor sugere hipóteses de relação entre as famílias por ele estudadas, de acordo com a estrutura do perforatório e vesícula acrossomal. A estrutura do perforatório formou três agrupamentos: cilíndrico em Discoglossidae, cônico (= cone subacrossomal) em Pelobatidae, Leptodactylidae, Bufonidae, Rhinodermatidae e Hylidae e ausente em Ranidae. A simetria da vesícula acrossomal formou também três grupos: simetria simples em Discoglossidae (*Bombina* e *Alytes*), Pelobatidae e Leptodactylidae (*Telmatobufo* e *Telmatobius*), simetria diferencial em Discoglossidae (*Discoglossus*), Leptodactylidae (*Batrachyla* spp., *Alsodes* e *Hylorina*), Bufonidae, Rhinodermatidae e Hylidae. Em Ranidae a disposição é assimétrica.

Mais de uma década depois, Lee & Jamieson (1992), ao descreverem a ultra-estrutura da família Myobatrachidae, inferiram uma série de implicações derivadas da comparação entre 42 espécies em 11 famílias de anuros analisadas até então. Os espermatozóides das três espécies descritas de Myobatrachidae são bastante semelhantes, a despeito das diferenças entre a biologia reprodutiva dos mesmos. Com relação à ordem, suas análises concluíram que diversos caracteres eram compartilhados pelos anuros, dentre eles o acrossoma cônico, a presença de material subacrossomal e a membrana ondulante "sustentada" por uma fibra axial. Os autores ainda sugerem que a subordem Bufonoidea (=Hyloidea) aparentemente seria monofilética devido à presença, em todas as espécies do grupo (à exceção de *Caudiverbera caudiverbera* e *Pleurodema thaul*), de um perforatório cônico subacrossomal. Esse não seria homólogo ao perforatório em bastão endonuclear devido a diferenças de posição e conformação das duas estruturas. Ainda, o perforatório cônico não seria homólogo ao cone subacrossomal visto que as duas estruturas diferem em elétron densidade, além do cone ser mais homogêneo e de apresentar ocasionais fibras

perpendiculares ao eixo da célula, ao passo que o perforatório cônico é sempre fibrilar e paralelo ao eixo da célula. Ainda, o cone apresenta um contato íntimo com o núcleo e a membrana do acrossoma, ao contrário da distribuição relaxada e livre do perforatório cônico. Essas justificativas endossaram a sugestão, pela primeira vez, de uma sinapomorfia de Bufonoidea.

Em seguida, Lee & Jamieson (1993), ao apresentarem dados a respeito da ultra-estrutura das famílias Bufonidae e Hylidae, reafirmaram o perforatório cônico como sinapomorfia dos Bufonoidea. Ainda, os dados substanciaram uma separação de Myobatrachidae e Eubufonoidea (Hylidae, Bufonidae e Leptodactylidae do novo mundo). A presença de um colar de mitocôndrias no último contrasta com a distribuição no primeiro, onde essas organelas encontram-se associadas à fibra axial. Mais adiante os autores afirmam que a morfologia constante do espermatozóide em Bufonoidea, apesar de permitir a inferência de relações suprafamiliais, acabava por impedir a comparação entre famílias.

Jamieson *et al.* (1993) reavaliaram a ultra-estrutura de *Ascaphus truei*, previamente descrita por James (1970). Além de corroborar o perforatório cônico como sinapomorfia de Bufonoidea, os autores sugeriram que a condição dessa espécie seria plesiomórfica para anuros, devido à presença de um perforatório em bastão localizando em um canal nuclear. Ainda, a complexidade do espermatozóide dessa espécie substanciaria uma fecundação originalmente interna. A aparente correlação entre fecundação em ambientes aquáticos e simplicidade do espermatozóide (Rouse & Jamieson, 1987) levou os autores a sugerir que a condição original em anuros seria a fecundação interna, e que, conseqüentemente, famílias derivadas com reprodução aquática possuiriam espermatozóides mais simples (como é o

caso de Ranidae).

Kwon & Lee (1995) endossaram conclusões anteriores. Segundo os autores, a ultra-estrutura do espermatozóide separava as famílias de anuros em três grupos: Ascaphidae+Discoglossidae com caracteres plesiomórficos, Myobatrachidae +Bufonidae+Hylidae+Leptodactylidae com Myobatrachidae sendo ancestral do grupo e Ranidae+Pipidae+Rhacophoridae como o grupo mais derivado devido à ausência de elementos de cauda e ângulo de 140° entre os centríolos.

Em um compêndio sobre a filogenia baseada na ultra-estrutura do espermatozóide em vertebrados, Jamieson (1999) caracteriza o espermatozóide de anuros e avalia as tendências centrais da evolução dessa célula. Nesse trabalho o autor classifica diversas estruturas do espermatozóide como sinapomorfias, simplesiomorfias e autapomorfias a partir da comparação entre os membros de Lissamphibia e os outros vertebrados. Archaeobatrachia seria parafilético e Neobatrachia seria monofilético e unido pela ausência de um perforatório endonuclear e presença de um perforatório cônico. O autor também menciona a possibilidade de homologia entre cone subacrossomal e o perforatório cônico, visto que ambos ocupam a mesma posição. Por ser uma revisão, o capítulo volta a levantar conclusões prévias a respeito das relações entre várias famílias [como a separação de Myobatrachidae e Eubufonoidea previamente proposta por Lee & Jamieson (1992)].

Em virtude da boa disponibilidade de dados para Archaeobatrachia, Scheltinga *et al.* (2001) fizeram uma análise filogenética preliminar para avaliar as relações dentro da ordem anura. Usando cinco caracteres do espermatozóide de uma salamandra, seis espécies (em cinco famílias) de Archaeobatrachia e uma espécie de Neobatrachia. As conclusões desse

trabalho mostraram que *Leiopelma hoshtetteri* seria provavelmente o anuro mais próximo de salamandras. Assim, devido à presença de uma série de caracteres plesiomóficos, como a fibra justa-axonemal no oitavo par de microtúbulos, essa espécie e não *Ascaphus truei* seria o anuro mais basal.

Com isso, vemos a possibilidade da utilização da ultra-estrutura do espermatozóide para análises filogenéticas. Entretanto, alguns problemas com a base de dados ainda impedem uma avaliação melhor desses caracteres de modo que possamos saber se há sinal filogenético nos mesmos, bem como em que nível taxonômico eles são mais úteis.

1.5 Motivação

A necessidade de conhecer as relações históricas entre as famílias de qualquer táxon em estudo reside na forte influência que a filogenia exerce sobre estudos comparativos. A falta de uma hipótese de relações consistente para as famílias de anuros implica numa deficiência na nossa compreensão a respeito da ordem. A introdução de novos grupos de caracteres (moleculares) mostrou-se muito frutífera e promissora. Os últimos 15 anos mostraram um avanço crescente do uso da ultra-estrutura do espermatozóide para a análise filogenética de diversos grupos, como insetos (Jamieson, 1987), peixes (Jamieson, 1991) e, recentemente, répteis (Jamieson, 1995; Teixeira *et al.*, 1999a; Teixeira *et al.*, 1999b).

Com apenas uma exceção (Scheltinga *et al.*, 2001), todas as sugestões até aqui colocadas para as relações entre as famílias de anuros usando ultra-estrutura do espermatozóide são resultado de comparações baseadas na análise de caracter por caracter. A cladística moderna, entretanto, desencoraja esse tipo de abordagem. Com efeito, o

enfoque mais adequado passa pela construção de uma tabela de caracteres e subseqüente análise filogenética dos mesmos, com todos os dados, desse modo, sendo avaliados em conjunto. Só após essas análises pode-se sugerir, por exemplo, se um caracter é uma sinapomorfia ou uma simplesiomorfia dos táxons em questão. Contudo, uma análise filogenética usando caracteres dos espermatozóides atualmente não é viável devido à ausência de dados a respeito de muitas famílias.

Desse modo, a chance de contribuir com o número de famílias de anuros descritas e desse modo possibilitar a avaliação do uso da ultra-estrutura do espermatozóide para a reconstrução filogenética de anuros motivaram a presente tese.

1.6 Objetivos

A partir do que foi anteriormente exposto, os principais objetivos desta tese consistem em:

- Descrever a ultra-estrutura do espermatozóide de anuros de três famílias (Dendrobatidae, Microhylidae e Pseudidae) ainda não estudadas;
- Sugerir, a partir de comparações com as espécies já descritas, possíveis implicações para a filogenia e a biologia reprodutiva em famílias de anuros, tendo em vista os dados aqui acrescidos.
- Propor um grupo de caracteres da ultra-estrutura do espermatozóide passíveis de serem utilizados em uma futura análise filogenética de anuros.

2. ARTIGOS

A presente tese resultou na produção de quatro manuscritos submetidos e em fase final de preparação:

1) **Garda, A. A.**, Colli, G.R., Aguiar-Júnior, O., Recco-Pimentel, S. M. and Báo, S. N. The ultrastructure of the spermatozoa of *Epipedobates flavopictus* (Dendrobatidae, Anura, Amphibia), with comments on its evolutionary significance. (Submetido).
2) Aguiar-Júnior, O., **Garda, A. A.**, Lima, A P., Colli, G. R., Báo, S. N. and Recco-Pimentel, S. M.. The biflagellate spermatozoon of the dart-poison frogs *Epipedobates femoralis* and *Colostethus* sp. (Anura, Dendrobatidae). Journal of Morphology (No Prelo).
3) **Garda, A. A.**, Colli, G.R., Costa, G. C. and Báo, S. N. The spermatozoa of the family Pseudidae (Anura, Amphibia), with a test of the hypothesis of correlation between ultrastructure and reproductive modes in anurans. (Submetido).
4) Scheltinga, D. M., Jamieson, B. G. M., Bickford, D. P., **Garda, A. A.**, Báo, S. N. and McDonald, K.R. Morphology of the spermatozoa of the Microhylidae (Anura, Amphibia). *Acta Zoologica* (Stockholm) (No Prelo).

Os artigos 1 e 2 fazem parte também da tese de Odair Aguiar Júnior e estão apresentados em ambas dissertações não apenas por serem artigos produzidos em colaboração, mas principalmente por serem utilizados em abordagens distintas e gerarem conclusões diferentes nas duas teses em questão.

FAMÍLIA DENDROBATIDAE

The ultrastructure of the spermatozoa of *Epipedobates flavopictus* (Amphibia, Anura, Dendrobatidae), with comments on its evolutionary significance

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Running title: Sperm ultrastructure of *Epipedobates flavopictus*.

Keywords: Sperm, Ultrastructure, Anura, Dendrobatidae, *Epipedobates*.

Acknowledgements: We thank Ariovaldo A. Giaretta, Cristiane G. Batista, and Osmindo R. Pires Jr. for providing specimens, an anonymous reviewer for helpful suggestions on an earlier version of the manuscript, and all the staff of the Laboratório de Fisiologia Animal at Universidade de Brasília. This work was supported by Fapesp, by a master's fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES to AAG, and by research fellowships from CNPq to GRC (# 302343/88-1) and SNB (# 520130/97-9).

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Abstract – We describe, for the first time, the spermatozoon ultrastructure of a dendrobatid frog, *Epipedobates flavopictus*. Mature spermatozoa of *E. flavopictus* are filiform, with a moderately curved head and a proportionally short tail. The acrosomal vesicle is a conical structure that covers the anterior portion of the nucleus. A homogeneous subacrosomal cone lies between the acrosome vesicle and the nucleus. The nucleus contains a nuclear space at its anterior end, and electron-lucent spaces and inclusions. No perforatorium is present. In the midpiece, the proximal centriole is housed inside a deep nuclear fossa. Mitochondria are scattered around the posterior end of the nucleus and inside the undulating membrane in the anterior portion of the tail. The tail is formed by an U-shaped axial fiber connected to the axoneme through an axial sheath, which supports the undulating membrane. The juxta-axonemal fiber is absent. The spermatozoon of *E. flavopictus* has several characteristics not observed before in any anurans, such as a curved axial fiber, absence of a juxta-axonemal fiber, and presence of mitochondria in the typical undulating membrane. Our results endorse the view that, in anurans, the conical perforatorium and subacrosomal cone are homologous and that Dendrobatidae should be grouped within Bufonoidea rather than Ranoidea.

Introduction

The phylogenetic relationships among families of Anura are still largely unresolved (Duellman & Trueb, 1986; Ford & Cannatella, 1993). Groups widely accepted as monophyletic have often been challenged with new phylogenetic reconstructions and the continuous accumulation of new data. For example, Hillis et al. (1993) using 28S fragments of rRNA found Neobatrachia to be polyphyletic. Contents of groups such as Bufonoidea and Ranoidea are in a constant state of flux because of the addition and exclusion of families, such as Dendrobatidae (Ford, 1993; Ford & Cannatella, 1993). At the family level, the resolution of most phylogenetic trees is very poor, the relations between most clades being largely unresolved, while two of the major families (Leptodactylidae and Ranidae) are generally considered polyphyletic (Ford & Cannatella, 1993).

Reasons for this lack of resolution range from the limited utility of external morphology (Inger, 1967) to the great paucity of data for tropical lineages. Analyses using alternative data sets, such as molecular markers (De Sá & Hillis, 1990; Hillis et al., 1993; Hay et al., 1995), have slowly added new insights to the problem but also have refuted well-established clades. Filling the gaps on existing data sets and exploring new kinds of characters are important ways to improve phylogenetic hypotheses among anurans (Ford, 1993).

The ultrastructure of spermatozoon has been used as an alternative data set to investigate the phylogeny of many taxa such as fishes (Jamieson, 1991; Tanaka et al., 1995), amphibians (Lee & Jamieson, 1992, 1993; Jamieson et al., 1993; Scheltinga et al., 2001), reptiles (Jamieson, 1995; Teixeira et al., 1999a,b), and invertebrates (Jamieson, 1987). An advantage of sperm ultrastructure data is that they provide more conservative characters for groups with highly derived body plans, such as Amphisbaenia, which cannot

be scored for some traditional morphological traits (Teixeira et al., 1999b). Spermatozoon ultrastructure data have also been useful in clarifying relationships among Polyplacophora, where traditionally used characters are either too conserved or too variable (Buckland-Nicks, 1995). Spermatozoon morphology, therefore, seems to be useful for groups where, for some reason, external morphology cannot be scored, either because of evolutionary conservativeness (as in some traits of Polyplacophora) or specialization (as for Amphisbaenia).

Some conjectures on anuran phylogeny have been made based upon spermatozoon ultrastructure and the cladistic significance of some characters has been investigated. For example, the conical perforatorium has been proposed as a tentative synapomorphy of Bufonoidea (Lee & Jamieson, 1993), whereas the presence of an undulating membrane or a rod-shaped perforatorium have been scored as symplesiomorphies of Anura. Yet, due to the paucity of data on spermatozoon morphology for several families, no comprehensive cladistic analysis of sperm ultrastructure data, such as those made for squamate reptiles (Jamieson, 1995; Teixeira et al., 1999b) and fishes (Tanaka et al., 1995), has been conducted for anurans (but see Scheltinga, 2001).

The ultrastructure of sperm can therefore provide an alternative data set for the phylogenetic analysis of amphibians. Unfortunately, until now only about half of the families of Anura have had the spermatozoa of at least one species described (Kwon & Lee, 1995; Jamieson, 1999) and several characters mentioned in the literature are poorly defined. Consequently, there is a need both for the detailed description of the sperm ultrastructure in families not yet studied and for the continued data accumulation on families already studied. Herein we describe, for the first time, the ultrastructure of the spermatozoon of a member of the family Dendrobatidae, *Epipedobates flavopictus*, from the central Brazilian

Cerrado. Further, we discuss the evolutionary significance of the results regarding the evolution of the subacrosomal cone in anurans and the phylogenetic position of dendrobatid frogs.

Material and Methods

We collected 2 individuals of *Epipedobates flavopictus* on September 1998, at Minaçu, Goiás, Brazil (13°38' S, 48°15' W), and 1 individual on November 1995 at Serra do Cipó, Minas Gerais, Brazil, during the reproductive season. We killed animals by rubbing xylocaine onto the abdomen skin and deposited them at the Coleção Herpetológica da Universidade de Brasília (CHUNB 09581, 09582) and Museu de História Natural "Prof. Adão José Cardoso", Universidade Estadual de Campinas (ZUEC 11434). We removed testes by dissection and placed them in a Petri dish with phosphate buffer (PBS) pH 7.2 and saline solution. We cut testes into small pieces and fixed them overnight at 4°C in a solution of 2.5% glutaraldehyde, 5mM $CaCl_2$, and 5% sucrose in 0.1M sodium cacodylate buffer pH 7.2. After rinsing in sodium cacodylate buffer, we postfixed testes for 1h in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5mM $CaCl_2$ in 0.1M sodium cacodylate buffer pH 7.2, and left them overnight in a solution of 0.5% uranyl acetate for "in block" contrast. We proceeded with dehydration in a series of ascending acetone (30-100%) and embedded the material in Spurr's epoxy resin. We made ultrathin sections with glass and diamond knifes on a Leica Reichert ultramicrotome and stained the sections with uranyl acetate and lead citrate. We observed the sections in a Jeol® 100C transmission electron microscope and took micrographs at 80kV. We also made light microscope

observations of spermatozoa under interferential contrast using a Zeiss® Axiophot microscope.

Results

Under the light microscope, the spermatozoon is filiform, approximately 59 μm long, with a short tail (ca 33 μm) when compared to the head region (Fig. 1A). The head is curved and the midpiece is very short and not clearly visible. An undulating membrane is distinguishable in the tail, and the axoneme is seen describing a very sinuous path along the axial fiber.

Acrosome Complex

The acrosome of *Epipedobates flavopictus* sperm is located at the anterior portion of the head and is composed of a single and narrow vesicle, filled with a homogeneous material of low electron density (Figs. 1B-E). Under the acrosome vesicle, the subacrosomal cone forms a conical cap that reaches the anterior portion of the nucleus. In cross-section, the acrosome and subacrosomal cone are circular (Figs. 1C-F). The acrosome vesicle surrounds the anteriormost portion of the nucleus, below which the nucleus thickens and is enveloped only by the subacrosomal cone (Figs. 1B-F).

Nucleus

Below the nuclear envelope, a nuclear space that probably results from the condensation of chromatin process is seen (Figs. 1B,D-E). The nucleus is circular in transverse section (Fig. 1G) and conical in longitudinal section (Fig. 1B). The anterior portion of the nucleus is enveloped by the acrosome complex and gradually tapers (nuclear shoulders absent) to a

point within the subacrosomal cone (Figs. 1E-F). The chromatin is highly condensed and electron-dense. Despite the high degree of condensation, several small electron-lucent nuclear lacunae and inclusions are seen (Figs. 1G-H).

Midpiece

The midpiece is the transitional region between the spermatozoon head and tail, containing the nuclear fossa, proximal and distal centrioles, axoneme, mitochondria, and paraxonemal rod (sensu Jamieson et al., 1993) (Fig. 1J). The proximal centriole is seen in Figure 1I inside the nuclear fossa and surrounded by pericentriolar material. The posteriormost portion of the nucleus is curved, forming a deep nuclear fossa that completely surrounds the proximal centriole and the pericentriolar material (Figs. 1J-K and 2A). The paraxonemal rod inserts into the nuclear fossa, reaching the proximal centriole, and is embedded in the pericentriolar material (Fig. 2A). The distal centriole lies at an approximate angle of 50° with respect to the longitudinal axis of the spermatozoon (Fig. 1K). Numerous round mitochondria are seen scattered in the midpiece (Figs. 1I-K and 2A). They completely surround the posterior region of the nucleus and extend into the anterior portion of the tail (Figs. 1K and 2B).

Tail

In transverse section, the tail of *Epipedobates flavopictus* sperm consists of the axoneme, undulating membrane and axial fiber (Fig. 2B). The axial fiber is U-shaped and extends itself through an electron dense structure that supports the undulating membrane. We name this extension of the the axial fiber as "axial sheath", being formally defined as the

connection between the axial fiber and the axoneme (or juxta-axonemal fiber, when it is present). In *E. flavopictus* the axial sheath is directly connected to the axoneme through the doublet 3, without a juxta-axonemal fiber (Figs. 2B-C). Posteriorly, the axial sheath gradually shrinks and finally ends, the axoneme continuing with the remnant of the axial fiber and, later, alone for a short distance (Figs. 2C-E). While the axial sheath shrinks, the axial fiber maintains its curved shape until very near its end (Fig. 2D). Further posteriorly, only the axoneme is observed (Fig. 2E).

Mitochondria are observed inside the undulating membrane in the anterior portion of the tail (Figs. 2B,F). At the end of the tail, the undulating membrane no longer contains mitochondria and the plasma membrane is closely adhered to the axial fiber and axial sheath (Fig. 2G). In Figure 2H the intermediate piece of a spermatid is seen. Contrary to the condition seen in the mature spermatozoan, the mitochondria are organized in a mitochondrial collar around the flagellum.

Discussion

The basic structure of the spermatozoon of *Epipedobates flavopictus* (Fig.3) is similar to that of most neobatrachians (Ford, 1993) described to date, such as *Bufo arenarum* (Burgos & Fawcett, 1956), *B. marinus* (Swan et al., 1980; Lee & Jamieson, 1993), *Melanophryniscus cambaraensis* (Báo et al., 2001), *Odontophrynus cultripes* (Báo et al., 1991), *Lepidobatrachus laevis* (Waggener & Carroll, 1998), and *Pachymedusa dacnicolor* (Rastogi et al., 1988). However, it possesses several traits not seen in these other species and which may possibly be shared with other dendrobatids.

Epipedobates flavopictus has a subacrosomal cone below the acrosomal vesicle. We advance that this is homologous with the subacrosomal cone of *Ascaphus truei* and the

conical perforatorium of bufonoids, contrary to the proposition of Lee & Jamieson (1992; 1993) and Jamieson et al. (1993). These authors indicated that the subacrosomal cone seen in *A. truei*, urodeles, and basal amniotes is absent in all other anurans they examined and that in bufonoids a similar structure, the conical perforatorium, lies beneath the acrosome vesicle. Further, they provide four reasons why the conical perforatorium is not homologous with the subacrosomal cone of *A. truei*: (1) a rod-shaped endonuclear perforatorium exists in *A. truei*; (2) the close proximity of the subacrosomal cone, in its posterior region, to the nucleus and acrosome vesicle in *A. truei*, whereas in bufonoids the conical perforatorium lies free in the subacrosomal space; (3) the homogeneous nature of the subacrosomal material in *A. truei*, whereas loose bundles of coarse fibers running parallel to the nucleus are seen in bufonoids; and (4) the subacrosomal cone in *A. truei* is less electron-dense than the acrosome vesicle, while in bufonoids the conical perforatorium is more electron-dense than the acrosome vesicle. Later, Jamieson (1999) suggested that the conical perforatorium may be homologous with the subacrosomal cone, but provided no rationale to his proposition.

We regard, like James (1970) and Jamieson (1999), that the conical perforatorium and the subacrosomal cone are homologous based on what follows. First, argument (1) above is a syllogism: bufonoids have a conical extranuclear perforatorium whereas *Ascaphus truei* has a rod-like, endonuclear perforatorium; hence, since the function of supporting the spermatozoon head is performed by the perforatorium, the subacrosomal cone of *A. truei* cannot be homologous with the conical perforatorium of bufonoids. Similarity of function, however, is not a requirement for homology (Lauder, 1994). Lee & Jamieson (1992; 1993) and Jamieson et al. (1993) were probably influenced by the earlier work of Burgos & Fawcett (1956), the first to suggest that the coarse strands of dense

material, observed around the tapering end of the nucleus in the spermatozoon of *Bufo arenarum*, form a perforatorium. Had Burgos & Fawcett (1956) chosen a different name (without implying a function) for the same structure, say "subacrosomal cone", argument (1) above would vanish.

Second, arguments (2), (3), and (4) above are not independent. The more detached aspect of the presumed conical perforatorium relative to the nucleus and acrosome vesicle, and its higher electron density in bufonoids are a direct consequence of its fibrous arrangement. Moreover, in the bufonoids *Myxophyes fasciolatus* (Lee & Jamieson 1992, Fig. 3E), *Odontophrynus cultripes* [Báo et al. 1991, Figs. 12 and 13, mislabelled as acrosome (A)], and *Melanophryniscus cambaraensis* (Báo et al., 2001, Fig. 4) the presumed conical perforatorium is much more homogeneous, forming a almost continuous layer in transverse section around the nucleus, with no coarse fibers being observed. We regard this condition as intermediate between that found in *Ascaphus truei* and the state typical of most bufonoids.

Finally, if the view of Lee & Jamieson (1992; 1993) and Jamieson et al. (1993) is correct, six steps would be required during the evolution of anurans, when mapping the characters conical perforatorium and subacrosomal cone onto a current phylogeny of the group (Hay et al., 1995) (Fig. 4). According to this view, the conical perforatorium was absent in the common ancestor of anurans and salamanders and evolved once in the "bufonoid" lineage (Fig. 4A). Furthermore, the subacrosomal cone was originally absent in anurans and evolved independently twice in the group (Fig. 4B). Conversely, if James (1970) was correct in regarding the conical perforatorium of bufonoids as homologous with the subacrosomal cone of *Ascaphus truei*, then only four evolutionary steps are required and the presence of the subacrosomal cone would be plesiomorphic for bufonoids (Fig.

4C). If inferring genealogy rests on the principle of parsimony, i.e. choosing genealogical hypothesis that minimize requirements for homoplasies (Farris, 1982), then James' view is to be preferred.

The acceptance of the hypothesis proposed by James (1970) implies that the condition seen in the acrosome of *Bombina* (Furieri, 1975), *Discoglossus* (Sandoz, 1975), and *Xenopus* (Bernardini et al., 1986), where the subacrosomal cone is absent, is not intermediate between *Ascaphus truei* and bufonoids as suggested by Lee & Jamieson (1993, Fig 7). In addition, the proposition made by Lee & Jamieson (1993) and Jamieson (1999) that the conical perforatorium is a synapomorphy of bufonoids is not supported. Instead, the replacement of a perforatorial rod (as in *Ascaphus* and basal amniotes) with a modified fibrilar subacrosomal cone is a bufonoid synapomorphy.

The nucleus is highly compact in mature spermatozoa of *Epipedobates flavopictus*, with nuclear lacunae and inclusions being frequently observed. The nuclear lacunae are probably formed during the condensation of chromatin. They are typically electron-lucent, with no material inside, and are usually of small diameter, as seen in *Ascaphus truei* (Jamieson et al., 1993). Nuclear inclusions contain a clear but not completely electron-lucent substance and are usually bigger than lacunae, as observed in *Rana clamitans* (Poirier & Spink, 1971).

The numerous and randomly distributed mitochondria distinguish *Epipedobates flavopictus* from several other Neobatrachia so far examined, which usually have a mitochondrial collar, as in Bufonidae (Burgos & Fawcett, 1956; Swan et al., 1980; Lee & Jamieson, 1993) and Leptodactylidae (Pugin-Rios, 1980; Báo et al., 1991). The presence of mitochondria creates a large separation between the axial sheath and the plasma membrane in the anterior tail region of *E. flavopictus*. In all other amphibians with an undulating

membrane, the plasma membrane is closely adhered to the axial sheath. Interestingly, early spermatids of *E. flavopictus* have a mitochondrial collar detached from the midpiece (Fig. 2H), as in Bufonidae (Burgos & Fawcett, 1956; Swan et al., 1980; Lee & Jamieson, 1993).

The reduction of the mitochondrial collar during the spermiogenesis, the absence of a juxta-axonemal fiber, and the somewhat degenerated structure of the axial fiber in *Epipedobates flavopictus* (Fig. 2H) agree with the proposition made by Lee & Jamieson (1993) that a reduction in complexity is a major trend in the evolution of anuran spermatozoa.

Our results suggest that the family Dendrobatidae should be placed within the "bufonoid" lineage, as proposed by several authors (Hedges & Maxson, 1993; Hillis et al., 1993; Hay et al., 1995). The acrosome structure resembles that seen in Leptodactylidae (Pugin-Rios, 1980; Amaral et al., 1999; Báo et al., 2001), Bufonidae (Burgos & Fawcett, 1956; Lee & Jamieson, 1993), Hylidae (Rastogi et al., 1988; Lee & Jamieson, 1993; Meyer et al., 1997), and Myobatrachidae (Lee & Jamieson, 1992). All of these families share a plesiomorphic condition of the acrosome, where a subacrosomal cone lies below the conical acrosome vesicle, as in the archaeobatrachian *Ascaphus truei* (Jamieson et al., 1993) and some Urodeles (Picheral, 1967). This feature differs markedly from the condition seen in ranoids such as Ranidae (Poirier & Spink, 1971; Pugin-Rios, 1980; Yoshizaki, 1987) and Rhacophoridae (Mainoya, 1981; Mizuhira et al., 1986; Jamieson, 1999), where the acrosome vesicle sits on top of the nucleus and the subacrosomal cone is absent. Similarly, the subacrosomal cone is also absent in some archaeobatrachians, such as Pelobatidae (James, 1970) and Pipidae (Reed & Stanley, 1972; Bernardini et al., 1986). Furthermore, despite some peculiarities, such as the shape of the axial fiber and the absence of a juxta-axonemal fiber, the tail of *Epipedobates flavopictus* is similar to that generally

observed in bufonoids, where an axial fiber is connected to the axoneme through an axial sheath inside an undulating membrane. All ranoids so far studied (Ranidae, Rhacophoridae, and Microhylidae) possess a tail with only the axoneme.

Several studies have empirically acknowledged the utility of sperm ultrastructure in systematics for different taxonomic groups, but the significance of anuran sperm ultrastructure still needs to be evaluated under the scope of sound phylogenetic techniques. To do so, characters must be continuously evaluated and families yet to be fully studied (e.g. Sooglossidae, Centrolenidae, Microhylidae, Rhinophrynidae, Mantellidae, Hyperoliidae, and Brachycephalidae) must be investigated in order to built a consistent data set that will enable cladistics analyses.

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Figure legends

Figure 1: Spermatozoa of *Epipedobates flavopictus*. (A) Light microscopy showing whole spermatozoon with head (h) and flagellum (f). X 1250. (B-K) Transmission electron micrographs of head and midpiece. (B) Longitudinal section of the head region showing the end of the acrosome vesicle (arrowheads). X 38000. (C-F) Transverse sections of the head region showing the reduction of the acrosome and enlargement of the nucleus. X 116000, X 100000, X 94000, X 54000, respectively. (G-H) Transverse and longitudinal sections of the nucleus showing the lacunae (l) and inclusions (i). X 40000, X 25000, respectively. (I) Oblique section of the midpiece. Note the scattered distribution of mitochondria. X 50000. (J and K) Longitudinal view of the midpiece at the level of the distal centriole and paraxonemal rod, respectively. X 28000, both. Abbreviations: ac= acrosome vesicle; af= axial fiber; ax= axoneme; c= subacrosomal cone; dc= distal centriole; m= mitochondrion; n= nucleus; ns= nuclear space; pc= proximal centriole; pm= pericentriolar material; pr= paraxonemal rod; u= undulating membrane. Scale bars: A, 20μm; B and I, 0.2μm; C, 0.05μm; D and E, 0.1μm; F and G, 0.3μm; H, J and K, 0.5μm.

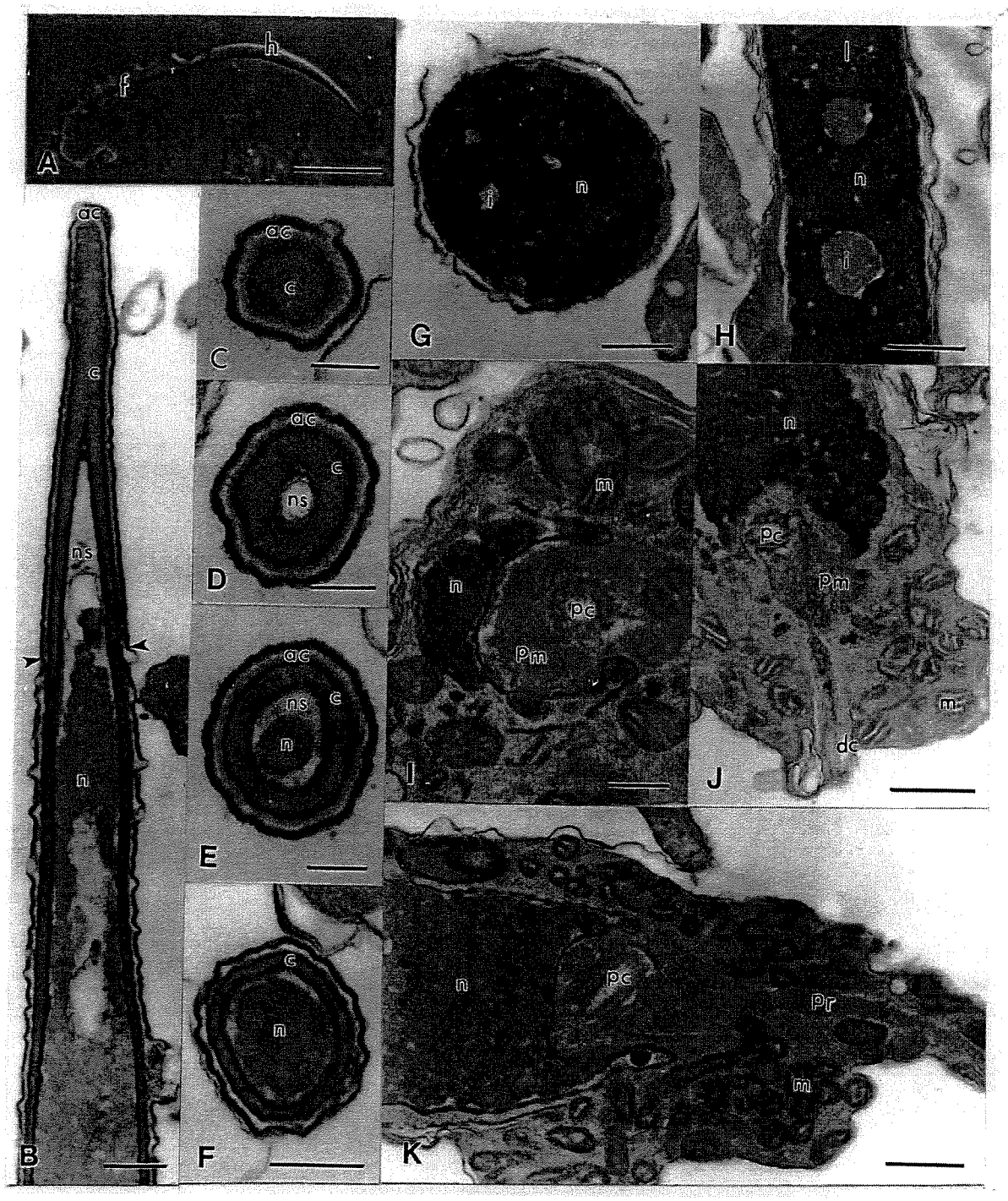
Figure 2: Spermatozoa of *Epipedobates flavopictus*. Transmission electron micrographs of the midpiece and tail. (A) Longitudinal section through the midpiece showing the insertion of the paraxonemal rod in the nuclear fossa (asterisk). X 42000. (B) Transverse section of the tail showing the S-shaped paraxonemal rod (U-shaped axial fiber and axial sheath) and mitochondria inside the undulating membrane. X 24000. (C) Transverse section of the posterior portion of the tail; note the absence of mitochondria. X 60000. (D and E) Reduction and disappearance of the axial fiber. X 82000, X 420000, respectively. (F)

Longitudinal section of the anterior region of the tail. Note the loose disposition of the membrane with respect to the axial sheath and the presence of mitochondria inside the undulating membrane. X 48000. (G) Longitudinal section of the tail at its posterior portion, with no mitochondria and a close association of the membrane and the axial sheath. X 27300. (H) Section of spermatid showing presence of mitochondrial collar in the early development of the sperm. X 40300. Abbreviations: af= axial fiber; as: axial sheath; ax= axoneme; m= mitochondrion; n= nucleus; pc: proximal centriole; pm= pericentriolar material; pr= paraxonemal rod; u= undulating membrane. Scale bars: A, F and H, 0.3μm; B and G, 0.5μm; C and D, 0.2μm; E, 0.05μm.

Figure 3: Diagrammatic representation of the spermatozoon of the dendrobatid frog *Epipedobates flavopictus*.

Figure 4: Reconstruction of the evolution of sperm ultrastructure characters of *Epipedobates flavopictus*. Phylogeny of anurans from Hay *et al.* (1995). A: evolution of conical perforatorium, according to Jamieson et al. (1993) and Lee & Jamieson (1993), number of steps = 4. B: evolution of subacrosomal cone, according to Jamieson *et al.* (1993) and Lee & Jamieson (1993), number of steps = 2. C: preferred hypothesis for the evolution of the subacrosomal cone, when considering the conical perforatorium homologous to the subacrosomal cone in anurans, number of steps = 4. Data for families used in the analysis was obtained from the present work (Dendrobatidae) and from the following literature: Caudata (Selmi et al., 1997), Pipidae (Reed & Stanley, 1972; Bernardini et al., 1986), *Bombina* (Furieri, 1975; Pugin-Rios, 1980), Discoglossidae

(Pugin-Rios, 1980; Lee & Kwon, 1996), *Ascaphus* (James, 1970; Jamieson et al., 1993), Leiopelmatidae (Scheltinga et al., 2001), Pelobatidae (James, 1970; Asa & Phillips, 1988), Pelodytidae (Pugin-Rios, 1980), Ranidae (Poirier & Spink, 1971; Pugin-Rios, 1980), Microhylidae (*Chiasmocleis albopunctata*, pers. obs.), Myobatrachidae (Lee & Jamieson, 1992), Leptodactylidae (Pugin-Rios, 1980; Báo et al., 1991; Waggener & Carroll, 1998; Amaral et al., 1999), Rhinodermatidae (Pugin-Rios, 1980), Bufonidae (Burgos & Fawcett, 1956; Lee & Jamieson, 1993; Báo et al., 2001), and Hylidae (Rastogi et al., 1988; Lee & Kwon, 1992; Lee & Jamieson, 1993).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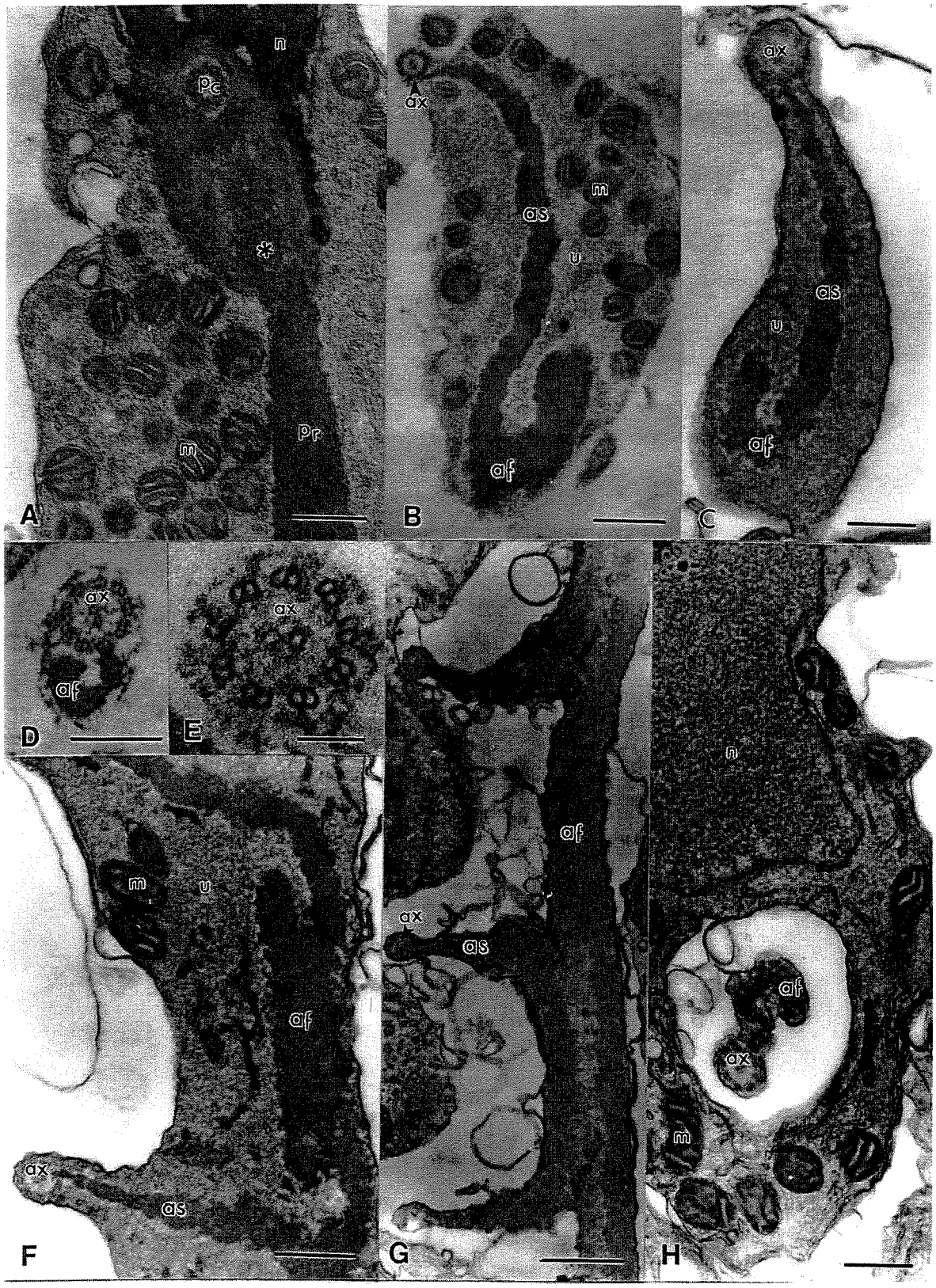
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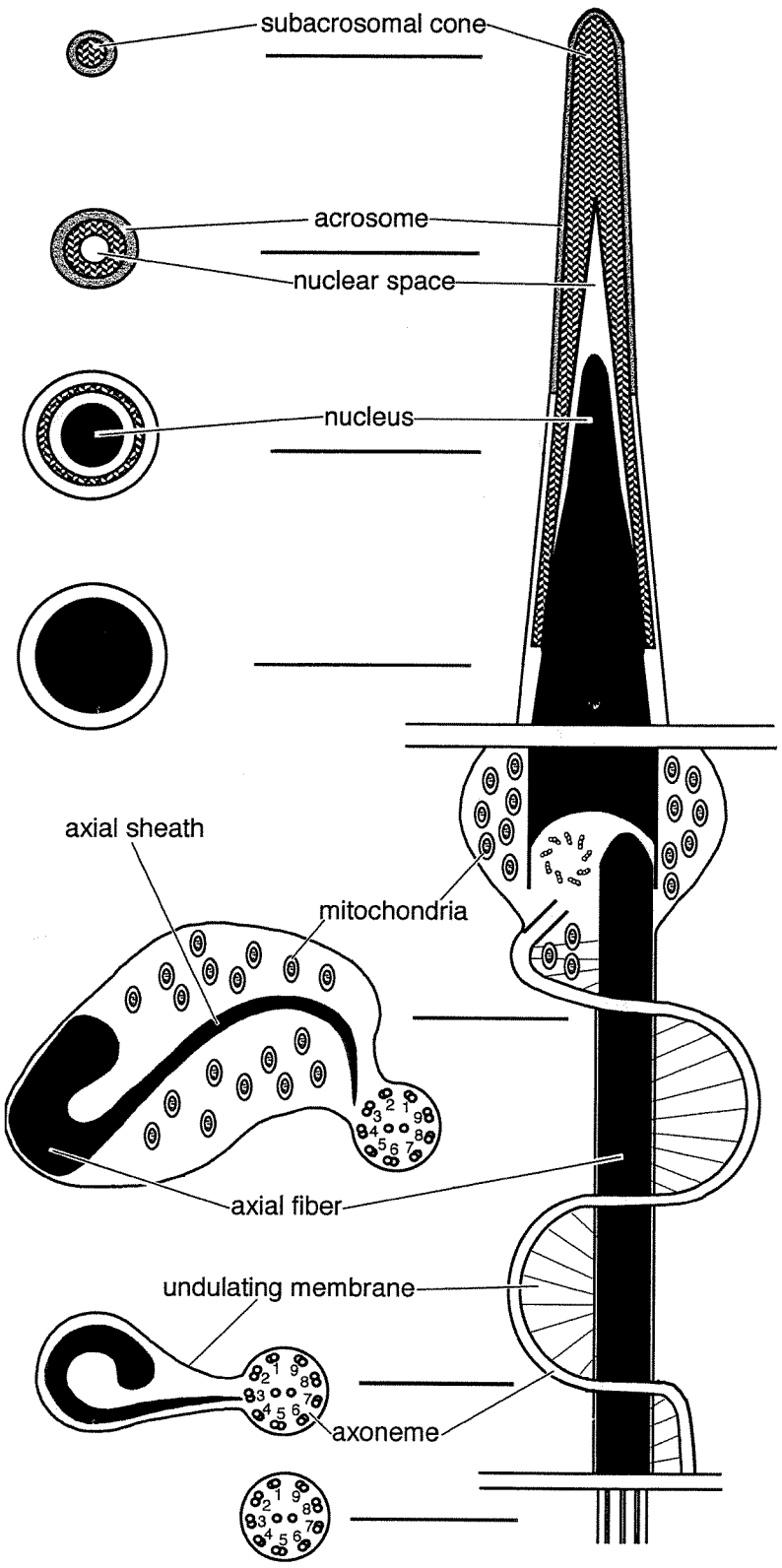
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undulating membrane
axoneme

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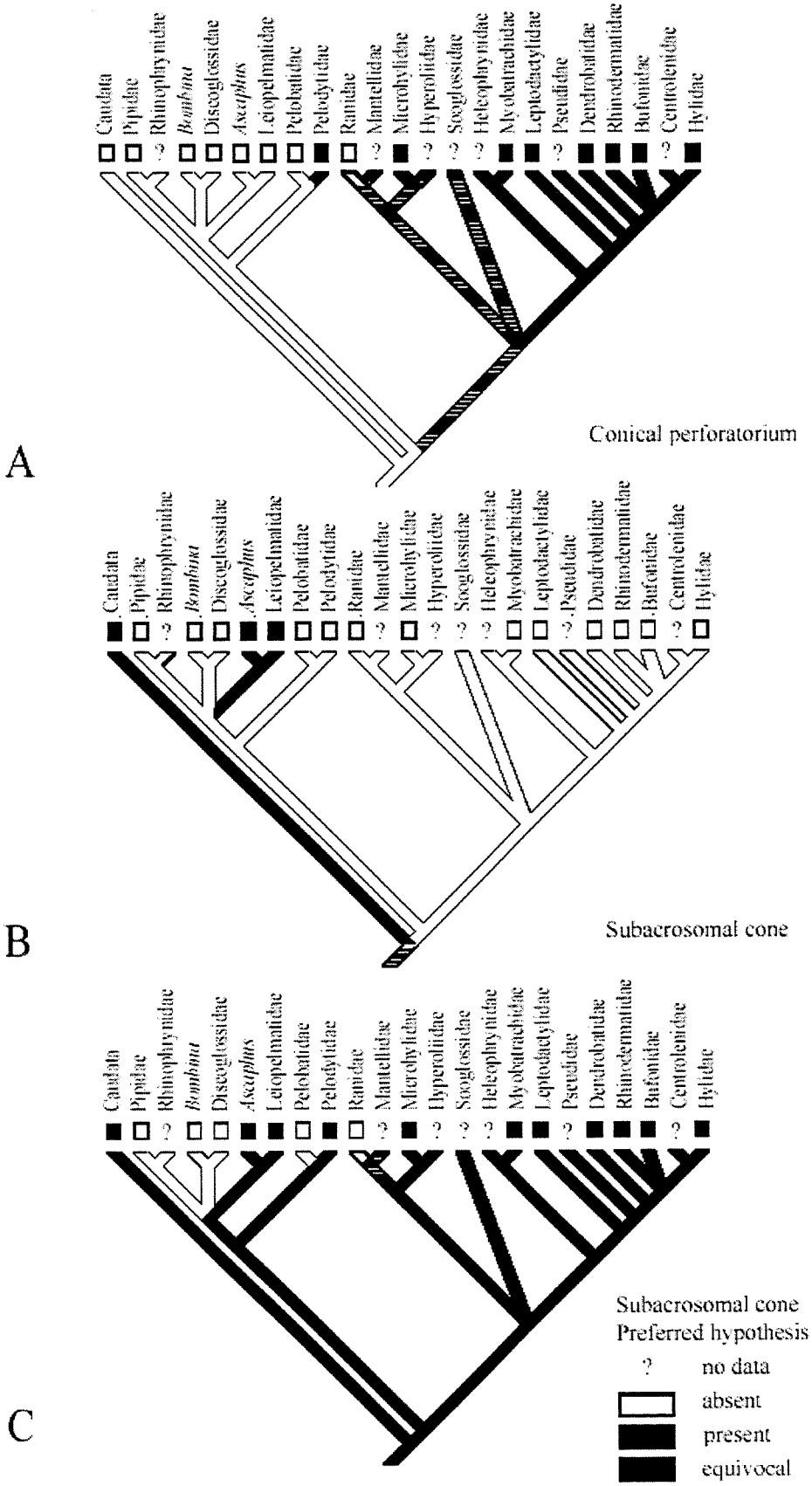
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Microhylidae
Hyperoliidae
Sooglossidae
Heleophrynidae
Myobatrachidae
Leptodactylidae
Pseudidae
Dendrobatidae
Rhinodermatidae
Bufonidae
Centrolenidae
Hylidae
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? no data
absent
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The Biflagellate Spermatozoon of the Poison-Dart Frogs *Epipedobates femoralis* and *Colostethus* sp. (Anura, Dendrobatidae)

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Text pages: 22

Figures: 3

ABBREVIATED TITLE: Biflagellarity in Two Dendrobatid Frogs

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KEY WORDS: sperm ultrastructure, biflagellarity, Anura, Dendrobatidae, *Epipedobates*, *Colostethus*.

ABSTRACT: This study describes the spermatozoa of the dendrobatids *Epipedobates femoralis* and *Colostethus* sp. using light and transmission electron microscopy. Both species possess a biflagellate spermatozoon, an unusual characteristic only previously reported in two anuran species belonging to the families Leptodactylidae and Racophoridae. The acrosomal complex of both species consists of a conical acrosomal vesicle and a subacrosomal cone, both of which cover the anterior portion of the nucleus, but to differing extents. In the midpiece, the centrioles are disposed parallel to each other and to the cell axis and give rise to two axonemes. Two paraxonemal rods were also seen entering the nuclear fossa. Both flagella are surrounded by a single mitochondrial collar. Each flagellum is formed by an axial fiber connected to the axoneme by an axial sheath; juxta-axonemal fibers are absent. Our data seems to support that *Epipedobates femoralis* is to be placed in a separate clade possibly related to *Colosthetus* and that these two speciose genera may not be monophyletic.

The systematic relationships of poison-dart frogs (Family Dendrobatidae) have been studied intensively (reviewed in Ford, 1993; Burton, 1998). Despite the well-accepted monophyly of dendrobatids, their intergeneric affinities remain obscure (Myers et al., 1991; Ford and Cannatella, 1993; Vences et al., 2000). According to Clough and Summers (2000), some members of this family cannot be classified in any particular genus because of the absence of diagnostic characters. These problematic species have undergone several taxonomic revisions that have resulted in different generic arrangements and the creation of new genera. This was the case of some species formerly placed in *Colostethus* and *Epipedobates*, but now allocated in *Nephelobates*, *Manophryne*, *Phobobates*, and *Allobates* (Myers, 1987; Zimmerman and Zimmermann, 1988; La Marca, 1992, 1994).

Colostethus is the largest dendrobatid genus, with approximately 100 described species (Frost, 2000). According to Grant et al. (1997), most species groups of *Colostethus* are not firmly established because they are defined by a combination of character states widespread in other groups, rather than by unambiguous synapomorphies. Since the genus *Colostethus* is defined by symplesiomorphic characters, some regard it as paraphyletic (Lynch, 1982).

Epipedobates is defined mainly by plesiomorphic characters and may or may not be a natural group, as argued by Myers (1987), who partitioned the genus *Dendrobates* (*sensu* Silverstone, 1975) into four new genera, including *Epipedobates*. Most species of *Epipedobates* were formerly classified into other genera such as *Dendrobates*, *Phyllobates* and *Colostethus* (Frost, 2000), thus reflecting the difficulty in the taxonomic assignment of these dendrobatids.

Various data sets have been used to investigate dendrobatid systematic relationships, including cytogenetic and molecular markers (Morescalchi, 1973; Rasotto et

al., 1987; Bogart, 1991; Hillis et al., 1993; Hay et al., 1995; Summers et al., 1999; Clough and Summers, 2000; Vences et al., 2000) and also behavioral characteristics (Zimmermann and Zimmermann, 1988; Toft, 1995). Such analyses have been useful in introducing new parameters to complement the classic morphological traits (Noble, 1931; Lynch, 1971, 1973; Duellman and Trueb, 1986; Ford, 1993; Ford and Cannatella, 1993; Burton, 1998) which can be misleading because of their limited number and the ambiguous and erroneous reporting of some character states (Ford, 1993).

More recently, sperm ultrastructure has been used in phylogenetic analyses of various taxa, including fishes (Mattei, 1991; Jamieson and Leung, 1991), reptiles (Jamieson, 1995; Teixeira et al., 1999 a, b), platyhelminths (Justine, 1991), and insects (Jamieson et al., 1999). Several studies have also shown that sperm ultrastructure is also useful for phylogenetic inference in anurans (Lee and Jamieson, 1992, 1993; Jamieson et al., 1993; Kwon and Lee, 1995; Meyer et al., 1997; Scheltinga et al., 2001).

In this paper, we describe the ultrastructure of testicular spermatozoa from *Epipedobates femoralis* (=*Allobates femoralis*, *sensu* Zimmermann and Zimmermann, 1988) and *Colostethus* sp. We report the presence of two complete flagella in the spermatozoon of these species, a feature not previously reported for other anuran species, although two simple flagella have already been described to one leptodactylid and one racophorid species (Pugin-Rios and Garrido, 1981;Wilson et al, 1991 - apud Jamieson, 1999). Finally, in light of the new data, we also discuss the affinities of *E. femoralis* with the genus *Colostethus*.

MATERIALS AND METHODS

Two adult male *Epipedobates femoralis* were collected by Albertina P. Lima in a 10,000 ha plot of tropical rainforest in the Reserva Florestal Adolfo Ducke, 25 km northeast of Manaus, Amazonas, Brazil (03° 08' S, 60° 04' W). Two specimens of *Colostethus* sp. were collected by Janalee P. Caldwell, Laurie J. Vitt, and Robson A. Souza in Guajará-Mirim, Rondônia, Brazil (10° 19' S, 64° 33' W). The specimens of *E. femoralis* were deposited in the Museu de História Natural "Prof. Adão José Cardoso", Universidade Estadual de Campinas, Brazil (ZUEC 11750, 11753), whereas the specimens of *Colostethus* sp. were deposited in the Sam Noble Oklahoma Museum of Natural History, University of Oklahoma, USA (OMNH 37001, 37002).

For light microscopic observations, spermatozoa from glutaraldehyde-paraformaldehyde fixed smears were stained with Giemsa (10% pH 6.8) and examined with an Olympus BX60 microscope. For transmission electron microscopy, fragments of testes from *Epipedobates. femoralis* and *Colostethus* sp. were prefixed in a solution of 2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2, at 4 °C overnight. The samples were then postfixed for 60 min in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer before staining *en bloc* with 0.5% uranyl acetate for 2 h. After dehydration in an ascending series of acetone, the samples of *E. femoralis* were embedded in Epon and polymerized at 60 °C for 48 h. Alternatively, the material of *Colostethus* sp. was embedded in Spurr resin after the same process of fixation. Ultrathin sections were stained with uranyl acetate (3% in water) for 30 min and then for 8 min in lead citrate following Reynolds (1963), with three rinses in distilled water after each solution. Grids were examined with a LEO 906 or JEOL 100C electron microscopes at 60kV or 80 kV.

RESULTS

Spermatozoa of *Epipedobates femoralis* and *Colostethus sp.* contain two flagella that are inserted into a short midpiece (Fig. 1A, B). The nucleus and tail of *E. femoralis* are approximately 19.5 μm and 27.0 μm long, respectively. The paired flagella and mitochondrial collars are particularly evident in transverse sections through a spermatocyst (Fig. 1B).

Colostethus sp.

The acrosomal tip is blunt in longitudinal section, and a conical acrosomal vesicle covers the anterior portion of the head and extends to the beginning of the nucleus, where a large nuclear space can be seen (Fig. 2A–C). Below the acrosomal vesicle, the subacrosomal cone ensheathes the nucleus (Fig. 2A, D). Above the nuclear membrane, the subacrosomal cone is traversed throughout its length by a canal, referred to here as the subacrosomal canal (Fig. 2A, B). In transverse section, at the anteriormost portion of the acrosome, the subacrosomal canal is surrounded by the subacrosomal cone and the detached acrosomal vesicle (Fig. 2B). More posteriorly, the nuclear space is surrounded by the subacrosomal cone that adhers closely to the nuclear membrane (Fig. 2C). Subsequently, the nucleus is surrounded by the subacrosomal cone alone and, eventually, only by the plasma membrane (Fig. 2D, E). The transition from the tip of the acrosome to the end of the subacrosomal cone is smooth, with no nuclear shoulders.

In the midpiece, and two paraxonemal rods enter the shallow nuclear fossa (Fig. 2F–I). The centrioles are disposed parallel to the longitudinal axis, with an axoneme showing the typical 9+2 pattern of microtubules emerging from each centriole (Fig. 2G, H).

The axial sheath (sensu Garda et al., submitted) and axial fiber coalesce near the point of insertion into the nuclear fossa to form the paraxonemal rod that projects slightly into the nuclear fossa (Fig. 2I). Several mitochondria are arranged around both flagella within the mitochondrial collar (Fig. 2J).

The tail is composed of a comma-shaped axial fiber connected to the axoneme through an axial sheath; the plasma membrane is detached from both the axial sheath and axial fiber and no juxta-axonemal fiber is present (Fig. 2J). Further back, the axial sheath shortens, the undulating membrane disappears, and finally only the axial fiber is observed (Fig. 2K, L). Finally, the axial fiber vanishes and the axoneme continues alone for a short distance.

Epipedobates femoralis

The acrosomal vesicle consists of a thin, narrow, membrane-bound vesicle containing homogeneous material of moderate electron-density and covers approximately one third of the nucleus (Fig. 3A). The subacrosomal cone extends beyond the limits of the acrosomal vesicle, and consists of low-density material (Fig. 3A-C). A space containing very low-density material (the nuclear space), is delimited by the nuclear membrane and chromatin (Fig. 3A, B).

The chromatin is not wholly compact but is condensed into large lumps (Fig. 3A). Transverse sections of the posterior end of the nucleus show that the chromatin is more compact (Fig. 3D). The nuclear fossa is occupied throughout its length by the two centrioles that are arranged parallel to each other and give rise to two independent axonemes (Fig. 3E)., The paraxonemal rod (*sensu* Jamieson et al., 1993) extends from the origin of the axoneme, as shown by transverse sections of the midpiece (Fig. 3F).

As in *Colostethus* sp., each flagellum consists of an axoneme with the typical 9+2 pattern, an undulating membrane, and an axial fiber. The later is a comma-shaped structure connected to the axoneme through the axial sheath (Fig. 3G, H). In the proximal region of the tails, the mitochondria form a sheath around the axoneme, axial fiber, and undulating membrane (Fig. 3G).

DISCUSSION

The basic structure of the sperm of *E. femoralis* is very similar to that of *Colostethus* sp.. The structure of the acrosomal complex of the two species is similar to that observed in most neobatrachians, including *Bufo arenarum* (Burgos and Fawcett, 1956), *Batrachyla* spp. (Garrido et al., 1989), *Odontophrynus cultripes* (Báo et al., 1991), *Litora* spp. and *Cyclorana* spp. (Meyer et al., 1997), *Physalaemus* spp. (Amaral et al., 1999), and *Pseudopaludicola falcipes* (Amaral et al., 2000). The conical shaped acrosomal vesicle is shared by several anuran species (Kwon and Lee, 1995), and is a plesiomorphic trait of sarcopterygians (Jamieson, 1999). The homogeneous nature of the subacrosomal cone differs from the longitudinal bundles of fibers observed in the so-called "conical perforatorium" of bufonoid anurans (Jamieson et al., 1993). In agreement with Garda et al. (unpublished data), we assumed that the subacrosomal cone is homologous with the conical perforatorium of bufonoids. The comma-shaped axial fiber and the absence of a juxta-axonemal fiber appear to be common features of dendrobatids.

The placement of the Dendrobatidae has shifted between the neobatrachian groups Ranoidea and Bufonoidea (Ford and Cannatella, 1993; Hillis et al., 1993, Ruvinsky and Maxson, 1996; Emerson et al., 2000). Beside the similarities in the acrosomal complex, which differ from that observed in ranoid groups (Kwon and Lee, 1995), the mitochondrial

collar surrounding the axonemes in the species studied here is characteristic of most bufonoids, for which it is considered a synapomorphic characteristic (Lee and Jamieson, 1992). However, the presence of this characteristic does not necessarily imply affinities between bufonoids and dendrobatids because Scheltinga et al. (2001) reassessed such characteristics and argued that there is no arrangement of mitochondria that can be convincingly regarded as plesiomorphic for the Anura. Even within *Epipedobates*, an alternative pattern was found by Garda et al. (submitted) in *E. flavopictus* sperm, in which the mitochondria are randomly distributed within the undulating membrane.

Biflagellate spermatozoa have been described in several animal groups. In fishes, for example, biflagellate sperm have evolved at least six times, without any link to phylogeny or internal fertilization (Mattei, 1988; Jamieson and Leung, 1991). The presence of two free flagella, widespread in Turbellaria, has been considered a plesiomorphic characteristic of platyhelminths with a tendency towards mono- and aflagellate conditions (Hendelberg, 1969; Justine, 1991). Franzén (1982) described the only case of biflagellarity in Annelida (the polychaete *Tomopteris helgolandica*). Among molluscs, biflagellate spermatozoa are known in two bivalves, *Corbiculla flamae* and *C. leana*, where they are considered a modification of the primitive monoflagellar condition related to the specialized mode of reproduction found in these species (Komaru and Konishi, 1996). In insects, where sperm structure is highly diversified, a biflagellate condition is observed in some Hemiptera (Jamieson et al., 1999).

Within the Caudata of Lissamphibia, Austin and Baker (1964) suggested the presence of two flagella and two undulating membranes in *Pseudobranchus striatus axanthus*, a sirenid salamander. However, such analysis was restricted to light microscopy and the figures shown by these authors are not convincing as to the presence of such a peculiar tail. So

much so, that later on Selmi et al. (1997), through an ultrastructural study of salamandrids sperm emphasized that sperm ultrastructure of Sirenidae species was still unknown. In anurans, a number of studies have reported the existence of biflagellarity. In the Chilean leptodactylid *Telmatobufo australis*, the presence of two flagella was inferred to be a primitive characteristic related to the fertilization environment, although the exact mode of fertilization of this species was unknown (Pugin-Rios and Garrido, 1981). Mainoya (1981) indicated that anurans may have sperm tails with either 1 or 2 flagella, and erroneously cited Nicander (1970) who mentioned no case of biflagellarity in anurans. Lee and Jamieson (1993) argued for a general trend towards the simplification of sperm among the Anura (see also Jamieson et al., 1993), and suggested that the biflagellarity in *Rhacophorus* (Rhacophoridae), *Scaphiophus* (Pelobatidae), and *Telmatobufo* (Leptodactylidae) was apomorphic. This view was reinforced by Jamieson (1999) for *Chiromantis*, another racophorid species. Although Mainoya (1981) asserted that sperm cells of *Chiromantis xerampelina* had only one tail flagellum with two axial filaments, Jamieson (1999) based on the report of Wilson et al. (1991), emphasized that a pair of free flagella comprise the tail piece in this species. However, *Rhacophorus* (Mizuhira et al., 1986) actually have two axonemes embedded in a matrix of hundreds of microtubules, forming a single tail rather than two free flagella. Likewise, the pelobatids *Scaphiophus holbrookii* (James, 1970) and *Megophrys montana* (Asa and Phillips, 1988) have a single tail with two axonemes. Waggener and Carrol (1998) also reported a biaxonemal condition in *Lepidobatrachus laevis*, but this conclusion was apparently based on a misinterpretation of micrographs: the presence of two axonemes united by membrane (fig. 5A-F in their report) resulted, in fact, from the same axoneme being hit twice by the same cut in the undulating tail; their figure 5G corroborates the common pattern found in neobatrachians, where the tail is formed by

the axoneme, juxta-axonemal fiber, axial sheath, and axial fiber. We propose that true biflagellarity is a condition in which the two axonemes are independent and isolated by the plasma membrane. Thus, biflagellar sperm in anurans have been observed only in *Telmatobufo australis* (Pugin-Rios and Garrido, 1981), *Chiromantis xerampelina* (Wilson et al., 1991 - apud Jamieson, 1999) and some dendrobatids (this study). The sperm of *Epipedobates femoralis* and *Colostethus* sp. represent the first case in the Anura of two complete flagella, with an undulating membrane and axial rod, because the spermatozoa of *Telmatobufo australis* and *Chiromantis xerampelina* have two simple flagella.

It seems premature to ascribe any phylogenetic significance of the biflagellarity to the Anura as a whole. However, within Dendrobatidae the biflagellarity may support the placement of *E. femoralis* in a separate clade (*Allobates femoralis*, sensu Zimmemann and Zimmermann, 1988) possibly related to *Colostethus*, as previously indicated by molecular analysis (see Vences et al., 2000; Clough and Summers, 2000), which placed *A. femoralis* outside the clade containing the other toxic dendrobatids. Dietary data collected by Toft (1995) and Caldwell (1996) are also in agreement with such idea. The genus *Colostethus* is characterized by the lack of skin toxins found in the remaining dendrobatids (Bogart, 1991; Toft, 1995) and is considered a basal lineage to the monophyletic assemblage of toxic genera (Myers et al., 1991). The biflagellarity of *E. femoralis* – also a non-toxic dendrobatid, as pointed by Caldwell (1996) – contrasts with the condition found in other congeneric species, such as *E. flavopictus* (Garda et al., submitted), *E. trivittatus* and *E. hahneli* (Aguiar-Jr. et al., unpublished data). Hence, our results also seem to suggest that these two speciose genera (*Epipedobates* and *Colostethus*) may not be monophyletic in agreement with that proposed by Vences et al. (2000) through molecular data. Further ultrastructural studies of other species of *Epipedobates* should confirm whether close

relatives of *E. femoralis* are to be retained in the genus, as noted by Myers et al. (1991). In addition, an analysis of *Aromobates nocturnus* – the presumed sister taxon of all other dendrobatids (Myers et al., 1991)– may shed light on the significance of biflagellarity in the Dendrobatidae. Also, we suggest that as many as possible genera need to be studied before the taxonomic significance of this character can be stated, considering the scarcity of sperm ultrastructure data of dendrobatids.

ACKNOWLEDGMENTS

The authors thank Prof. Lino Neto for technical suggestions and valuable comments. This work was supported by the Brazilian agencies FAPESP (Proc. 99/05602-0), CNPq (Proc. 300965/00) and CAPES.

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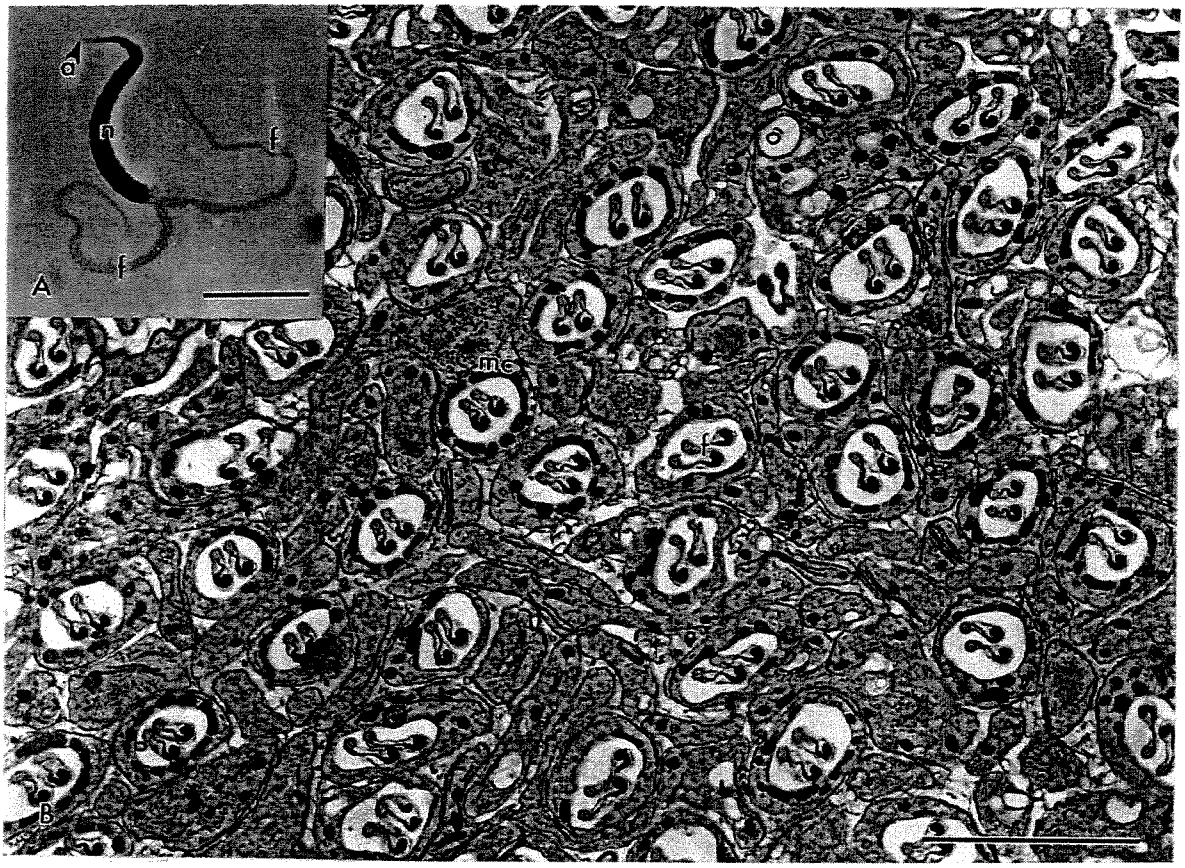
LEGENDS

Fig. 1. A: The spermatozoa of *Epipedobates femoralis* showing the presence of two independent flagella, both with an undulating membrane (L.M.). B: A spermatocyst of *Colostethus* sp. in the intermediate piece where several pairs of flagella surrounded by mitochondrial collars can be seen (T.E.M.). a, acrosome; f, flagellum; L.M., light microscopy; mc, mitochondrial collar; n, nucleus; T.E.M., transmission electron microscopy. Scale bars: A 10 μm; B 3 μm;

Fig. 2. Spermatozoa of *Colostethus* sp. A: Longitudinal section of the acrosome showing the end of the acrosomal vesicle (arrowheads). Also note the canal throughout the subacrosomal cone's length and the nuclear space. (B-E) Transverse sections of the headpiece showing reduction of the acrosomal vesicle and subacrosomal cone and progressive enlargement of the nucleus. F: Insertion of both paraxonemal rods independently in the nuclear fossa of a spermatid. G, H: Implantation of both axonemes in the nuclear fossa and the corresponding parallel centrioles in the nuclear fossa. I: Insertion of the auxiliary fibers in the nuclear fossa showing the coalescence of the fibers forming the paraxonemal rod near the insertion. The paraxonemal rod penetrates the nuclear fossa only slightly (arrowhead). J-L: Transverse sections of the tail showing the presence of the mitochondrial collar proximally and its subsequent disappearance, as well as the reduction in the length of the undulating membrane. Note the constant curved shape of the axial fiber. a, acrosomal vesicle; af, axial fiber; as, axial sheath; ax, axoneme; c, centriole; mc, mitochondrial collar; n, nucleus; nf, nuclear fossa; ns, nuclear space; p, paraxonemal rod; s, subacrosomal canal; sc, subacrosomal cone; u, undulating membrane. Scale bars: A-E, G and L 0.2 μm; F, H and I-K 0.5 μm.

Fig. 3. Spermatozoa of *Epipedobates femoralis*. A: Longitudinal section of the acrosomal region. Note the absence of the subacrosomal canal. B-D: Progressive enlargement of the nucleus and reduction of the acrosomal vesicle and subacrosomal cone. E: Insertion of both axonemes into the nuclear fossa. F: Sagittal section through the insertion of the axonemes showing a centriole and an early axoneme with the adjacent auxiliary fiber. G-H: Transversal sections of the tails showing the mitochondrial collar and its subsequent disappearance. a, acrosomal vesicle; af, axial fiber; as, axial sheath; ax, axoneme; c, centriole; m, mitochondria; n, nucleus; nf, nuclear fossa; ns, nuclear space; sc, subacrosomal cone; u, undulating membrane. Scale bars: A, D, F and G 0.5 μm; B 0.25μm, C and H 0.2 μm; E 1 μm.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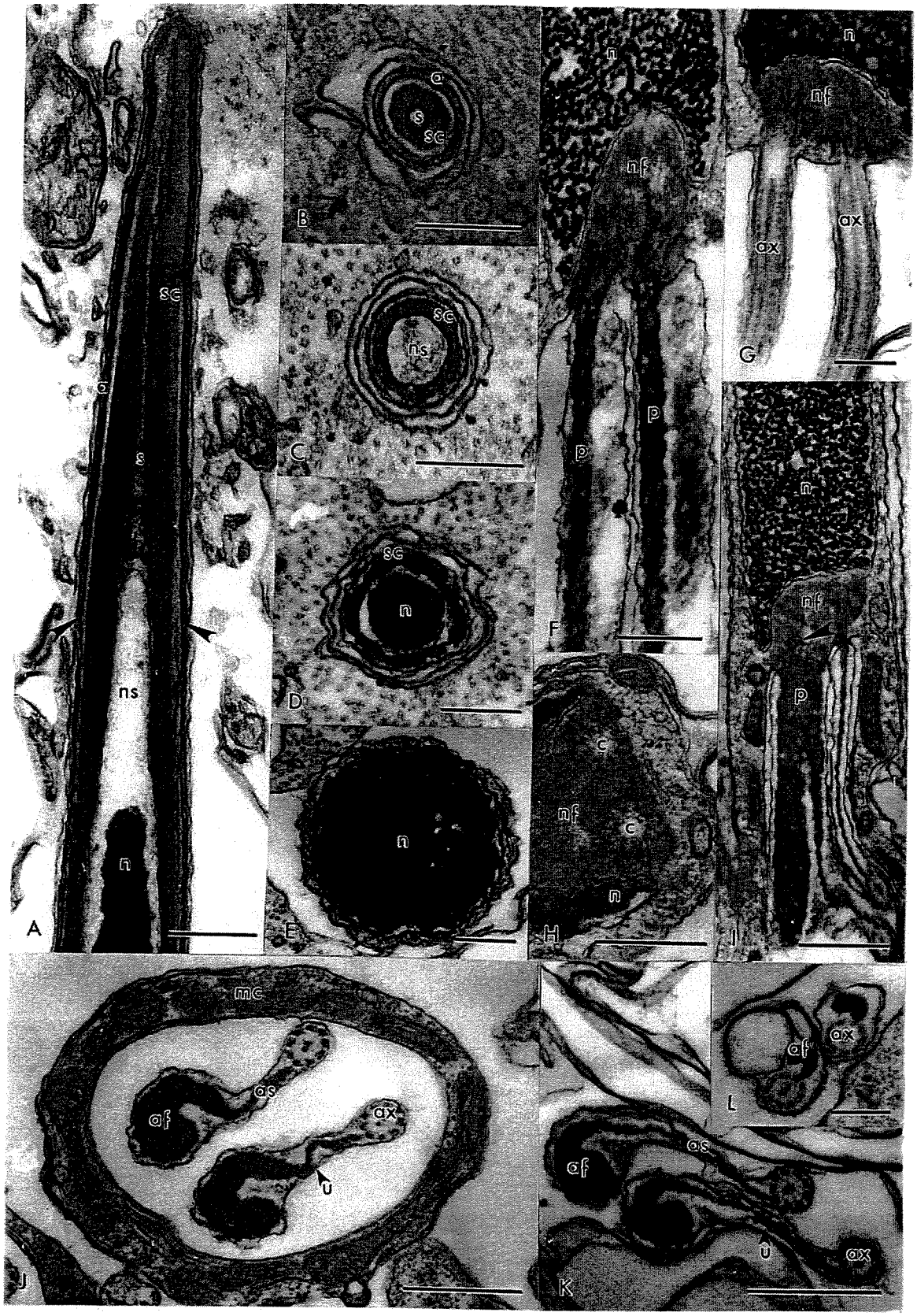
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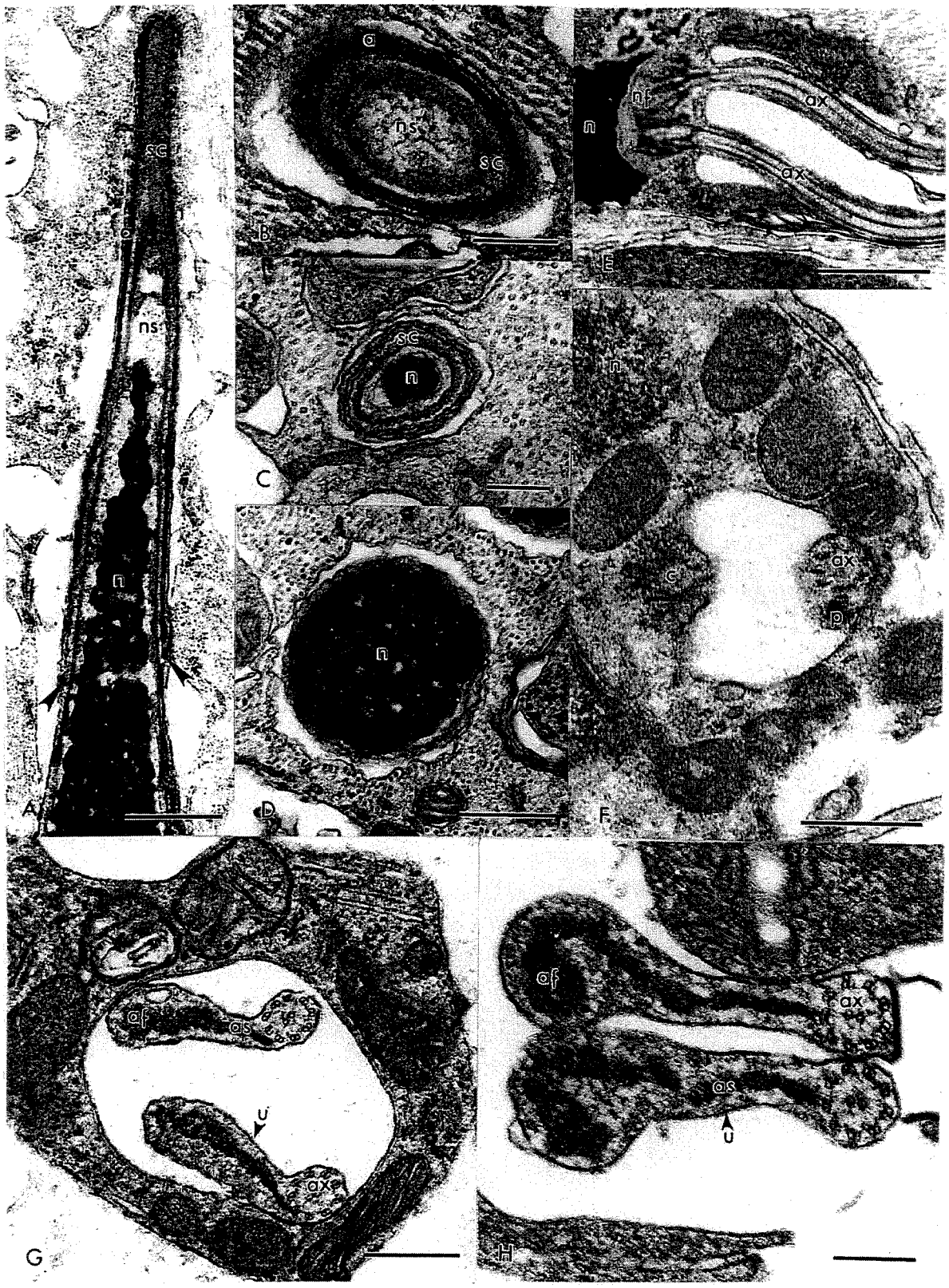
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The spermatozoa of the family Pseudidae (Anura, Amphibia), with a test of the hypothesis of correlation between ultrastructure and reproductive modes in anurans

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Running title: Sperm ultrastructure of the family Pseudidae. — *Zoologica Scripta*, *00*, 000-000.

Garda *et al*, (0000)

Abstract

Garda, A.A., Costa, G.C., Colli, G.R. & Báo, S.N. (0000). Sperm ultrastructure in the family Pseudidae (Anura, Amphibia) with phylogenetic considerations. *Zoologica Scripta*, *00*, 000-000.

We describe, for the first time, the sperm ultrastructure of the two genera of Pseudidae. According to sperm ultrastructure, the five species herein described can be separated in three groups: one containing *Pseudis paradoxa*, *P. bolbodactyla*, and *P. tocantins*, the second containing *P. minuta*, and the third containing *Lysapsus laevis*. The intermediate piece is similar in all species and auxiliary fibers and the undulating membrane are absent. In *Pseudis* a subacrosomal cone and a multilaminar structure (*P. minuta*) or a granular material (*P. paradoxa* group) is seen above the nucleus. *Lysapsus laevis* has only remnants of the subacrosomal cone. All species have peripheral fibers in the axoneme. Our results suggest that Pseudidae may be more related to Leptodactylidae than Hylidae, by virtue of shared characters with some Telmatobiinae. We performed a Concentrated Changes Test that rejected the hypothesis of correlation between presence of undulating membrane and fertilization environments in anurans.

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Introduction

The family Pseudidae is characterized by exclusively aquatic and semi-aquatic species restricted to South America, east of the Andes (Duellman & Trueb 1986). Due to their habits, these frogs have several morphological adaptations, such as large and protuberant eyes, robust hindlimbs, and fully webbed feet (Duellman & Trueb 1986). Another marked feature of the group are the giant tadpoles of *Pseudis*, that much exceed the size of the adults (Bokermann 1967). The family currently comprises 9 species, three in the genus *Lysapsus* and six in the genus *Pseudis* (Frost 2000). Recent taxonomic works with the family include the description of *Pseudis cardosoi* (Kwet 2000) and the revalidation and description of new species in the *P. paradoxa* group (Caramaschi & da Cruz 1998).

Despite the small number of species involved, little is known about the phylogenetic relationships of pseudids with other anurans. Earlier works placed *Lysapsus* and *Pseudis* either in Leptodactylidae (Noble 1922) or Hylidae (Parker 1935). Savage and Carvalho (1953) erected the family Pseudidae, arguing that the group is closely related to Leptodactylidae, based on similarities of sacral diapophyses, and that the presence of intercalary toe elements in Hylidae and *Lysapsus* results from convergence. This view was challenged by Burger (1954), who argued that the accessory phalanx of Pseudidae may be homologous to the intercalary elements of Hylidae (Parker's hypothesis) and that *Lysapsus*, with a smaller intercalary bone, can be viewed as the link between an ancestral hylid and *Pseudis* (i.e., *Pseudis* is derived in respect to *Lysapsus*). Later works have simply acknowledged Parker's hypothesis, without the addition of new data (e.g., Griffiths 1963). Recently, the use of cladistic methods on morphological (Duellman & Trueb 1986; Ford &

Cannatella 1993) and molecular (Hay *et al.* 1995) data sets has also supported the view that Pseudidae is more related to Hylidae than to Leptodactylidae. Intercalary elements are the only synapomorphy that supports the clade uniting Centrolenidae, Hylidae, and Pseudidae (Lynch 1973; Duellman 1975; Duellman & Trueb 1986; Ford & Cannatella 1993). Indeed, Ford and Canatella (1993) stated that "... we are not aware of other derived features that would unite pseudids to any other neobatrachians."

Since the various efforts to reconstruct the phylogeny of amphibian families have not led to overwhelming conclusions, some authors suggested the use of alternative data sets (Wake 1993), such as neuroanatomical characters (Wilkinson 1997) and vocal sac structure (Tyler 1971). Electron microscopy has opened a huge and completely new field of study in morphology in the past 50 years. The study of internal oral features of anuran larvae has greatly benefited from the use of electron microscopy (Wassersug & Heyer 1988). Furthermore, the ultrastructure of sperm has been used to investigate phylogenetic relationships among fishes (Jamieson 1991) and reptiles (Jamieson 1995b; Teixeira *et al.* 1999). Some problems, however, must be surpassed before sperm ultrastructure characters are profitably used in phylogenetic analyses of anurans, including: 1) the paucity of data, mainly the lack of descriptions for many neotropical families (such as Brachycephalidae, Centrolenidae, and Pseudidae); 2) the quality of descriptions, since many were made to highlight abnormal or atypical features, rather than to provide detailed accounts of sperm ultrastructure needed for systematic purposes. Nevertheless, some works offer good descriptions of anuran sperm morphology, covering new families and providing relevant phylogenetic discussions (e.g., Lee & Jamieson 1992; Jamieson *et al.* 1993; Lee & Jamieson 1993; Scheltinga *et al.* 2001).

Observations of amphibians sperm have led to some important generalizations, such as the suggestion of a correlation between the ultrastructural morphology of these cells and fertilization habitat in anurans (Jamieson *et al.* 1993; Lee & Jamieson 1993). This hypothesis suggests that the occurence of complex sperm in basal anurans, salamanders, and caecilias, as well as the high diversity of modifications in several derived anuran families, are good evidences to an ancestral condition with a complex sperm in amphibians. Complex sperm cells are correlated to internal fertilization in molluscans (Rouse & Jamieson 1987) and Lee and Jamieson (1993) suggest that similarly sperm ultrastructure supports internal fecundation as a primitive condition for amphibians. However, these propositions have not yet been rigorously tested, either for molluscans or anurans. Recent developments in phylogenetic analysis made available a number of techniques to test for correlated evolution patterns among discrete characters (Madisson 1990; Read & Nee 1995; Harvey & Gutberlet Jr 2000).

Herein we describe, for the first time, the ultrastructure of spermatozoa of the family Pseudidae, contemplating five species: *Lysapsus laevis*, *Pseudis bolbodactyla, P. minuta*, *P. paradoxa*, and *P. tocantins*. We also discuss the phylogenetic significance of sperm morphology of the family and perform a test of the hypothesis of correlated evolution between sperm ultrastructure and reproductive modes of anurans (Jamieson *et al.* 1993; Lee & Jamieson 1993).

Material and methods

We used the following species in the study: *Lysapsus laevis*, from Amapá, Amapá, Brazil (Coleção Herpetológica da Universidade de Brasília, CHUNB 14104, 14123, 14129, 14130); *Pseudis bolbodactyla*, from Pirenópolis, Goiás, Brazil (CHUNB 08320, 08321); *P. minuta*, from Eldorado do Sul, Rio Grande do Sul, Brazil (Museu de Zoologia da Universidade Estadual de Campinas, ZUEC 11581); *P. paradoxa*, from Poconé, Mato Grosso, Brazil (CHUNB 13857, 13858); and *P. tocantins*, from Palmas, Tocantins, Brazil (CHUNB 11238) and Santa Terezinha, Mato Grosso, Brazil (CHUNB 10370). We killed animals through the administration of xylocaine onto the abdomen, removed testis by dissection, placed on a Petri dish with phosphate buffer and saline solution (PBS) pH 7.2, cut into small pieces and fixed overnight at 4 C in a solution of 2.5% glutaraldehyde, 5 mM $CaCl_2$, and 5% sucrose in 0.1 M sodium cacodylate buffer pH 7.2. After rinsing in sodium cacodylate buffer, we postfixed the material for 1 h in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer pH 7.2 and left it overnight in a solution of 0.5% uranyl acetate for "in block" contrastation. We proceeded with dehydration in a series of ascending acetone (30-100%) and embedded the material in Spurr's epoxy resin. We made ultrathin sections with glass and diamond knifes on a Leica Reichert Supernova ultramicrotome, stained with uranyl acetate and lead citrate, as follows: 30 min in uranyl acetate and 7 min in lead citrate or, alternatively, 1 min in lead citrate, 6 min in uranyl acetate, and 4 min in lead citrate (Scheltinga *et al.* 2001). We observed sections in a Jeol 100C transmission electron microscope and took micrographs at 80kV. We made light microscope observations in macerates of testis under interferential contrast, using a Zeiss Axiophot microscope.

To evaluate the hypothesis of correlated evolution between presence of undulating membrane and fertilization environments of anurans we performed a Concentrated Changes Test (CCT) (Madisson 1990). The test determines whether losses of undulating membrane (dependent character) are concentrated on branches that show aquatic oviposition sites (independent character), by giving the probability that the observed number of changes in the dependent character would occur by chance. We used the phylogenetic hypothesis of anuran evolution proposed by Hay *et al* (1995), based on DNA sequences of mitochondrial 12S and 16S ribosomal RNA genes. Since the topology presents two trichotomies [((*Bombina*, Discoglossidae), (Pipidae, Rhynophrynidae), (*Ascaphus*, Leiopelmatidae)) and ((Centrolenidae, Hylidae), (Bufonidae), (Rhinodermatidae))], we evaluated the 9 alternative topologies that could be derived from it. If the reconstruction of undulating membrane evolution (dependent character) was equivocal in a given topology, we counted gains and losses of the dependent character in all most parsimonious reconstructions (MPRs), using the Equivocal Cycling option of MacClade v.4.03 (Maddison & Maddison 1992). This resulted in performing the CCT on 21 most parsimonious reconstructions (MPRs) of the evolution of undulating membrane. Presence of undulating membrane was recorded for each family from the following sources: Caudata (Selmi *et al.* 1997), *Ascaphus truei* (Jamieson *et al.* 1993), *Bombina* (Folliot 1979), Discoglossidae (Pugin-Rios, 1980), Pipidae (Reed & Stanley 1972), Leiopelmatidae (Scheltinga *et al.* 2001), Pelobatidae (James 1970), Pelodytidae (Pungin-Rios 1980), (Poirier & Spink 1971), Microhylidae (Jamieson 1999), Myobatrachidae (Lee & Jamieson 1992), Leptodactylidae (Pungin-Rios 1980; Báo *et al.* 2001), Pseudidae (present work) Dendrobatidae (Garda *et al,* unpublished data), Rhinodermatidae (Pungin-Rios 1980), Bufonidae (Burgos & Fawcett 1956; Swan *et al.* 1980; Lee & Jamieson 1993) and Hylidae (Pungin-Rios 1980; Kwon & Lee 1995; Meyer *et*

al. 1997). For excellent compilations of anuran sperm ultrastructure see Pungin-Ríos (1980), Jamieson *et al* (1993), and (Jamieson 1995a). Anuran reproductive modes follow Duellman (1985) and Duelman & Trueb (1986). The CCT was conducted in MacClade v. 4.03 (Maddison & Maddison 1992), using the Exact Count option to calculate probabilities.

Results

The general structure the sperm of Pseudidae is shown in Figure 1A. No undulating membrane is present. The head length is approximately 12.24 μm and the tail is approximately 40.28 μm long in *Pseudis bolbodactyla*. The ultrastructure of the sperm of the five species is described below. Since *P. paradoxa*, *P. bolbodactyla* and *P. tocantins* are very similar, they are described together. *P. minuta* and *Lysapsus laevis* are described next due to their peculiarities.

Pseudis bolbodactyla, P. paradoxa and P. tocantins

The acrosome vesicle is blunt in longitudinal section (Fig. 1B) and is composed of two distinct portions: the inner portion is thicker and shorter, barely reaching the level of the nucleus; the outer portion is thinner and longer (Fig. 1B). Below the acrosome vesicle, a subacrosomal cone with loose fibrilar structure covers the anteriormost portion of the nuclear rostrum (Fig. 1B-E). The subacrosomal cone has a sprinkled appearance, suggesting that fibrils are disposed approximately parallel to the longitudinal axis (Fig. 1C-E). A concentration of granules occurs between the subacrosomal cone and the tip of the nucleus (Fig. 1B-D).

The transition from the acuminate point of the nuclear rostrum to the cylindrical portion of the nucleus is smooth, no nuclear shoulders being observed (Fig. 1B). The chromatin is never completely compacted, even in mature cells, there being several nuclear lacunae (Fig. 1B, F). A shallow nuclear fossa is present at the posterior end of the nucleus (Fig. 1G), where the proximal centriole lies (Fig. 1H). The distal centriole lies in a nearly perpendicular position relative to the proximal centriole, reaching the most anterior portion of the mitochondrial collar (Fig. 1G). Several moderately electron-dense peripheral fibers adhere to each microtubule doublet at the anteriormost portion of the axoneme (Fig. 1G, J).

The midpiece contains several mitochondria (6 to 8) inside a mitochondrial collar that surrounds the anterior region of the flagellum (Fig. 1I). Neither an undulating membrane nor a paraxonemal rod is observed, as well as no auxiliary fiber is seen along with the axoneme or in posterior portions of the midpiece (Fig. 1I, and Fig. 1J inset).

Pseudis minuta and Lysapsus laevis

The sperm of *Pseudis minuta* resembles that of other pseudids in the midpiece and flagellum, there being differences in the head region, specially in the acrosome complex. The acrosome vesicle is similar to that of the other pseudids, being divided in two weel-marked portions, the inner being thicker than the outer, but the inner portion never reaches the level of the nucleus (Fig. 2A). The subacrosomal cone is also disposed as a cap over the nucleus with fibrils running parallel to the longitudinal axis (Fig. 2A) and sprinkled in transverse section (Fig. 2B). A multilaminar structure is observed below the subacrosomal cone and above the nuclear membrane, in a position equivalent to the granular material in other *Pseudis* (Fig. 2A, B). Another distinct feature of the sperm of *P. minuta* is the length of the portion between the tip of the nucleus to the tip of the acrosome vesicle: while attaining 1.61 μm in *P. minuta* (SD = 0.23, n = 11), it is 1.29 in *P. paradoxa* (SD = 0.27, n = 9), 0.98 μm in *P. bolbodactyla* (SD = 0.19, n = 12), 0.93 μm in *P. tocantins* (SD = 0.12, n = 14), and 0.21 in *Lysapsus laevis* (SD = 0.13, n = 10). Differences among species means were significant ($F_{4,51} = 75.21$, $p < 0.001$), and the following groups were formed based on Tukey multiple comparisons tests ($p < 0.05$): (*P. minuta*), (*P. paradoxa*), (*P. bolbodactyla* + *P. tocantins*), and (*L. laevis*).

In *Lysapsus laevis*, the acrosome is also divided into inner and outer portions, but the subacrosomal cone is very reduced, the acrosome vesicle being very close to the tip of the nucleus (Fig. 2C, D). In transverse sections, the acrosome is seen in around the nucleus but the subacrosomal cone can not be discerned (Fig. 2D). As in the other pseudids, the chromatin is not fully compacted, even in mature sperm.

In *Lysapsus laevis* and *Pseudis minuta* the midpiece is very similar to the other pseudids, with a mitochondrial collar and moderately electron-dense peripheral fibers near the implantation of the flagellum (Fig. 2E, F).

Concentrated Changes Test (CCT)

The distributions of undulating membrane and fertilization environment states are depicted in Figure 4. In none of the 21 CCTs there was a significant probability of correlated evolution between the two variables (Fig. 5).

Discussion

Despite the relatively simple sperm of pseudids (Fig. 3), the genera *Lysapsus* and *Pseudis* can be readily distinguished through the acrosome complex ultrastructure: the fibrilar subacrosomal cone in *Pseudis* contrasts markedly with the nearly absent acrosome in *Lysapsus laevis*. Considering that the subacrosomal cone is plesiomorphic in anurans (Jamieson *et al.* 1993; Lee & Jamieson 1993; Scheltinga *et al.* 2001) and that there is a general trend in anurans towards the simplification of sperm, through the loss of structures owing to the secondary reversion to external fertilization (Jamieson *et al.* 1993), the condition of the subacrosomal cone in *Lysapsus* is apparently derived. This contradicts the hypothesis that *Lysapsus* may represent the link between an early hylid ancestor and the genus *Pseudis* (Burger 1954).

Based on similarities in the ultrastructure of sperm, two groups can be recognized among the species of *Pseudis*: one containing *P. paradoxa*, *P. bolbodactyla*, and *P.*

tocantins and another containing *P. minuta*. The presence of granular material under the subacrosomal cone and above the nucleus in the first three species contrasts with the presence of a multilaminar structure in *P. minuta* in the same region. Further, in *P. minuta* the length of the portion between the tip of the nucleus to the tip of the acrosome vesicle is significantly larger than in the other congeneric species.

Several studies document the occurrence of polymorphism in sperm ultrastructure within families of anurans. These intra-familial variations are prevalent in Leptodactylidae, which is the largest anuran family and by far the family with more species described. Still, considerable consistency in sperm ultrastructure has been found within some genera, such as *Litoria* (Meyer *et al.* 1997), *Bufo* (Burgos & Fawcett 1956; Swan *et al.* 1980), and *Rana* (Poirier & Spink 1971; Pungin-Rios 1980). The differences we observed among the two genera of Pseudidae are somewhat subtle, when compared for example to those between *Caudiverbera* (Pungin-Rios 1980) and *Odontophrynus* (Báo *et al.* 1991), for example. The polymorphism in sperm ultrastructure may be a consequence of the different types of fertilization environments or even the paraphyly of the family under study. These possibilities can only be evaluated after adequate descriptions of all genera in the family and the development of sound phylogenetic hypotheses for anuran families. Our results provide a good example of distinction between genera through the use of sperm ultrastructure characters. Likewise, differences observed at the light microscopy level supported the splitting of *Hyla* and *Ololygon* (=*Scinax*) (Fouquette & Delahoussaye 1977). Hence, the use of sperm characters seems valuable in systematic studies at this taxonomic level and works dealing with different genera within the same family are warranted (such as Meyer *et al.* 1997).

The systematic affinities of pseudids have been debated since the early 20th century. The group has been assigned a closer relationship either to Leptodactylidae (Noble 1922; Savage & de Carvalho 1953) or to Hylidae (Parker 1935; Lynch 1973; Duellman & Trueb 1986). Pungin-Rios (1980) described four sperm morphotypes in Leptodactylidae. Sperm type two, present in *Telmatobius* and *Telmatobufo*, is characterized by the absence of undulating membrane and auxiliary fibers in the tail, the presence of the subacrosomal cone, and a double (*Telmatobufo australis*) or single (*Telmatobius hauthali*) flagellum. Moreover, *T. australis* and *T. hauthali* possess peripheral fibers adjacent to the peripheral doublets of microtubules, as those described herein for Pseudidae. Auxiliary fibers are also absent in other Telmatobiinae, such as *Caudiverbera caudiverbera* (Pungin & Garrido 1981). Yet other genera of Telmatobiinae have well developed auxiliary fibers in the tail, such as *Odontophrynus cultripes* (Báo *et al.* 1991), *Batrachyla* spp. (Garrido *et al.* 1989), and *Alsodes vittatus* (=*Eusophus vitattus*) (Pungin-Rios 1980). An intermediate condition is seen in *Eleutherodactylus*, where only an auxiliary fiber is seen beside the axoneme and no undulating membrane is observed (Garda, pers. obs.). Conversely, hylids sperm generally have an undulating membrane and axial and ujxta-axonemal fibers, while lacking peripheral fibers (Rastogi *et al.* 1988; Lee & Jamieson 1993; Meyer *et al.* 1997). The only hylids reported until now without the undulating membrane are *Hyla japonica* (Lee & Kwon 1992) and *H. meridionalis* (Pungin-Rios 1980), being similar to *Eleutherodactylus*. Therefore, the absence of undulating membrane and auxiliary fibers in the tail and the presence of peripheral fibers in the axoneme corroborate a closer relationship of Pseudidae with Leptodactylidae. This arrangement was first proposed by Noble (1931), who placed the subfamily Pseudinae as part of Bufonidae (sensu lato), in association with *Telmatobius*, *Eleutherodactylus*, and *Cycloramphus* (all members of Telmatobiinae).

However, one must be careful when considering the absence of sperm ultrastructure characters. Along with the subacrosomal cone, the undulating membrane and auxiliary fibers (axial and juxta-axonemal fibers) are some of the most widespread features in anurans sperm. The presence of auxiliary fibers and undulating membrane has been related both to phylogeny and to fertilization environments (Jamieson & Scheltinga 1993; Lee & Jamieson 1993). Tail elements in the anuran sperm were presumably inherited from ancestral fishes, since they are present in dipnoans (Jamieson 1999). Yet, as defined by Rouse and Jamieson (1987), animals which rely totally or partially on aquatic habitats for fertilization have sperm with particular characteristics, named aquasperm. Since fertilization in Pseudidae occurs totally in free water, its sperm may be classified as ect-aquasperm (sensu Rouse & Jamieson 1987). The definition fits both the environment where fertilization occurs and the sperm ultrastructure observed in the group. Animals with external fertilization are thought to have very simplified sperm. Therefore, the presence of auxiliary fibers and undulating membrane in the tail seems to be plesiomorphic and the absence of these elements may be convergent in anurans that have secondarily adopted fertilization habits intimately dependent on water.

Indeed, other anurans with external fertilization in water show similar sperm morphology. This is the case of *Xenopus laevis* (Pipidae) (Bernardini *et al.* 1986), *Rana spp.* (Ranidae(Poirier & Spink 1971; Pungin-Rios 1980), *Scaphiopus holbrook* (Pelobatidae) (James 1970), and *Cophixalus inornatus* (Microhylidae) (Jamieson 1999). In the family Rhacophoridae, where fertilization takes place on vegetation above water, neither auxiliary fibers nor an undulating membrane are present (Mainoya 1981; Mizuhira *et al.* 1986), but hundreds of microtubules adjacent to the double axoneme are seen (Jamieson 1999). These reductions in sperm characters may imply that loss of caudal

elements is intimately linked to reproduction in water, rather than inherited as an ancestral condition, since the animals mentioned are very distant in any phylogeny of Anura (Fig.4).

Jamieson *et al* (1993) and Lee & Jamieson (1993) postulated that the complex sperm observed in basal lineages of anurans, caecilians, and salamanders is an evidence that internal fertilization is ancestral in the group. This view is in agreement with Rouse & Jamieson (1987), who defined sperm morphotypes of polychaetes in relation to fertilization environment. Rouse & Jamieson (1987) observed in polychaets an apparent correlation between the reduction of sperm characters and the type of fertilization environment, classifying sperm that fertilize eggs in water as *aquasperm* and sperm that fertilize eggs inside females as *introsperm*. The same trend was attributed to amphibians and since ancestral families generally present a complex sperm, the authors suggested that internal fertilization is ancestral in anurans. Indeed, ancestral anurans (and also salamanders) such as *Ascaphus truei* (Jamieson *et al.* 1993), *Leiopelma hochstetteri* (Scheltinga *et al.* 2001), and *Discoglossus pictus* (Sandoz 1969) generally have complex sperm. Still, this hypothesis was never tested in such a way that phylogenetic influences were taken into account. Our results, however, failed to support the hypothesis of correlated evolution between undulating membrane and fertilization environment. The reconstruction of character evolution reveals several independent losses of the undulating membrane in anuran lineages (Fig. 4). It is possible that this character is correlated to other features, such as the ultrastructure of egg layers. Also, the possibility that no correlation at all exists between sperm ultrastructure and environment conditions cannot be dismissed. Recent works have accumulated a great deal of information on the ultrastructure of anuran sperm. This growing data set will open new avenues of research both in phylogenetic

reconstruction and comparative analysis, but only the use of sound methodology will allow the full exploitation of its potential.

Acknowledgements

We would like to thank Reuber A. Brandão and Ayrton Péres Júnior for field assistance. Antonio Sebben for supplying the material of *Pseudis paradoxa*, Shirlei Recco-Pimentel for the material of *P. minuta*, and Ruscaia Dias Teixeira for the material of *Lysapsus laevis*. We are also in debt with Herman Nuñez who provided help in accessing bibliographic material. This work was supported by a master's fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES to AAG, by a "Iniciação Científica" fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (PIBIC-CNPq-UnB) to GCC, and by research fellowships from CNPq to GRC (# 302343/88-1) and SNB (# 520130/97-9).

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Figure Legends

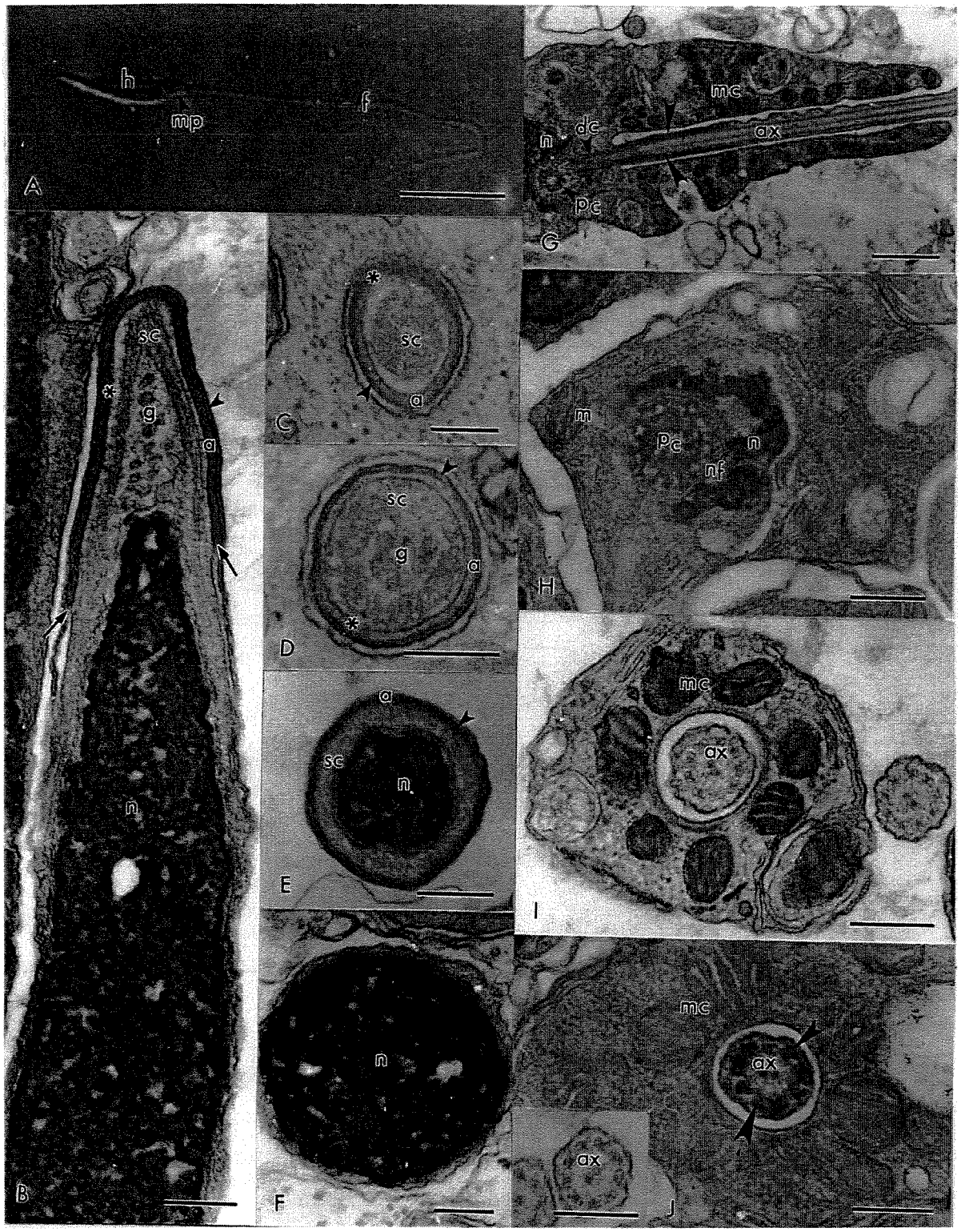
Figure 1. *Pseudis bolbodactyla*, *P.paradoxa*, and *P. tocantins*. A: interferential contrast showing the sperm cell with head, midpiece and flagellum. B: longitudinal section of the acrosome with internal (asterisk) and external (arrowheads) regions of the acrosome vesicle, subacrosomal cone, and granular region (arrows show the end of the internal region of the acrosome). C-F: serial transverse sections of the acrosome complex showing both parts of the acrosome vesicle (internal marked with asterisk and external marked with arrowheads), as well as the sprinkled appearance of the subacrosomal cone and the granular material above the nucleus. G: longitudinal section of the intermediate piece showing the proximal and distal centrioles and the mitochondrial collar. Note the peripheral fibers (arrowheads) in the anterior portion of the axoneme. H: transversal section through the nuclear fossa showing the proximal centriole. I: transverse section of the intermediate piece with mitochondrial collar and axoneme. J: anterior transverse section of the intermediate piece with mitochondrial collar and axoneme with peripheral fibers. Inset: terminal region of the flagellum. Legends: ax: axoneme; dc: distal centriole; f: flagellum; g: granular material; h: head; mc: mitochondrial collar; mp: midpiece; m: mitochondrion; n: nucleus; nf: nuclear fossa; pc: proximal centriole; sc: subacrosomal cone. Scale bars: A = 10 μm; B-D, F, H-J, and inset = 0.2 μm; E = 0.3 μm; G = 0.5 μm.

Figure 2. *Pseudis minuta*. A: longitudinal section through the acrosome, showing the multilaminar structure (star) above the nucleus (asterisk shows the internal region of the acrosome and arrowhead shows the external region). B: transverse section through the acrosome showing the multilaminar structure (star) and the sprinkled appearance of the subacrosomal cone. *Lysapsus laevis*. C: longitudinal section through the acrosome, with the proximity between the acrosome vesicle and nucleus shown (asterisk shows the internal region of the acrosome and arrowhead shows the external region). Note that the subacrosomal cone is nearly absent and only some putative remnants are observed (open stars). D: transverse section of the acrosome complex showing the anterior portion of the acrosome vesicle around the nucleus. E: longitudinal section through the intermediate piece showing the mitochondrial collar and peripheral fibers (arrowheads) near the proximal region of the axoneme. F: transverse section in the proximal region of the intermediate piece where peripheral fibers are clearly seen (arrowheads). Legends: ax: axoneme; dc: distal centriole; mc: mitochondrial collar; n: nucleus; nf: nuclear fossa; pc: proximal centriole; sc: subacrosomal cone. Scale bars: A and B = 0.3 μm; C, D, and F = 0.2 μm; E = 0.5 μm.

Figure 3. Schematic drawing of the sperm of the family Pseudidae.

Figure 4. Mapping of the absence of undulating membrane and auxiliary tail fibers in the cladogram proposed by Hay *et al* (1995) for the relationships of anuran families.

Figure 5. Distribution of probabilities resulting from a Concentrated Changes Test (CCT) to test the correlation between absence of undulating membrane and fertilization environment.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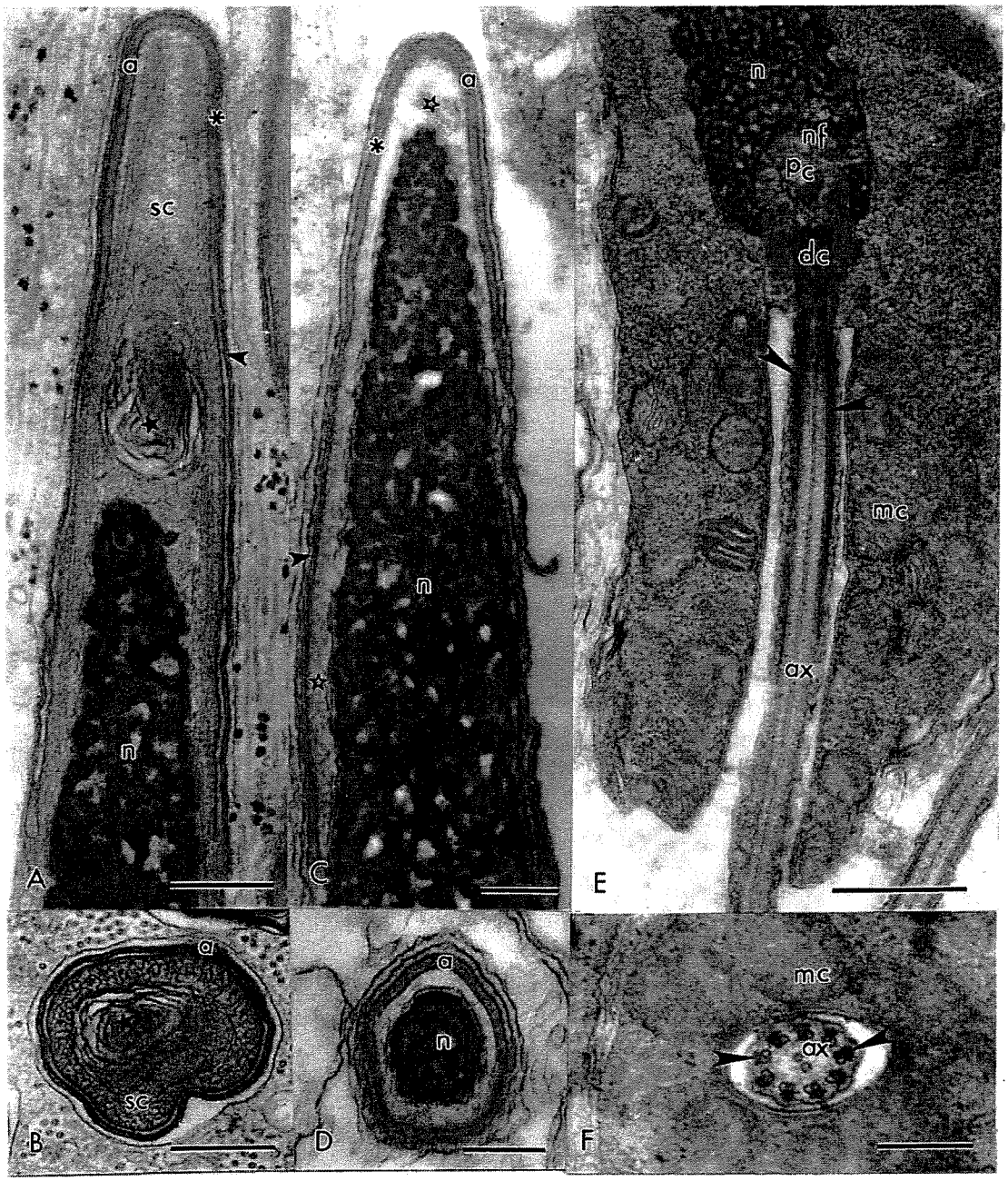
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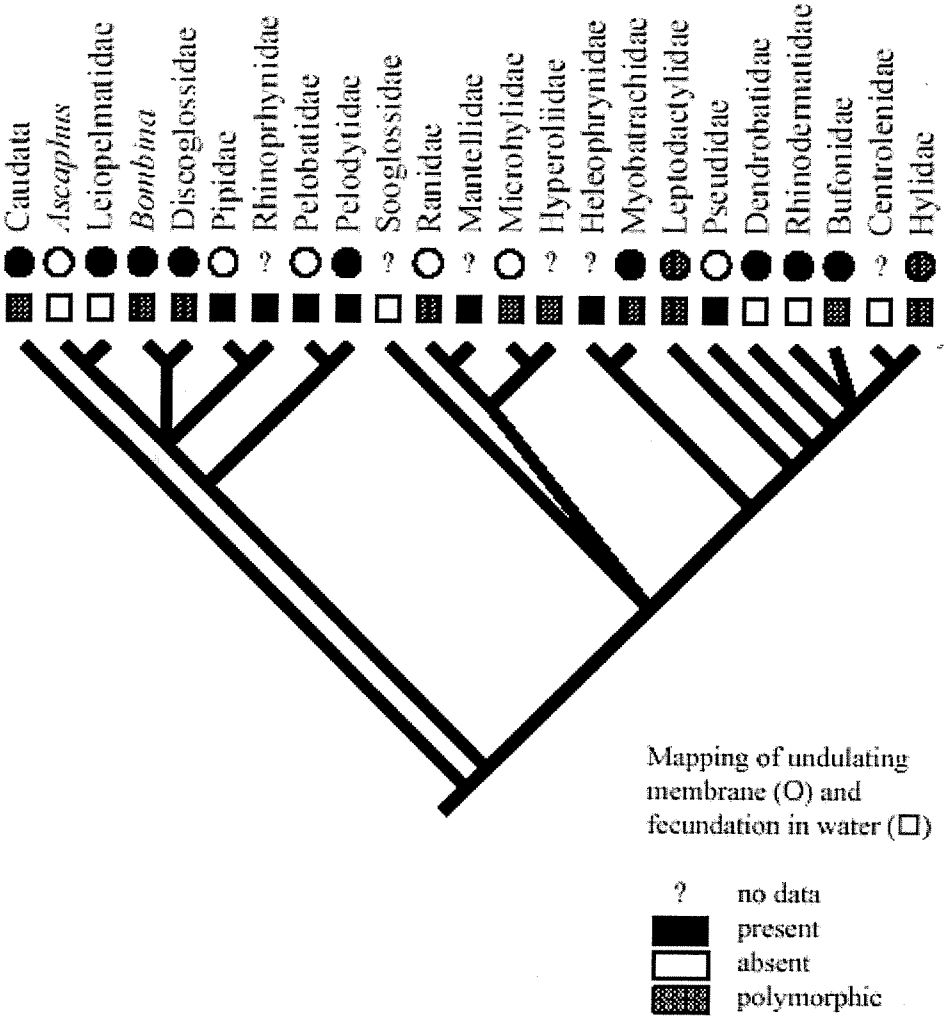
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Mantellidae
Microhylidae
Hyperoliidae
Heleophrynidae
Myobatrachidae
Leptodactylidae
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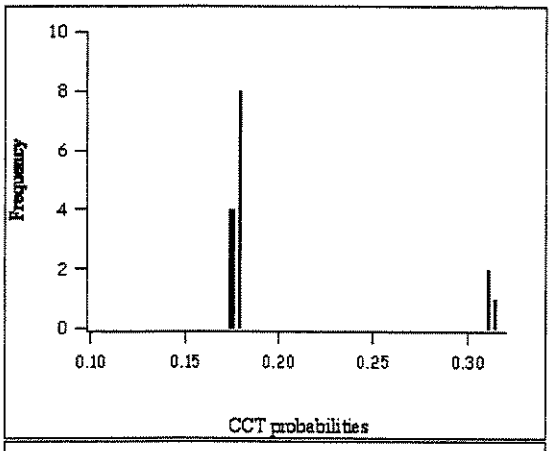
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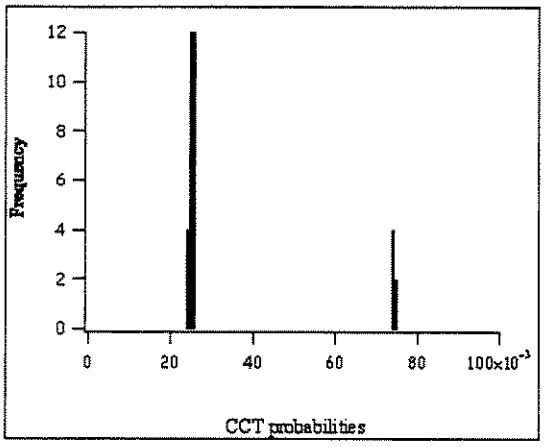
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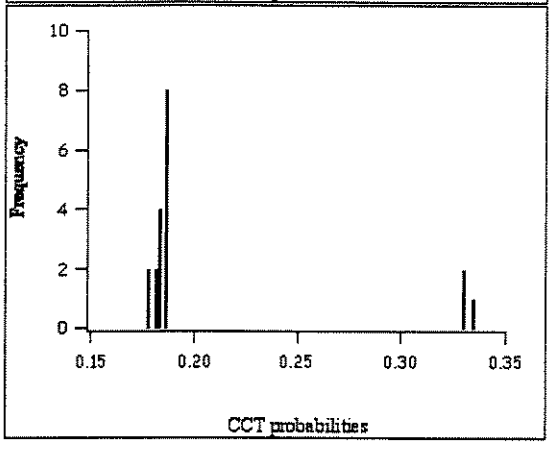
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Morphology of the spermatozoa of the Microhylidae (Anura, Amphibia)

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Running Head: Microhylid spermatozoa

Abstract

Scheltinga, D.M., Jamieson, B.G.M., Bickford, D.P., Garda, A.A., Báo, S.N. and McDonald, K.R. 200#. Morphology of the spermatozoa of the Microhylidae (Anura, Amphibia).-*Acta Zoologica* (Stockholm) ##: ###-###

Microhylid spermatozoa show the autapomorphic condition of possessing a thin post-mitochondrial cytoplasmic collar. Their spermatozoa are apomorphic in several respects. They have lost the distinct nuclear shoulder, endonuclear canal, and axial perforatorium observed in urodeles, caecilians and primitive frogs, possess a conical perforatorium, and apomorphically lack any fibres associated with the axoneme. The spermatozoa of *Cophixalus*, however, differ in several respects from those of the other microhylids examined. *Cophixalus* spermatozoa are longer in almost all measurements, the acrosome vesicle is cylindrical and does not completely cover the putative perforatorium, the perforatorium is asymmetrical and composed of fine fibres, the nucleus is strongly attenuated and narrower, and the mitochondria are elongate. The absence of fibres associated with the axoneme is an apomorphic condition shared with the Ranidae, Rhacophoridae, and Pipidae.

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Key Words: spermatozoa, ultrastructure, microhylid, frog, phylogeny, reproduction

Introduction

Frogs have the most diverse reproductive modes of all terrestrial vertebrates (Duellman and Trueb, 1994). These modes occur along trajectories toward terrestriality and can include placement of eggs out of water (e.g. in foam nests, on leaves overhanging streams, or completely

terrestrial nests), direct development (all development occurring within an egg capsule instead of a free-living aquatic tadpole stage) and parental care of eggs, tadpoles, and/or froglets. Frogs breed in a variety of habitats from aquatic to fossorial, terrestrial, and even arboreal. Any of the numerous combinations of these factors could influence selection of sperm motility and function.

The family Microhylidae is an enigmatic frog family with the highest generic diversity (>65 genera), most extreme morphological differences (from squat, nearly spherical fossorial and burrowing frogs, to slender and fully arboreal tree frogs), and greatest geographic disjunctions within the subfamilies of any other frog family (Parker, 1934; Savage, 1973; Frost, 1985; Burton, 1986; Wu, 1994; Duellman, 1999). Of the ten recognized subfamilies, four (Asterophryinae, Dyscophinae, Genyophryninae, and Microhylinae) are included in the present account. These frogs occur across Wallacea and South America. The Wallacea frogs in our study are from Australia (Genyophryninae: *Cophixalus ornatus* and *C. mcdonaldi*) and New Guinea (Genyophryninae: *Cophixalus* sp. A, *Liophryne schlaginhaufeni*, and *Sphenophryne cornuta*; Asterophryinae: *Callulops* sp. and *Hylophorbus rufescens*) through the Indo-Malaysian archipelago (Microhylinae: *Microhyla ornata*) to mainland S.E. Asia (Dyscophinae: *Calluella guttulata*). The South American frogs we examined are from Brazil (Microhylinae: *Ctenophryne geayi*, *Dermatonotus muelleri*, and *Elachistocleis ovalis*). The family Microhylidae has been considered a problematic frog family not only in terms of the evolutionary relationships within the family, but between it and other families of frogs as well (Burton, 1986; Ford and Cannatella, 1993; Wu, 1994; Hay *et al.*, 1995). Placement of the family relative to other frogs has ranged from primitive (Inger, 1967) to highly derived (Blommer-Schlosser, 1993; Wu, 1994; Emerson *et al.*, 2000).

Diagnostic synapomorphies for the Microhylidae have been elusive. Mandibular arch structure, carefully examined by Haas (2001), while providing several apomorphic characters, did

not provide characters diagnostic of all members of the family. The supposedly diagnostic type II tadpole does not define the family but a unique microhylid tadpole has nevertheless been defined (Wassersug, 1989). However, the microhylid frogs of New Guinea and Australia lack tadpoles, constituting the only microhylid subfamilies with all members displaying direct development (Asterophryinae and Genyophryninae). The Dyscophinae and Microhylinae taxa in this paper follow the typical frog life cycle, having aquatic eggs and tadpoles (Altig and McDiarmid, 1999).

Taxonomy and classification of the microhylid frogs have been and continue to be problematic. The only modern treatment of the entire family since Parker's (1934) opus is by Wu (1994), who utilized 188 morphological and osteological characters from all ten subfamilies of the microhylid frogs. Unfortunately, Wu's estimates of phylogeny were weakened by homoplasy and extremely low measures of data consistency (his "preferred cladogram": CI = 0.088, RI = 0.545, RC = 0.048). Considering the extremely high levels of homoplasy and lack of robustness in Wu's proposed cladograms, there remains progress to be made on a well-supported hypothesis of phylogeny for the group. An example of the status of the current taxonomy and systematics of the microhylid frogs is the recent work of Zweifel (2000), where frogs formerly placed in the genus *Sphenophryne* (Genyophryninae), were partitioned into three other genera (e.g. *Liophryne*) without evidence to hypothesize evolutionary relationships.

Spermatozoa provide an important non-traditional source of data that can be, and has been, used to better resolve relationships among various animal taxa (see Jamieson, 1999*a*, *b*, 2000*a*, *b*), including amphibian lineages (Jamieson *et al.*, 1993; Kwon and Lee, 1995; Meyer *et al.*, 1997; Scheltinga *et al.*, 2001; Selmi *et al.*, 1997). As part of a larger study using spermatozoal morphology in the phylogenetic reconstruction of the Amphibia, we here provide the first description of sperm of the anuran family Microhylidae.

Materials and Methods

Transmission Electron Microscopy

Wallacea specimens. Five calling male *Cophixalus ornatus* (Fry, 1912) were collected from several locations in far north-eastern Queensland, Australia between 10 February 1995 and 31 January 1997. Two male *Cophixalus mcdonaldi* Zweifel, 1985 (N73642, N73634) from Alligator creek, Bowling Green Bay National Park, Queensland, Australia on 13 August 1999. One male *Cophixalus* sp. (BSFS11900) from Aerosele, Herowana, Eastern Highlands Province, Papua New Guinea on 4 October 1998. A single male specimen of *Hylophorbus rufescens* Macleay, 1878 (BSFS11805) from the Pio River, Papua New Guinea on 11 August 1998. One male *Callulops* sp. from Wara Sera, Chimbu Province, Papua New Guinea on 3 September 1998 (BSFS11784). Three male *Liophryne* (=*Sphenophryne*) *schlaginhaufeni* (Wandolleck, 1911) from Aerosele camp, Herowana, Eastern Highlands Province, Papua New Guinea on 6 October (BSFS11904), 15 October (BSFS11965) and 23 October (BSFS11992) 1998. A single male *Sphenophryne cornuta* Peters and Doria, 1878 (BSFS11808) from Papua New Guinea on 12 August 1998.

Frogs were killed with either a lethal injection of anesthetic or immersed in a saturated chlorobutanol solution shortly after capture. Upon dissection, testes were quickly removed and fixed for TEM in 3% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2) at 4°C for at least two hours before being transported at ambient temperature to Brisbane, Australia, for processing and sectioning.

Museum specimens. Testes of a single specimen of *Calluella guttulata* (Blyth, 1856 "1855") (USNM103454) collected from Chantabun, Thailand on 8 May 1937 and a single *Microhyla ornata* (Dumèril and Bibron, 1841) (USNM278471) collected from N. P. Royal Thai air force

base, Nakhon Phanom, Thailand on 18 April 1969 were removed from animals in the Smithsonian Institution, Washington D.C., USA, collection. The specimens are of unknown initial fixation but have been stored in ethanol. Testes of these specimens were taken to Brisbane in 80% ethanol, where they were rehydrated through a descending ethanol series before being placed into 3% glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2) overnight before processing and sectioning as for normal glutaraldehyde-fixed tissues taken from the Wallacea specimens, as described below.

In Brisbane the testis material was cut into $1mm^3$ pieces and rinsed in 0.1M sodium phosphate buffer (three changes, each of 15 min); post-fixed for 80 min in similarly buffered 1% osmium tetroxide; rinsed in buffer (three changes, each of 15 min); dehydrated through an ascending ethanol series (20%, 40%, 60%, 80%, 90%, 100% (two changes); 30 min in each); and infiltrated (50% for 1 h; 75% for 1 h; 100% for 2 h; 100% for 12 h) and embedded (baked overnight at 60°C) in Spurr's epoxy resin (Spurr, 1969). Sections were cut with a diamond knife on a LKB 2128 UM IV microtome. Thin sections, 50-80nm thick, were collected on carbon stabilized, colloidin-coated, 200µm mesh copper grids, stained for 30 s in Reynold's lead citrate (Reynolds, 1963), rinsed in distilled water, then placed in 6% aqueous uranyl acetate for 4 min, rinsed in distilled water, and stained for a further 2 min in lead citrate before final rinsing. Electron micrographs were taken on an Hitachi 300 electron microscope at 75kV and a JEOL 100-s electron microscope at 60kV.

South American specimens. Five calling male *Elachistocleis ovalis* (Schneider, 1799) (CHUNB 13027, 13029, 12780, 12781, 12782) were collected from Parque Nacional de Águas Emendadas Planaltina, DF, Brazil, on 4 October 1999. Two calling male *Dermatonotus muelleri* (Boettger,

1885): one (CHUNB 12912) from Parque Nacional Grande Sertão Veredas, Formoso de Minas, MG, Brazil on 1 October 1998 and one (CHUNB 15250) from Palmas (Lajeado hydroelectric plan), TO, Brazil in November 1999. Four male specimens of *Ctenophryne geayi* Mocquard, 1904: two from near Porto Velho (Samuel hydroelectric plan), RO, Brazil, in 1992 and two (CHUNB 22584 and 22590) from Guajará-Mirim, RO, Brazil on 29 December 2000.

Animals used for light microscopy were initially fixed in formaldehyde and stored in 70% ethanol. Those used for electron microscopy were killed by rubbing xylocain onto the abdomen, testes were removed and cut into small pieces before being fixed in a solution of 2% glutaraldehyde, 2% paraformaldehyde, 3% sucrose and $CaCl_2$ at 5mM in cacodylate buffer at 4°C overnight. The testes material was then rinsed in cacodylate buffer (four changes, each of 15 min); post-fixed for 1 h in 1% osmium tetroxide and 0.8% potassium ferricyanide; stained *in block* overnight with an aqueous solution of uranyl acetate; rinsed in cacodylate buffer (four changes, each of five min); dehydrated through an ascending acetone series (30%, 50%, 70%, 90%, 15 min each and 100% three changes, each of 10 min) and infiltrated (30% for 6 h; 50% for 12 h; 60% for 6 h; 100% for 6 h) and embedded (24 h at 60°C) in Spurr's epoxy resin. Sections were cut with a diamond knife on a Leica Reichert Supernova ultramicrotome. Thin sections, 70-95nm thick, were collected on 200µm mesh copper grids, stained for 30 min in 3% uranyl acetate, rinsed in distillated water, then placed in Reynold's lead citrate for 8 min and rinsed again in distilled water. Electron micrographs were taken on an Jeol 100C electron microscope at 80kV.

Light Microscopy

Due to the limited amount of testes-material obtained for *Microhyla ornata* and *Hylophorbus*

rufescens, the sperm of these two species was not examined by light microscopy. Light microscopic observations and photographs of the spermatozoa of all the other species listed above were made using an Olympus BH2 microscope with Nomarski interference contrast and an attached OM-2 camera.

Results

The spermatozoa of all microhylids examined here are of similar structure and are therefore described together, with any differences noted.

Light microscopy

The testicular spermatozoa of all species examined are filiform and lack an undulating membrane associated with the tail (Fig. 1). The spermatozoa of *Cophixalus* spp, particularly *Cophixalus ornatus* and *C. mcdonaldi*, are extremely elongate, while those of *Liophryne* and *Sphenophryne* are moderately long and *Calluella*, *Callulops*, *Ctenophryne*, *Dermatonotus*, and *Elachistocleis* are relatively short (Fig. 1; Table 1). As most of the samples have been stored in fixative for several years the spermatozoa have become fragile. This may explain the large amount of variation seen in the measurements taken. Therefore, as the sperm are prone to breaking, the larger values given for the range may be more accurate of "typical" live sperm. Measurements of head (acrosome complex and nucleus), midpiece (neck and mitochondrial sheath), tail (and/or tail+midpiece combined) and total length are given in table 1. In some species it was impossible to distinguish between the end of the midpiece and the beginning of the tail. Thus, no measurements of midpiece length are given for these species. To allow comparisons between species, measurements of the tail+midpiece combined length are given for all species.

Transmission electron microscopy

The spermatozoon of *Cophixalus ornatus* is depicted diagrammatically in Fig. 2, and can be referred to throughout for all *Cophixalus* species, while Fig. 3 diagrammatically depicts *Elachistocleis ovalis* and can also be referred to for non-*Cophixalus* species. The testes samples of *Calluella guttulata* and *Microhyla ornata* are of unknown initial fixation (probably formalin) and have been alcohol stored for many years, therefore some structures, particularly membranes, have broken down.

Acrosome complex. The acrosome complex of *Cophixalus* spp. differs from that found in the other microhylids examined. The acrosome complex is elongate (Table 2), and composed of a relatively short cylindrical acrosome vesicle which caps only the apical portion of the putative conical perforatorium (Figs 4A,C,D, 6E). The acrosome vesicle is membrane bound and filled with electron-lucent material (Figs 4A,F,G, 6H). The elongate perforatorium occurs within the subacrosomal space, is composed of fine longitudinal fibres which appear homogenous in transverse section, and overlies the attenuated nuclear tip (Figs 4A,D, 6E). Posteriorly, the perforatorium extends beyond the base of the acrosome vesicle and is closely adpressed to one side of the nucleus (Figs 4A,B,H, 6G).

The acrosome complex of *Calluella*, *Callulops*, *Ctenophryne*, *Dermatonotus*, *Elachistocleis*, *Hylophorbus*, *Liophryne*, *Microhyla*, and *Sphenophryne* are of similar structure to each other, being relatively short (Table 2), and composed of a conical acrosome vesicle and an underlying putative conical perforatorium which caps the tapered nuclear tip (Figs 5A,J, 6A,C). The acrosome vesicle is membrane bound, composed of electron-pale to electron-lucent material, and completely covers the perforatorium (Figs 5A, 6A,B). Posteriorly, the electron density of the acrosome vesicle changes from pale to lucent. The perforatorium is composed of coarse

longitudinal fibres and is more electron-dense than the acrosome vesicle (Figs 5A,C,D, 6A-C,K,L). The acrosome complex is symmetrically attached to the nucleus (Figs 5A,D,J, 6A,C,L).

Nucleus. The nucleus is conical. It varies from short (*Calluella*, *Callulops*, *Ctenophryne*, *Dermatonotus*, *Elachistocleis*, *Hylophorbus*, and *Microhyla*) to elongate (*Cophixalus*), and is composed of condensed chromatin (Figs 4I, 5E,J, 6A,C,D; Table 2). Anteriorly, the nucleus of *Cophixalus* spermatozoa tapers to a fine point within the acrosome complex (Figs 4B, 6E), whereas the apical tip of the nucleus is rounded in the other examined microhylids (Figs 5A, 6A,C). Numerous nuclear inclusions containing moderately electron-dense material are present (Fig. 6A,C). At the base of the nucleus is an asymmetrical fossa (the basal nuclear or centriolar fossa) in which the proximal centriole lies (Figs 4E, 5F,L,M). Distinct nuclear shoulders are absent. The nucleus increases throughout its length to a maximum diameter at the level of the basal nuclear fossa (Table 2).

Midpiece. The midpiece is here considered to include the centriolar region (neck) and the mitochondrial collar, but not the cytoplasmic collar. Values for midpiece length are given in Table 1. Within the nuclear fossa lies the proximal centriole which is surrounded by pericentriolar material that connects it to the nuclear fossa and distal centriole. The proximal centriole is orientated at 80° to the long axis of the nucleus (Figs 4E, 5J,L,M). The distal centriole is located posterior to the proximal centriole and is orientated with its length in the long axis of the spermatozoon. The distal centriole is continuous with the 9+2 axoneme of the flagellum. Both centrioles are composed of nine, circularly arranged, triplets of short microtubules (Figs 4E,J, 5G). Several mitochondria with prominent cristae form a sheath, the mitochondrial collar, surrounding the anterior portion of the axoneme. This sheath is attached only to the centriolar

region of the spermatozoon. A space (the cytoplasmic canal) separates the mitochondrial collar from the axoneme (Figs 4E,K,M, 5B,H,M,N, 6F,I). The mitochondria of *Cophixalus* differ from the approximately spherical form of other microhylids in being elongate. This is particularly evident in *Cophixalus mcdonaldi* where the mitochondria extend along the length of the midpiece in a spiral pattern (Fig. 4O).

Tail. For an undetermined length the axoneme is surrounded by a cytoplasmic collar, the two being separate by the cytoplasmic canal. The collar is composed of a thin layer of membrane bounded cytoplasm which extends posteriorly from the mitochondrial collar (Figs 4L,N, 5O). The cytoplasmic collar observed in *Calluella guttulata* and *Microhyla ornata* requires confirmation from suitably fixed material. The collar is longer and more highly developed in *Cophixalus* than the other microhylids examined. An axial fibre, undulating membrane, and juxta-axonemal fibres are absent (Figs 4K-N,P,Q, 5H,I,K,M-P, 6F,I,J,M,N).

Discussion

Examination of microhylid spermatozoa has produced a variety of characters which may be useful in elucidating both intra and inter-family relationships. The spermatozoa of *Cophixalus* differ in several respects from those of the other microhylids examined. *Cophixalus* spermatozoa are longer in almost all measurements, the acrosome vesicle is cylindrical and does not completely cover the perforatorium, the perforatorium is asymmetrical and composed of fine fibres, the nucleus is strongly attenuated and narrower, and the mitochondria are elongate. However, all microhylid spermatozoa studied to date show the autapomorphic condition of possessing a thin post-mitochondrial cytoplasmic collar, which supports their monophyly. Their spermatozoa are apomorphic in several respects. They have lost the distinct nuclear shoulders,

endonuclear canal, and axial perforatorium observed in urodeles, caecilians and primitive frogs (Furieri, 1975*a*, *b*; Jamieson *et al.*, 1993; Picheral, 1979; van der Horst *et al.*, 1991). The lack of any fibres associated with the axoneme is also an apomorphic character state as associated fibres are present in most amphibians, sarcopterygian fish, and amniotes (see Jamieson, 1999*a*). The presence of a putative conical perforatorium composed of longitudinal sheaves or fibres is questionably apomorphic and will be discussed below.

The gross morphology of the acrosome complex of microhylids is distinctly different from that of ranids and more similar to Bufonoidea and Pelodytidae in being conical (cylindrical in *Cophixalus*) and having the perforatorium composed of fibres (Lee and Jamieson, 1993; Pugin-Rios, 1980). However, questions remain as to the homology of the conical perforatorium. The conical perforatorium appears unlikely to be homologous with either the axial perforatorium and/or subacrosomal cone/periperforatorial material found in urodeles, caecilians, and some primitive frogs. The conical perforatorium develops much later in spermiogenesis, in a different position, and with a different ontogeny and final form (Abé *et al.*, 1981; Folliot, 1979; Lee and Kwon, 1992; Picheral, 1972; Rastogi *et al.*, 1988), and is probably a unique development of those anurans possessing it. However, the replacement of an axially located rod-like perforatorium with a conical, albeit putative, perforatorium, is a synapomorphy whether or not axial and conical perforatoria are homologous. The presence of a conical perforatorium in the archaeobatrachian Pelodytidae (Pugin-Rios, 1980) suggests that this developed relatively early in anuran evolution.

The relatively short acrosome vesicle compared to the perforatorium and asymmetrical nature of the perforatorium of *Cophixalus* is superficially similar to that seen in the hylid frog *Litoria longirostris* (pers. obs.). However, the acrosome complex of *L. longirostris* is highly modified, compared to that of other hylids, and may be selected for the penetration of a thick jelly mass which surrounds the terrestrial egg mass. In *L. longirostris* the acrosome vesicle is distinct and

well-developed while the perforatorium is not composed of distinct longitudinal fibres and does not surround the nuclear attenuation at any stage.

The Eubufonoidea are united by a single synapomorphy, the mitochondrial collar (Jamieson, 1999*a*; Lee and Jamieson, 1993). This is a thick collar-like cytoplasmic sheath that emanates from the centriolar region, is separated from the flagellum by a cytoplasmic canal, and contains the mitochondria. This synapomorphy led to the erection of the superfamily Eubufonoidea by Lee and Jamieson (1993). This condition is also seen in the microhylids though in these an autapomorphic, distinct cytoplasmic collar is seen in addition to the mitochondrial collar. A short poorly-developed mitochondrial collar is also found in the lungfish *Neoceratodus forsteri*, Leiopelmatidae, and Pipidae (Bernardini *et al.*, 1986; Jespersen, 1971; Scheltinga *et al.*, 2001). Thus, a mitochondrial collar appears to be plesiomorphic for anurans.

The large, elongate mitochondria observed in *Cophixalus* spermatozoa are, among other amphibians, known only from some foam nesting rhacophorids, in which the highly aberrant spermatozoa are corkscrew-shaped and biflagellate (Mizuhira *et al.*, 1986; Wilson *et al.*, 1991), and *Ascaphus truei* (Jamieson *et al.*, 1993). Numerous small ovoid mitochondria are characteristic of dipnoans and urodeles, and appear to be the primitive condition in anurans (Jamieson, 1999*a*; Picheral, 1979; Selmi *et al.*, 1997). The large elongate mitochondria appear to have arisen independently in each of the taxa *Ascaphus*, rhacophorids and *Cophixalus*.

The absence of any fibres associated with the axoneme in microhylids is an apomorphic condition for Amphibia. Associated fibres are present in urodeles, caecilians and most frogs (Kwon and Lee, 1995; Picheral, 1979; Pugin-Rios, 1980; van der Horst *et al.*, 1991). Their absence from the spermatozoa of the Ranidae, Rhacophoridae, Pipidae, and Microhylidae is an apomorphy shared by these families. On currently accepted relationships of anuran families (Duellman and Trueb, 1994; Hedges and Maxon, 1993), it appears that this may be convergence

(homoplasy) but the possibility that it may be a synapomorphy, indicating that loss was a monophyletic event, cannot yet be dismissed. Absence in some leptodactylids (Pisano and Adler, 1968; Pugin-Rios, 1980) is clearly secondary and independently derived.

Spermatozoal lengths vary greatly within the Anura and in particular within microhylids. A total length of 38.2μm in *Dermatonotus muelleri* makes it one of the shortest recorded while *Cophixalus ornatus* at up to 158μm is amongst the longest (*Telmatobufo australis* and *Leiopelma hochstetteri* reach approximately 240-250μm, while *Discoglossus pictus* attains a length of 2,500μm (Furieri, 1975*a*; Pugin-Rios, 1980; Scheltinga *et al.*, 2001). Lengths of various aspects of the spermatozoa may prove useful in examination of subfamial relationships. Spermatozoa of the Genyophryninae have longer head, tail and overall lengths while the Asterophryinae and Dyscophinae have a similar moderate head length, and the Microhylinae a short head length (Table 1).

Sperm characters provide an important data set which has been shown by this study to contribute valuable information to phylogenetic reconstruction. To the suite of characters defining an, albeit inconstant, unique larval type in other Microhylidae (Wassersug, 1984, 1989), sperm ultrastructure has added a further synapomorphy for the family: the presence of a thin post-mitochondrial cytoplasmic collar in the spermatozoon. It should be noted, however, that several subfamilies of the Microhylidae have yet to be examined for sperm ultrastructure. Further work describing the spermatozoal ultrastructure and dimensions of other microhylids is needed to determine the degree of variability within the family and to provide data which may assist in determining intrafamial relationships, particularly at subfamial level.

Acknowledgements

We thank Wayne E. Martin and Conrad Hoskin for assisting with the collection of Australian frogs, and George R. Zug and the staff at the Smithsonian Institution (Washington D.C., USA) for their assistance in accessing material in their collection. Illustrations are by DMS. This work was made possible by a Department Research Grant to DMS, an Australian Research Council grant to BGMJ, and Capes and CNPq financial assistance to AAG and SNB. The Wildlife Conservation Society (New York, USA) and the University of Miami supported field work by DPB in Papua New Guinea. The Research and Conservation Foundation of PNG is thanked for logistical field support to DPB while in PNG. The constructive comments of an anonymous reviewer are gratefully acknowledged.

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Table 1 Dimensions (mean and standard deviation (SD)) of spermatozoa taken from light and transmission electron microscopy

Species	Head	Midpiece	Tail	Tail & Midpiece	Total length
Asterophryinae					
Callulops sp.	14.9μm (n=8, SD=1.1)	-	-	37.8μm (n=6, SD=1.0)	52.9μm (n=7, SD=1.1) (50.7-54.3μm)
Dyscophinae					
Calluella guttulata	15.1μm (n=18, SD=1.3)	2.3μm (n=14, SD=0.4)	29.0μm (n=11, SD=1.0)	31.1μm (n=11, SD=1.1)	46.6μm (n=11, SD=1.4) (44.2-48.8μm)
Genyophryninae					
Cophixalus mcdonaldi	66.2μm (n=20, SD=3.6)	13.6μm (n=5, SD=0.8)	57.6μm (n=4, SD=4.9)	67.2μm (n=15, SD=4.5)	132.9μm (n=19, SD=6.0) (125.3-145.5μm)
Cophixalus ornatus	78.3μm (n=32, SD=4.5)	4.4μm (n=10, SD=1.0)	67.0μm (n=5, SD=3.3)	70.2μm (n=22, SD=3.6)	148.2μm (n=22, SD=5.2) (140.0-158.4μm)
Cophixalus sp.	40.4μm (n=25, SD=3.5)	-	-	45.4μm (n=18, SD=2.7)	84.9μm (n=19, SD=3.6) (79.2-93.9μm)
Liophryne schlaginhaufeni	22.9μm (n=25, SD=1.3)	5.0μm (n=7, SD=0.9)	45.3μm (n=5, SD=3.3)	47.4μm (n=18, SD=2.7)	69.0μm (n=21, SD=4.4) (62.6-79.2μm)
Sphenophryne cornuta	24.9μm (n=11, SD=1.2)	4.5μm (n=4, SD=0.3)	38.1μm (n=3, SD=1.1)	42.3μm (n=3, SD=1.7)	66.6μm (n=3, SD=1.0) (65.4-67.2μm)
Microhylinae					
Ctenophryne geayi	11.3μm (n=40, SD=0.9)	2.1μm (n=5, SD=0.4)	♀ 36.9μm	38.9μm (n=25, SD=2.8)	50.2μm (n=25, SD=2.5) (46.7-55.8μm)
Dermatonotus muelleri	11.3μm (n=30, SD=0.9)	1.8μm (n=2, SD=0.1)	♀ 25.1μm	27.0μm (n=25, SD=1.1)	38.2μm (n=27, SD=1.5) (35.7-42.2μm)
Elachistocleis ovalis	12.6μm (n=27, SD=1.0)	1.5μm (n=11, SD=0.5)	♀31.0μm	32.5μm (n=13, SD=5.2)	45.4μm (n=14, SD=4.6) (40.3-57.8μm)
Microhyla ornata	8.5μm (n=1)	-	-	-	-

n, number. Range given for total length.

Table 2 Dimensions (mean and standard deviation (SD)) of spermatozoa taken from transmission electron microscopy

Species	Acrosome complex	Nucleus length	Nucleus width (base)
Asterophryinae			
Callulops sp.	short	short	-
Dyscophinae			
Calluella guttulata	4.2μm (n=4, SD=0.2)	10.21μm (n=1)	1.02μm (n=5, SD=0.03)
Genyophryninae			
Cophixalus mcdonaldi	elongate	elongate	0.64μm (n=6, SD=0.02)
Cophixalus ornatus	elongate	elongate	0.6μm (n=6, SD=0.06)
Cophixalus sp.	elongate	elongate	0.75μm (n=2, SD=0.15)
Liophryne schlaginhaufeni	moderate	moderate	-
Sphenophryne cornuta	6.8μm (n=2, SD=0.6)	♀21.6μm	1.1μm (n=2, SD=0.14)
Microhylinae			
Ctenophryne geayi	3.0μm (n=7, SD=0.6)	♀9.4μm	0.89μm (n=3, SD=0.07)
Dermatonotus muelleri	2.8μm (n=6, SD=0.4)	♀10.3μm	0.95μm (n=3, SD=0.05)
Elachistocleis ovalis	2.6μm (n=4, SD=0.4)	11.7μm (n=1)	1.0μm (n=10, SD=0.15)
Microhyla ornata	3.8μm (n=2, SD=0.3)	6.7μm (n=1)	1.15μm (n=2, SD=0.07)

n, number. Acrosome complex: short ⊘ 5μm; moderate > 5μm but ⊘ 9μm; elongate > 9μm.

Nucleus length: short ⊘ 15μm; moderate > 15μm but ⊘ 25μm; elongate > 25μm.

Figure Legends

Fig. 1 -Light micrographs of the spermatozoa of: -**A**. *Cophixalus ornatus* (note anterior tip of acrosome broken off). -**B**. *Cophixalus* sp. (note tail broken off). -**C**, *Liophryne schlaginhaufeni* (note tail broken off). -**D**. *Sphenophryne cornuta*. -**E**. *Calluella guttulata*. -**F**. *Dermatonotus muelleri*. -**G**. *Ctenophryne geayi*. -**H**. *Elachistocleis ovalis*. All to the same scale as indicated. Abbreviations: h, head (acrosome and nucleus); mp, midpiece; t, tail.

Fig. 2 -Drawing of a spermatozoon of *Cophixalus ornatus*. Drawn from several TEM micrographs.

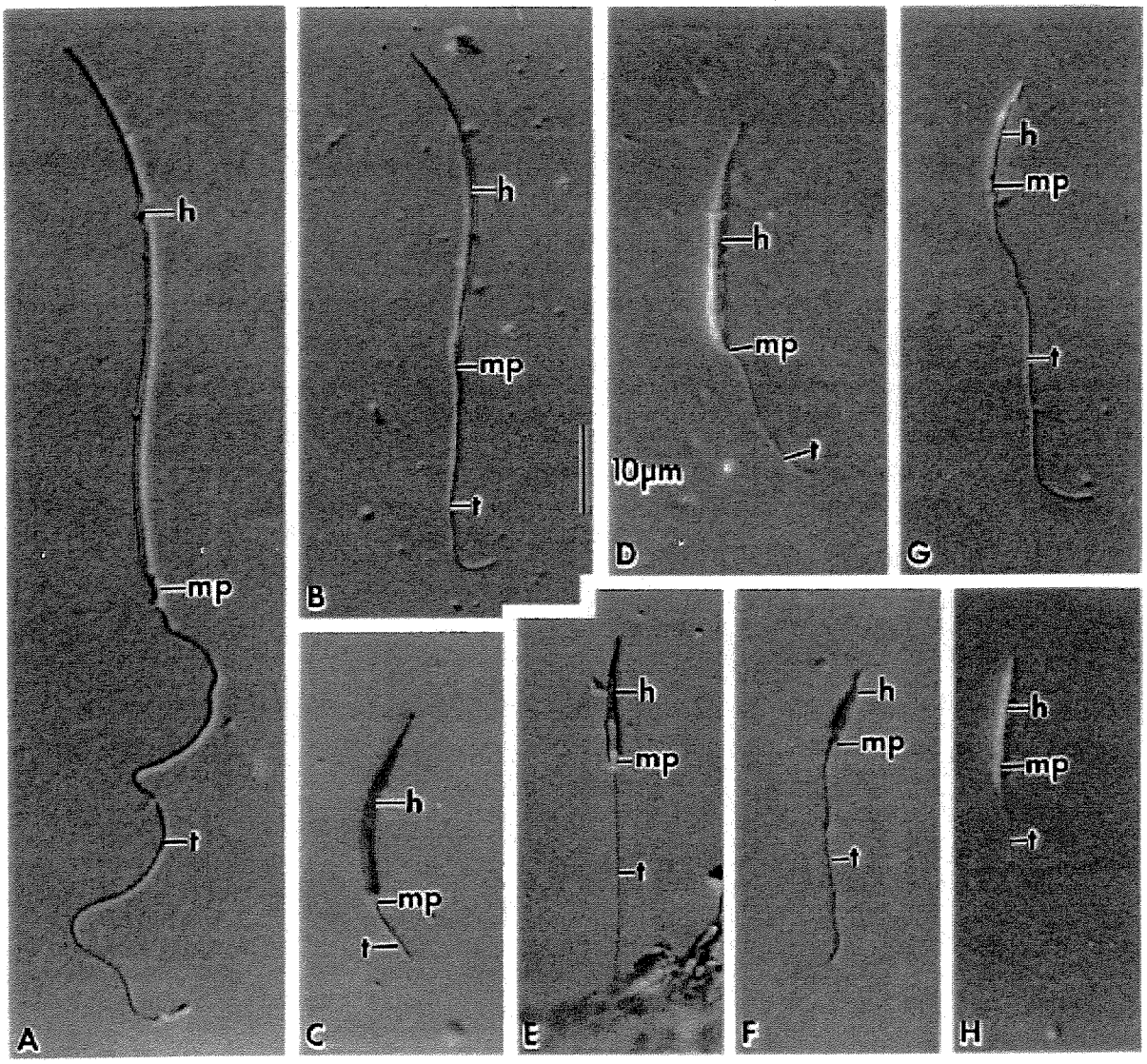
Fig. 3 -Drawing of a spermatozoon of *Elachistocleis ovalis*. Drawn from several TEM micrographs.

Fig. 4 -TEM micrographs of the spermatozoa of *Cophixalus mcdonaldi*. -**A**. Longitudinal section (L.S.) of the acrosome vesicle which surrounds the apical region of the perforatorium and nucleus. -**B**. L.S. through the anterior nucleus showing the perforatorium lying along one side of the nucleus only (see also H). -**C**. L.S. through the apical tip of the spermatozoon. -**D**. L.S. through the posterior region of the acrosome vesicle. -**E**. L.S. through the neck region showing the nuclear fossa, centrioles, axoneme, cytoplasmic canal and mitochondrial collar. **F-L**. Successive transverse sections (T.Ss) through the spermatozoon as indicated. -**F**. through the apical region of the acrosome complex. -**G**. through the posterior region of the acrosome vesicle. -**H**. through the posterior junction of the perforatorium and nucleus. -**I**. through the nucleus. -**J**. through the distal centriole. -**K**. through the midpiece. -**L**. through the cytoplasmic collar region

of the tail. -**M**. L.S. through the end of the mitochondrial collar (arrows). -**N**. L.S. through the cytoplasmic collar of the tail. -**O**. Oblique L.S. of the midpiece showing the elongate mitochondria's spiral pattern. -**P**. L.S. of the tail. -**Q**. T.S. through the tail as indicated. Note the absence of any associated fibres. All to the same scale as indicated. Abbreviations: a, axoneme; av, acrosome vesicle; cc, cytoplasmic canal; cy, cytoplasm; cyc, cytoplasmic collar; dc, distal centriole; f, nuclear fossa; l, nuclear inclusion; m, mitochondrion; mc, mitochondrial collar; n, nucleus; p, putative conical perforatorium; pc, proximal centriole; pm, plasma membrane.

Fig. 5 -TEM micrographs of the spermatozoa of **A-L** *Calluella guttulata*. -**A**. Longitudinal section (L.S.) of the apical region of a spermatozoon, showing the acrosome complex surrounding the anterior region of the nucleus. -**B**. Oblique L.S. of the neck region showing spherical mitochondria and the cytoplasmic canal. **C-I**. Successive transverse sections (T.Ss) through the spermatozoon as indicated. -**C**. through the apical region of the acrosome complex. -**D**. through the posterior region of the acrosome complex and anterior nucleus. -**E**. through the nucleus. -**F**. through the nuclear fossa and proximal centriole. -**G**. through the distal centriole. -**H**. through the midpiece. -**I**. through several tails. -**J**. L.S. of the nucleus. -**K**. L.S. of the tail. -**L**. L.S. of the neck region showing the nuclear fossa and centrioles. **M-P** *Hylophorbus rufescens*. -**M**. L.S. of the neck region showing the nuclear fossa, centrioles, axoneme, cytoplasmic canal and mitochondrial collar. -**N**. T.S. through the midpiece. -**O**. T.S. through the cytoplasmic collar region of the tail. -**P**. T.S. through the tail. A-I, K-P to the same scale as indicated. J to scale as indicated. Abbreviations: a, axoneme; av, acrosome vesicle; cc, cytoplasmic canal; cy, cytoplasm; cyc, cytoplasmic collar; dc, distal centriole; f, nuclear fossa; m, mitochondrion; mc, mitochondrial collar; n, nucleus; p, putative conical perforatorium; pc, proximal centriole.

Fig. 6 -TEM micrographs of the spermatozoa of **A-B** *Ctenophryne geayi*. -**A**. Longitudinal section (L.S.) of the apical region of a spermatozoon, showing the acrosome complex surrounding the anterior region of the nucleus. -**B**. Transverse sections (T.S.) through the apical region of the acrosome complex. **C** *Sphenophryne cornuta*. -**C**. L.S. of the acrosome complex. **D** *Ctenophryne geayi*. -**D**. T.S. through the nucleus. **E** *Cophixalus* sp. -**E**. L.S. of the acrosome complex. **F** *Ctenophryne geayi*. -**F**. T.S. through the midpiece. **G-I** *Cophixalus* sp. -**G**. L.S. through the anterior nucleus showing the perforatorium lying along one side of the nucleus (see also H). -**H**. T.Ss through the acrosome complex and nucleus. -**I**. T.S. through the midpiece. **J** *Ctenophryne geayi*. -**J**. T.Ss through the tail. **K** *Liophryne schlaginhaufeni*. -**K**. T.S. through the apical region of the acrosome complex. **L-M** *Sphenophryne cornuta*. -**L**. through the posterior region of the acrosome complex. -**M**. T.S. through the tail. **N** *Cophixalus* sp. -**N**. T.S. through the tail. Scales as indicated. Abbreviations: a, axoneme; av, acrosome vesicle; cc, cytoplasmic canal; l, nuclear inclusion; m, mitochondrion; n, nucleus; p, putative conical perforatorium.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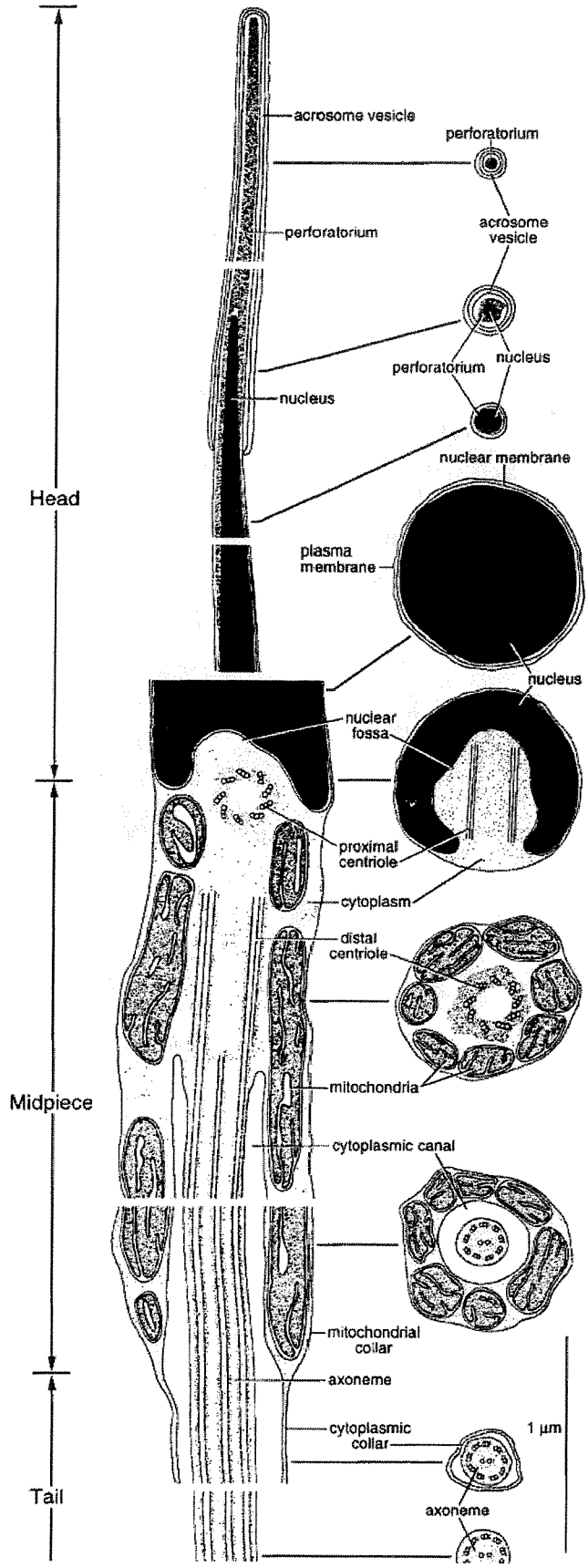
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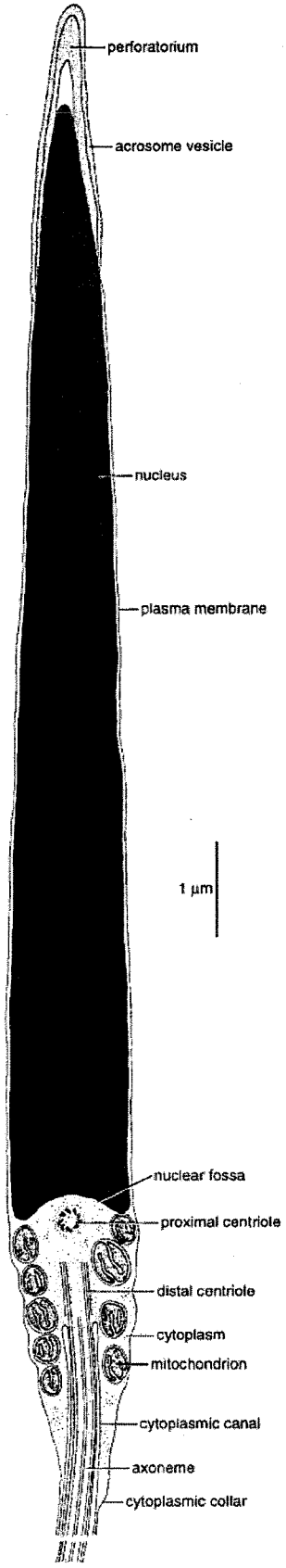
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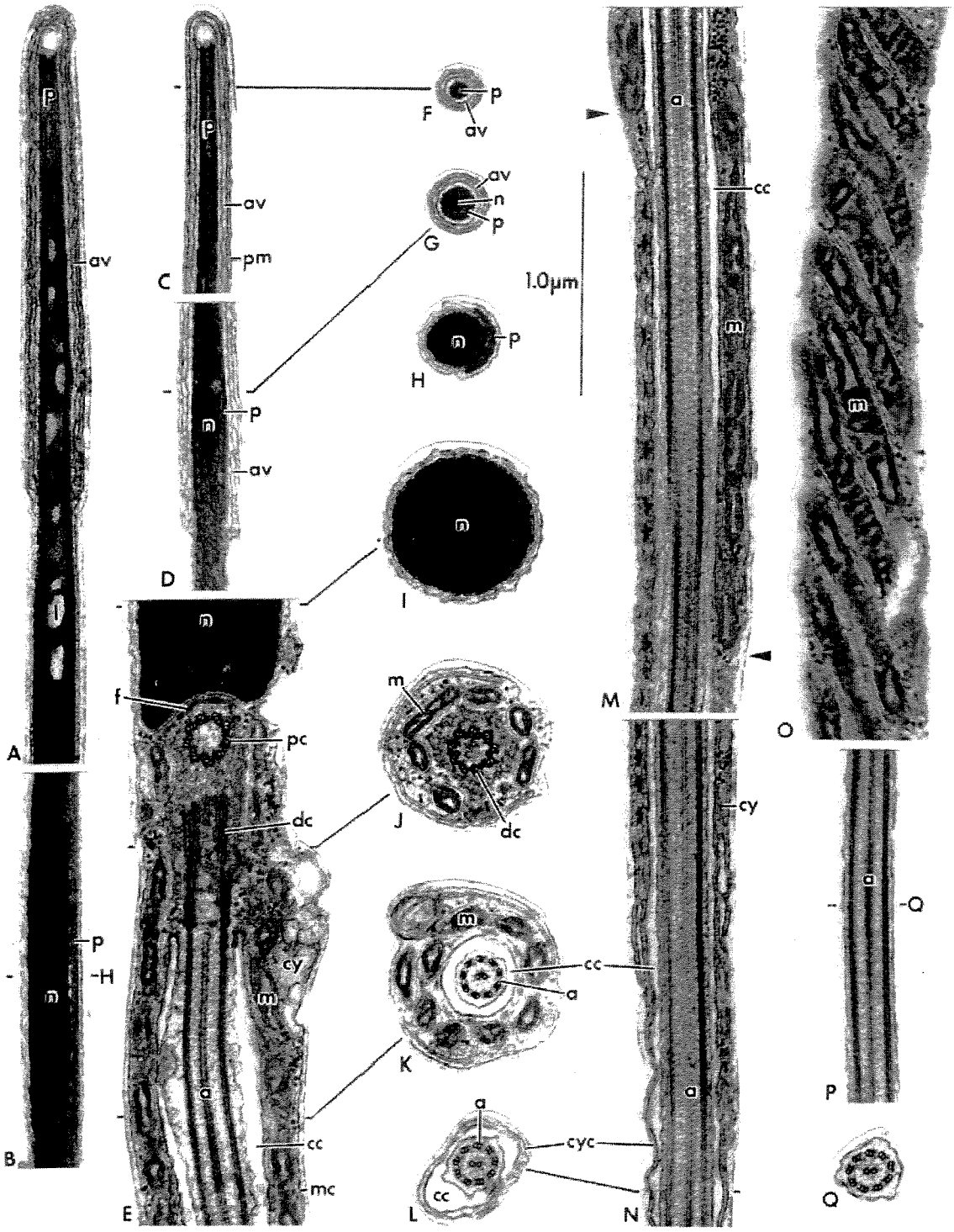
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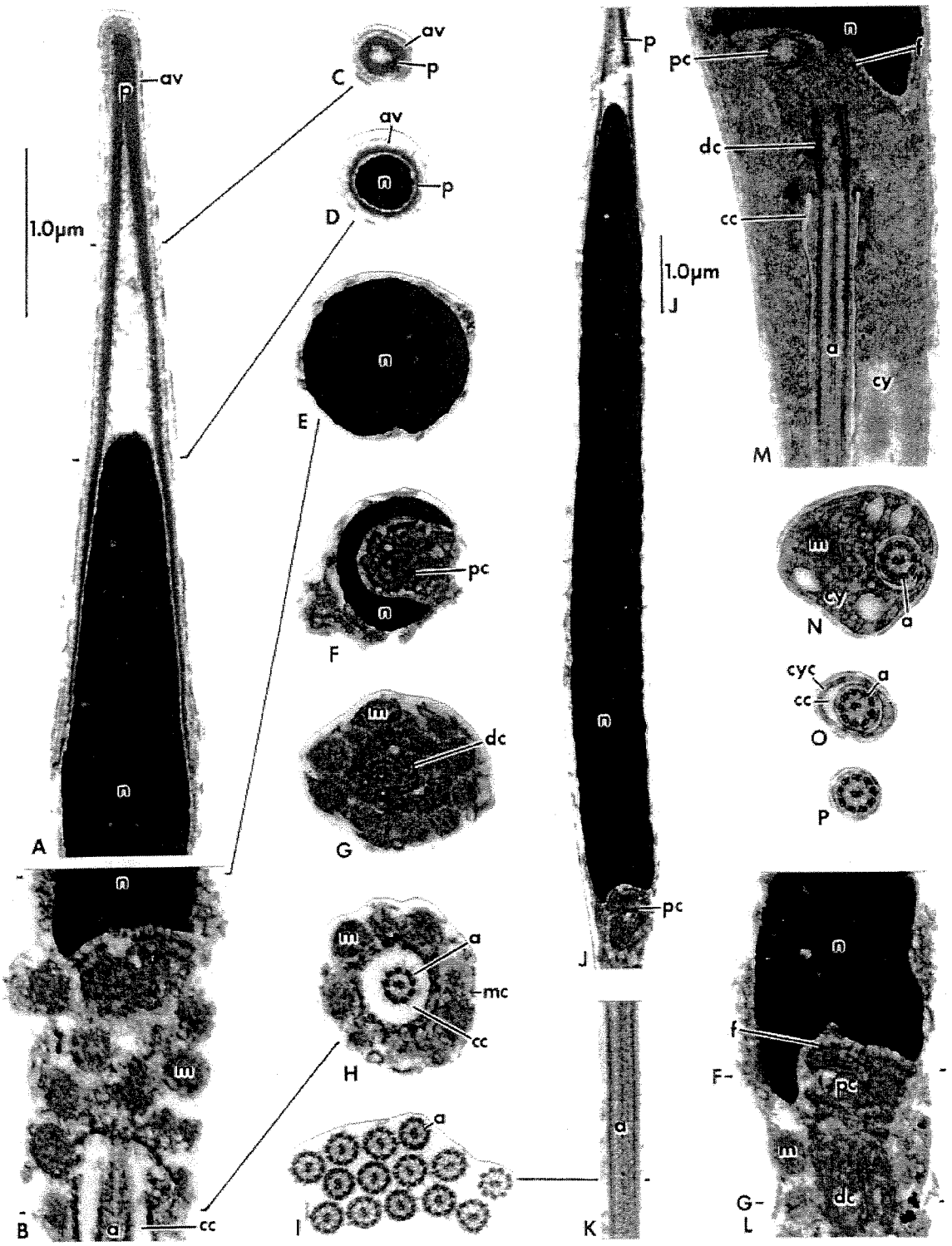
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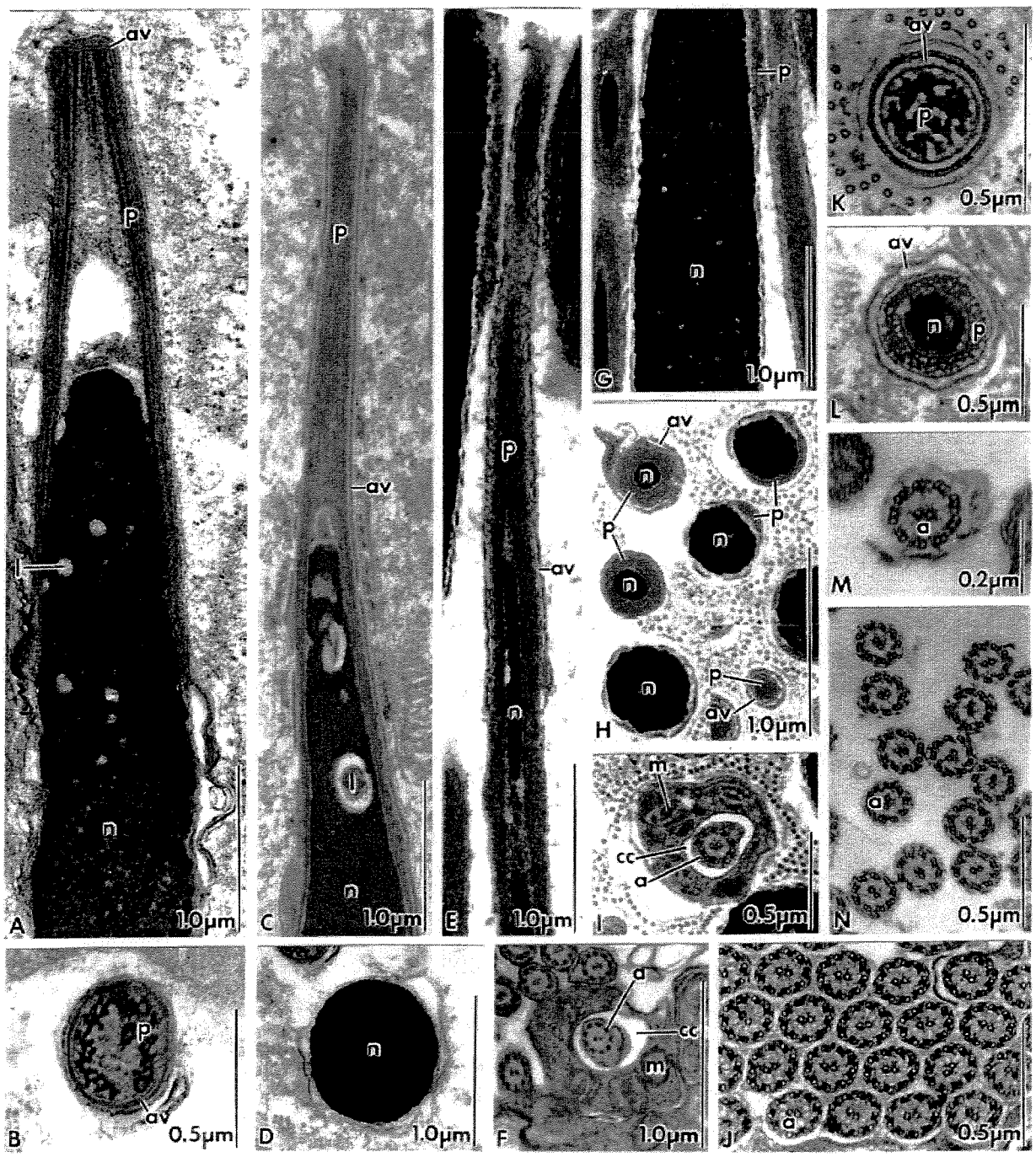
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3. Conclusões Gerais

Dendrobatidae

Os resultados dos trabalhos aqui apresentados sobre a família Dendrobatidae mostram que essa está mais relacionada à linhagem Bufonoidea em virtude da presença de um cone subacrossomal bem desenvolvido, colar de mitocôndrias e fibras auxiliares da cauda. Segundo Duellman & Trueb (1986), a família Dendrobatidae pertenceria à subfamília Ranoidea. Trabalhos mais recentes, todavia, também sugerem a colocação de Dendrobatidae em Bufonoidea (Vences *et al.*, 2000). O cone subacrossomal altamente desenvolvido em *Epipedobates flavopictus* sugere que as estruturas outrora tidas como análogas (cone subacrossomal e perforatório cônico) são na verdade homólogas devido ao menor número de passos que essa alternativa fornece.

A comparação entre os dois gêneros de dendrobatídeos (*Epipedobates* e *Colostethus*) sugere que *E. femoralis* seria mais próximo de *Colostethus* que de outros membros de *Epipedobates*, conforme sugerem resultados de trabalhos com filogenia molecular (Vences *et al.*, 2000).

Pseudidae

O espermatozóide da família Pseudidae tem uma ultra-estrutura simples e peculiar em muitos aspectos. Apesar da ausência de elementos auxiliares na cauda, a presença de um cone subacrossomal e a disposição perpendicular dos centríolos aproximam a família de Bufonoidea. A redução dos caracteres no espermatozóide de *Pseudis* e *Lysapsus* aparentemente endossa uma possível correlação entre ultra-estrutura e ambiente de fecundação (como observado em Pipidae e Ranidae), visto que essas três famílias

freqüentemente têm fecundação associada à água. Todavia, essa aparente correlação não foi estatisticamente significativa quando uma análise filogenética foi feita com esses dados. A presença de uma vesícula acrossomal subdividida em duas porções nas cinco espécies de pseudídeos analisadas e a ausência dessa condição em qualquer outro anuro estudado sugerem que essa seja uma possível sinapormofia da família. Ainda, a ausência de elementos auxiliares na cauda, bem como a presença de fibras periaxiais sugerem uma possível relação entre Pseudidae e alguns membros da família Leptodactylidae (*Telmatobius*), conforme anteriormente proposto (Noble, 1931).

Portanto, algumas hipóteses estabelecidas antes do presente trabalho não se mostraram consistentes sob a luz das novas descrições e metodologias aqui apresentadas. O perforatório cônico parece, na verdade, homólogo ao cone subacrossomal, ao contrário do que era previamente proposto (Jamieson *et al.*, 1993). Com isso, o mesmo não pode ser tomado como uma sinapormorfia de Bufonoidea. Ainda, a presença de um cone subacrossomal partido em fibras em Microhylidae endossa essa conclusão. A entrada do bastão paraxonemal na fossa nuclear em Dendrobatidae, por sua vez, vai de encontro a suposições anteriores de que essa característica seria uma sinapomorfia de Myobatrachidae (Lee & Jamieson, 1992).

Assim, fica clara a necessidade de investigação das famílias ainda não descritas, bem como uma amostragem maior de gêneros em famílias já estudadas. Só com essas análises será possível ter-se uma noção da variabilidade da ultra-estrutura do espermatozóide nas famílias de anuros. Ainda, o presente trabalho mostra como a inferência a respeito da condição de certos caracteres pode ser errônea se feita *a priori* de uma análise filogenética dos caracteres em questão.

Descrições detalhadas e bem informativas sobre todas as espécies são de suma

importância para complementar a base de dados e permitir a utilização desse dados para futuras análises filogenéticas. Com o intuito de estabelecer-se um padrão na análise da ultra-estrutura, seguem recomendações a respeito da análise dos caracteres de ultra-estrutura.

MICROHYLIDAE

A família Microhylidae, por sua vez, mostrou uma série de peculiaridades em relação ao grupo em que atualmente é alocada (Ranoidea: Rhacophoridae e Ranidae). Os outros membros dessa subfamília descritos até o momento não possuem cone subacrossomal, o qual está presente em todas as espécies analisadas de Microhylidae. Por outro lado, a ausência de elementos auxiliares na cauda (membrana ondulante e fibras) endossa uma maior proximidade entre essas três famílias. A presença de uma extensão do colar de mitocôndrias em todas as 11 espécies pertencentes a 4 subfamílias (Asterophrynae, Dyscophinae, Genyophryninae, Microhylinae). Essa característica pode ser considerada uma possível sinapomorfia da família, visto que as espécies analisadas têm uma distribuição geográfica bastante disjunta (Austrália, Asia e América do Sul).

CARACTERES A SEREM ANALISADOS

Para uma análise consistente sobre a ultra-estrutura do espermatozóide de anuros, é recomendado que a espécie em estudo seja ranqueada para os caracteres que seguem abaixo. Assim, cortes longitudinais e transversais de diversas regiões da célula são necessários, bem como um n razoável para cada região e a utilização de mais de um indivíduo de cada espécie em estudo.

SUGESTÃO DE CARACTERES DA ULTRA-ESTRUTURA DO ESPERMATOZÓIDE DE ANUROS

CABEÇA

[1] **Forma do espermatozóide**

(0) Linear

(1) Helicoidal

(2) Saca-rolhas

[2] **Forma da região acrossomal em secção transversal**

(0) Angulada

(1) Circular

[3] **Membranas Multilaminares**

(0) Presentes

(1) Ausentes

[4] **Forma do Acrossoma**

(0) Cilíndrico

(1) Arredondado

(2) Botão

[5] **Posição do Acrossoma**

(0) Apical

(1) Ao lado do núcleo

[6] **Estrutura do cone subacrossomal**

(0) Feixes individuais

(1) Maciço

[7] **Espaço subacrossomal**

(0) Ausente

(1) Presente

[8] **Zona epinuclear lúcida**

(0) Ausente

(1) Presente

[9] **Canal endonuclear contendo perforatório**

(0) Ausente

(1) Presente

[10] **Ombros nucleares**

(0) Ausente

(1) Presente

PEÇA INTERMEDIÁRIA

[11] **Ângulo entre os centríolos**

(0) Paralelo

(1) Oblíquo

(2) Perpendicular

[12] **Axonema**

(0) Um

(1) Dois

[13] **Fibras periféricas adjacentes aos microtúbulos externos**

(0) Presentes

(1) Ausentes

[14] **Distribuição das mitocôndrias**

(0) Em volta da fibra axial

(1) Colar de mitocôndrias

(2) Adjacentes ao axonema

[15] **Inserção do bastão paraxonemal**

(0) Entrando na fossa nuclear

(1) Não entrando na fossa nuclear

CAUDA

[16] **Bastão Paraxonemal**

(0) Presente

(1) Ausente

[17] **Membrana Ondulante**

(0) Presente

(1) Ausente

[18] **Fibra axial**

(0) Presente

(1) Ausente

[19] **Forma da fibra axial**

(0) Dilatada ao final da membrana ondulante

(1) Com diâmetro igual ao da lâmina axial

(2) Recurvado

[20] **Fibra justa-axonemal no terceiro par de microtúbulos**

(0) Presente

(1) Ausente

[21] **Fibra justa-axonemal no oitavo par de microtúbulos**

(0) Presente

(1) Ausente

[22] **Lâmina axial**

(0) Fina

(1) Espesso

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