UNIVERSIDADE ESTADUAL DE CAMPINAS

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ESTUDO ULTRAESTRUTURAL DE ESPERMATOZÓIDE DE SQUAMATA (REPTILIA), E SUA IMPORTÂNCIA NA ANÁLISE FILOGENÉTICA.

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

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RESUMO

A análise filogenética a partir da ultraestrutura do espermatozóide vem ganhando interesse dos biólogos celulares e sistematas. Este interesse é devido à ultraestrutura do espermatozóide ser uma fonte de informação valiosa para a análise filogenética, por fornecer dados novos e não tradicionais, e por ter uma natureza mais conservativa que os dados morfológicos tradicionais. Numa tentativa de contribuir para o entendimento da filogenia dos Squamata, a presente dissertação tem como finalidade fornecer novas informações sobre a ultraestrutura do espermatozóide. Sua proposta é descrever pela primeira vez a ultraestrutura do espermatozóide de quatro famílias de Squamata, utilizando microscopia eletrônica de transmissão; fazer comparações com outras famílias de Squamata e conduzir uma análise filogenética dos Squamata. Apesar do novo conjunto de dados ter produzido resultados insatisfatórios na análise filogenética de Squamata, ele revelou uma variedade de caracteres independentes e filogeneticamente estruturados para a análise filogenética.

ABSTRACT

The phylogenetic analysis based on sperm ultrastructure has proved to be very interesting to cellular and systematics biologists. This interest stems from the fact that the sperm ultrastructure provides an important new and non-traditional source of data, evolutionarily more conservative than the morphological characters traditionally used and, hence, this ultrastructure provides more informative data for phylogenetic analyses. In order to contribute to the knowledge of phylogenetic relationships among Squamata, this dissertation adds new information about sperm ultrastructure. It describes, for the first time, the sperm ultrastructure of four families of Squamata, using transmission electron microscopy; makes comparisons with other squamates, and finally conducts a phylogenetic analysis of the Squamata. The results show that data on the ultrastructure on spermatozoa of Squamata suffer from deficiencies and produce unsatisfactory results in terms of resolving the phylogenetic relationships of Squamata. Nevertheless, the detailed studies of the sperm ultrastructure in squamates uncovered an independent and phylogenetically structured source of characters that can profitably be used in phylogenetic analyses where other data sets are uninformative.

1. INTRODUÇÃO

A presente dissertação tem em Biologia Celular e Sistemática suas áreas de pesquisa. Mais precisamente, esta dissertação busca a melhor compreensão de uma nova e valiosa fonte de informações para a análise filogenética de Squamata, a ultraestrutura do espermatozóide.

1.1 TENDÊNCIAS EVOLUTIVAS DA ESTRUTURA DO ESPERMATOZÓIDE

O espermatozóide (*Sperma* - semente, *zoon* - animal, *eídeo* - forma) é geralmente uma célula pequena, compactada, altamente especializada para a tarefa de fertilizar um óvulo. É uma célula otimizada para transferir os genes paternos para o óvulo, processo essencial para a hereditariedade e fases posteriores do desenvolvimento do organismo. O espermatozóide é uma célula equipada com um flagelo que o impulsiona através de um meio aquoso e é livre de organelas citoplasmáticas, tais como ribossomos, retículo endoplasmático ou complexo de Golgi, as quais são desnecessárias para a tarefa de transferir seu DNA ao óvulo. Ele é livre, móvel e direcionado para dar rapidez e eficiência à fertilização. Esta definição refere-se apenas a espermatozóides de animais e plantas mais primitivas, pois em plantas superiores, suas células germinativas masculinas, denominadas de microgametas, não são livres e são reduzidas a pequenas células dentro do gametófito no grão de pólen. O espermatozóide é uma célula altamente complexa e tem passado por diversas modificações morfológicas de acordo com os processos evolutivos, de forma independente de outros tipos celulares (Baccetti & Afzelius, 1976).

A extensa diversidade morfológica entre os organismos é um resultado de milhões de anos de evolução, incluindo os processos de adaptação aos diversos habitats e nichos ecológicos. A sobrevivência e a propagação de espécies dependem essencialmente de adaptações reprodutivas que permitem a transferência do material genético e a fertilização (Anderson & Personne, 1973).

O gameta masculino é muito adaptado para fertilizar o ovócito sob diversas condições ambientais. O espermatozóide é um modelo de extrema diferenciação celular, com variações fenotípicas e genéticas bem marcantes. A diversidade da estrutura do espermatozóide está relacionada às demandas fisiológicas do ambiente em que ocorre a fertilização. Mesmo com sua ampla distribuição na natureza e da enorme variabilidade morfológica, os espermatozóides de invertebrados e de vertebrados são adaptados para exercer as seguintes funções: sobreviver em órgãos de estoque com pouca capacidade de oxigenação; alcançar e selecionar o gameta feminino; perfurar as barreiras do óvulo e depositar seu material genético dentro desta célula; e ativar o desenvolvimento do zigoto (Anderson & Personne, 1973). Logo, muitas de suas características estruturais estão relacionadas com as exigências do processo de fertilização, ou seja, são necessárias para penetrar e fecundar o óvulo (Baccetti & Afzelius, 1976).

A colonização do ambiente terrestre foi um passo muito importante na evolução de animais e plantas, que não seria possível sem a evolução das estratégias reprodutivas. Várias características na morfologia do espermatozóide podem ser traçadas de acordo com essas mudanças no modo da fertilização, sendo assim, o espermatozóide é um excelente indicador da biologia reprodutiva (Baccetti e Afzelius, 1976). A vida sobre a terra só foi possível naquelas espécies que já se reproduziam por fertilização interna e que possuíam espermatozóides apropriados (Baccetti, 1986).

A diversidade morfológica de animais e plantas é de uma forma geral bem maior entre os terrestres do que entre os aquáticos, pois estes últimos vivem num ambiente relativamente uniforme. Assim sendo, a diversidade estrutural de espermatozóide é bem maior em animais com fertilização interna do que em animais que se reproduzem por fertilização externa, em ambientes aquáticos. Espermatozóides depositados em meio aquático demonstram uma simetria radial e três regiões distintas: cabeça, peça intermediária e flagelo. São chamados de "primitivos" devido à forma simples e porque são característicos de animais que liberam os espermatozóides diretamente na água. Apenas os animais com fertilização externa possuem espermatozóides "primitivos" (Franzén, 1970), que são estruturalmente similares nas diversas espécies. Quando depositados em um ambiente aquático, no qual não há nutrientes metabolizáveis, eles dependem inteiramente de suas reservas intracelulares para a produção de energia, que provêm da oxidação de fosfolipídios mitocondriais e de estoques de glicogênio. O suprimento de energia é suficiente para suportar uma curta estadia no meio aquático, por isso, o espermatozóide deve ser depositado perto do óvulo (Anderson & Personne, 1973).

Na fertilização interna o espermatozóide tem que nadar ou ser transportado para alcançar o ovócito. Modelos mais complexos com estruturas específicas e características fisiológicas se desenvolveram para superar os riscos da fertilização interna, tais como: a presença de micróbios e outros intrusos nos ductos reprodutores femininos; a grande viscosidade do meio; o tempo de permanência do espermatozóide no corpo da fêmea que excede dias ou até meses; a distância que o espermatozóide tem que percorrer para alcançar seu destino. A adição de fibras locomotoras mais eficientes e mudanças no metabolismo celular proporcionam ao espermatozóide uma maior capacidade de sobreviver e de superar o aumento da viscosidade do meio. Esse espermatozóide conhecido como "modificado",

difere do tipo "primitivo" por possuir modificações em certas regiões da sua estrutura, como cabeça e peça intermediária e, em alguns casos (nematóides, aracnídeos e crustáceos decápodes), por possuir uma estrutura bastante distinta do protótipo (Favard & André, 1970; Franzén, 1970).

A fertilização interna oferece enormes vantagens para os espermatozóides: eles ficam protegidos do ambiente externo e o gasto de energia se reduz bastante. Porém, é um processo mais complexo, que exige a capacidade de atração e reconhecimento do parceiro certo. Com isso, o desenvolvimento de sistemas de sinais visuais, químicos, órgãos sensoriais e tecido nervoso torna-se essencial. As conseqüências desse desenvolvimento do ponto de vista celular são inesperados: os espermatozóides sofrem uma série de modificações em sua estrutura, dando origem a uma grande diversidade de formas e tamanhos (Baccetti & Afzelius, 1976).

Concluindo, os espermatozóides surgem, em animais primitivos, como células que apresentam flagelos e um complexo acrossomal formado por acrossoma e perforatório. A fertilização interna e, subseqüentemente, a vida sobre a terra seleciona os espermatozóides com formatos alongados e com uma organela citoesquelética acessória no flagelo. Nas formas superiores, o flagelo é mais longo para uma mobilidade perfeita e os mecanismos de copulação tornam-se tão aperfeiçoados que os padrões de axonema podem aparecer aberrantes, como por exemplo, os espermatozóides aflagelados. No topo dos grupos terrestres, são encontrados aparatos alternativos de mobilidade celular e uma variedade de complexos acrossomais, que variam de acordo com o tipo de estruturas dos envelopes dos óvulos (Baccetti, 1986).

1.2 ESTUDOS DA ULTRAESTRUTURA DO ESPERMATOZÓIDE EM SQUAMATA

O espermatozóide foi descoberto por van Leeuwenhoek por volta de 1677, quando relatou a existência do espermatozóide humano e esboçou sua morfologia. No entanto, o estudo mais acurado do espermatozóide e de suas estruturas desenvolveu-se apenas com o advento do microscópio eletrônico após a II Guerra Mundial (Baccetti & Afzelius, 1976; Baccetti, 1986).

Recentemente, o estudo ultraestrutural do espermatozóide ou a espermiogênese tem sido realizado em várias famílias de Squamata, grupo de répteis constituído por lagartos, cobras-de-duas-cabeças e serpentes (Jamieson 1995b; Jamieson *et al.* 1996; Oliver *et al.* 1996). Estudos ultraestruturais foram feitos nas seguintes famílias de Squamata: Chamaeleonidae, Gekkonidae, Iguanidae, Lacertidae, Phrynosomatidae, Polychrotidae, Pygopodidae, Scincidae, Teiidae, Tropiduridae, Varanidae, e Serpentes (Boidae, Colubridae, Elapidae, Viperidae).

Entre as espécies de Chamaeleonidae, o espermatozóide foi descrito detalhadamente em *Bradypodium karroicum* (Jamieson, 1995b) e em *Pogona barbata* (Oliver *et al.* 1996). A espermiogênese foi examinada em *Agama stellio* (Al-Hajj *et al.* 1987), *A. adramitana* (Dehlawi *et al.*, 1992), *Uromastyx philbyi* (Dehlawi & Ismail, 1990; Dehlawi *et al.*, 1990) e *Stenodactylus selvini* (Dehlawi & Ismail, 1991).

Em Gekkonidae, Furieri (1970) descreveu resumidamente o espermatozóide de *Lygodactylus picturatus* e fez referência a *Hemidactylus frenatus*, *H. mabouia* e *Tarentola mauritanica mauritanica*; Phillips & Asa (1993) estudaram a formação da peça intermediária no espermatozóide de *Sphaerodactylus cinereus*; e Jamieson *et al.* (1996) descreveram o espermatozóide de *Heteronotia binoei*.

Em Iguanidae, Saita *et al.* (1988a) descreveram o processo de espermiogênese em *Iguana iguana*, identificada como *I. delicatissima*.

Os espermatozóides de Lacertidae foram examinados ultraestruturalmente em *Algyroides alleni*, *Lacerta sicula campestris*, *L. lepida lepida*, *L. laevis* e *L. viridis* (Furieri, 1970), *L. vivipara* (Courtens & Depeiges 1985) e, com referência ao desenvolvimento da cabeça do espermatozóide, *Podarcis (=Lacerta) taurica* (Butler & Gabri, 1984).

Em 1967, Clark fez um breve relato sobre a espermiogênese de um membro da família Phrynosomatidae, *Phrynosoma coronatum*.

Na família Polychrotidae, Clark (1967) descreveu a espermiogênese do lagarto *Anolis carolinensis* e Furieri (1974) descreveu o espermatozóide maduro de *Pristidactylus (=Cupriguanus) scapulatus*.

Harding *et al.* (1994) apresentaram um estudo preliminar dos espermatozóides dos Pygopodidae *Aprasia repens*, *Delma tincta*, *Lialis burtonis* e *Pygopus lepidopus*; e Jamieson *et al.* (1996) descreveram com detalhes o espermatozóide de *L. burtonis*.

Estudos dos espermatozóides de Scincidae incluem: uma descrição do espermatozóide maduro de *Chalcides ocellatus tiligugu* (Furieri, 1970); um relato da espermiogênese e alguns dados sobre o espermatozóide epididimal da mesma subespécie (Carcupino *et al.*, 1989); estudos da espermiogênese em *Chalcides ocellatus* (Dehlawi, 1992; Ismail & Dehlawi, 1992); um breve relato do desenvolvimento da peça intermediária em *Eumeces laticeps* (Okia, 1990); descrições dos espermatozóides de *Nangura spinosa* (Jamieson & Scheltinga, 1993), *Tiliqua scincoides*, *Ctenotus taeniolatus* e *Anomalopus verreauxii* (Jamieson & Scheltinga, 1994), e *C. robustus*, *Cryptoblepharus virgatus*, *Lampropholis delicata*, e *Carlia pectoralis* (Jamieson *et al.*, 1996).

Nas espécies de Teiidae examinadas, o processo de espermiogênese foi estudado em *Cnemidophorus lemniscatus lemniscatus* por Del conte (1976) e a ultraestrutura do espermatozóide de *C. sexlineatus* foi detalhadamente descrita por Newton & Trauth (1990, 1992).

Em Tropiduridae, os espermatozóides de *Liolaemus austromendocinus* e *Prymaturus flagellifer (=palluma)* foram descritos por Furieri (1974) e o estudo da espermiogênese de *Tropidurus* cf. *torquatus* foi feito por DaCruz-Landim & DaCruz-Höfling (1977) e DaCruz-Höfling & DaCruz-Landim (1978).

Dentre os lagartos da família Varanidae, apenas uma espécie foi examinada em termos de ultraestrutura de espermatozóide, *Varanus gouldii* (Oliver *et al.*, 1996).

A ultraestrutura de espermatozóide das serpentes tem sido a mais estudada. Austin (1965) forneceu informações mais detalhadas da ultraestrutura do flagelo do espermatozóide de *Lampropeltis getulus*, *Coluber constrictor*, *Drymarchon corais*, *Crotalus adamanteus* (Colubridae), *Micrurus fulvius* (Elapidae), e *Constrictor* sp. (Boidae). Boisson & Mattei (1965, 1966) descreveram a espermiogênese em *Python sebae* (Boidae). Hamilton & Fawcett (1968) detalharam a região do pescoço e da peça intermediária em *L. getulus* e em *Constictor constrictor*. Saita *et al.* (1988b) descreveram a espermiogênese em *Coluber viridiflavius*; Phillips & Asa (1993) descreveram o processo de formação da peça intermediária em *Masticophis flagellum flagellum* (Colubridae); e Afzelius (1981) descreveu a organização dos microtúbulos nos flagelos dos espermatozóides de *Liophis miliaris*. Contudo, apenas Furieri (1965, 1970), descreveu a ultraestrutura do espermatozóide inteiro, fornecendo um relato geral de quatro espécies da família Colubridae, *Coluber viridiflavius viridiflavius*, *Natrix tesselata tesselata*, *N. natrix* e *Coronella austriaca*, e uma espécie da família Viperidae, *Vipera aspis aspis* até que o

espermatozóide de *Nerodia sipedon* foi descrito por Jamieson & Koehler (1994). Oliver *et al.*(1996) descreveram o espermatozóide de *Boiga irregularis*, *Stegonotus cucullatus* (Colubridae), *Oxyuranus microlepidotus* (Elapidae), and *Aspidites melanocephalus* (Boidae).

Os outros répteis não Squamata cuja ultraestrutura de seus espermatozóides foram estudadas são: Chelonia (Yasuzumi & Yasuda, 1968; Furieri, 1970; Phillips, 1970; Yasuzumi *et al.* 1971; De *et al.*, 1987; Sprando & Russell, 1988; Hess *et al.*, 1991); Sphenodontida (Healy & Jamieson, 1992; 1994; Jamieson & Healy, 1992); e Crocodilia (Saita *et al.*, 1987).

1.3 - ESTRUTURAS DO ESPERMATOZÓIDE DE SQUAMATA

Diferenças ultraestruturais de espermatozóide de répteis (Chelonia, Crocodilia, Sphenodontida e Squamata) têm sido observadas apenas ao nível de ordens e famílias (Furieri, 1970). Apesar de certa variabilidade encontrada, os espermatozóides de Squamata apresentam uma constituição básica surpreendentemente constante em diferentes famílias (Jamieson, 1995a; 1995b).

1.3.1 - COMPLEXO ACROSSOMAL

O complexo acrossomal situa-se na porção anterior da cabeça do espermatozóide e reveste cerca de 2/3 do núcleo. É formado na maioria dos animais pelo acrossoma e por uma substância subacrossomal, conhecida por perforatório. O acrossoma é uma organela rica em enzimas, que facilitam a entrada do espermatozóide para dentro do óvulo através da

zona pelúcida. É originado a partir do complexo de Golgi através da fusão de vários grânulos proacrossomais e seu conteúdo é liberado por exocitose durante a fertilização (Baccetti & Afzelius, 1976). É a única organela específica do gameta masculino. Localizado abaixo do acrossoma, encontra-se o perforatório, que difere do primeiro em estrutura e origem e sofre modificações quando o acrossoma entra em contato com o óvulo (Afzelius & Baccetti, 1976).

Acrossoma

Os acrossomas mostram uma considerável variação no tamanho, forma, complexidade e grau de compartimentalização. A organização do conteúdo acrossomal em compartimentos está relacionada à função do acrossoma na fertilização, mas seu significado ainda é pouco conhecido. No entanto, em mamíferos, a compartimentalização do acrossoma parece facilitar a liberação seqüencial das enzimas acrossomais solúveis. Na maioria das espécies, o acrossoma contém material tanto solúvel quanto insolúvel, algumas vezes organizados em compartimentos que são reconhecidos até ultraestruturalmente (Talbot, 1991).

Em Squamata, o acrossoma apresenta dois compartimentos visíveis ao microscópio eletrônico de transmissão, um externo, denominado de vesícula acrossomal e um interno, cone subacrossomal. A presença das capas acrossomais e do topo afilado do núcleo no interior da capa mais interna é uma condição plesiomórfica dos tetrápodes (Tabela 1), ou seja, é uma condição ancestral dos tetrápodes, que se manteve em Squamata. Em algumas famílias de Squamata examinadas, a vesícula acrossomal é formada por um material homogêneo e elétron-denso (Furieri, 1974; Da Cruz Landim and Da Cruz Höfling, 1977; Da Cruz Höfling and Da Cruz Landim, 1978). No entanto, em outras famílias ele aparece

TABELA 1. Taxonomia filogenética simplificada dos Tetrápodes atuais

- TETRÁPODA
  - ⇨ Amphibia (Lissamphibia)
    - → Anura
    - → Urodela
    - → Gymnophiona
  - ⇨ Amniota
    - → Chelonia (répteis)
    - → Diapsida
      - ➢ Aves
      - ➢ Crocodilia (répteis)
      - ➢ Shenodontidae (répteis)
      - ➢ Squamata (répteis)
        - ✓ Lagarto
        - ✓ Amphisbaenia
        - ✓ Serpente
    - → Synapsida
      - ➢ Mammalia

FONTE: Pough *et al.*, 1993.

dividido em um córtex estreito e elétron-lucente e uma medula larga e elétron-densa (Al-Hajj *et al.*, 1987; Dehlawi *et al.*, 1992; Jamieson, 1995b; Oliver *et al.*, 1996).

O cone subacrossomal, em todas as famílias estudadas, é formado por uma substância paracristalina, considerada uma condição derivada (modificada ao longo da evolução, ou seja, não é a característica presente no ancestral) unicamente encontrada nos Squamata, ou seja, uma sinapomorfia dos Squamata (Jamieson & Scheltinga, 1993).

Em corte transversal, o acrossoma pode ser circular (Furieri 1970, 1974; Saita *et al.*, 1988b; Jamieson & Scheltinga 1994; Jamieson *et al.* 1996; Oliver *et al.* 1996), ou deprimido (Charnier *et al.* 1967; Furieri, 1970; Butler & Gabri, 1984; Courtens &

Depeiges, 1985; Al-Hajj *et al.*, 1987; Dehlawi *et al.*, 1992; Jamieson 1995b; Oliver *et al.* 1996).

Na região anterior ao topo do núcleo e abaixo do cone subacrossomal, geralmente há um canal estreito, conhecido por zona epinuclear lúcida, preenchida por uma substância elétron-lucente. Há três estados possíveis para esse caracter nas famílias examinadas: ausente, pouco desenvolvido e muito desenvolvido. A ausência da zona epinuclear lúcida é uma condição plesiomórfica, ou seja, é uma condição original, presente no ancestral comum dos Squamata, que foi alterada resultando em uma condição nova (presença da zona epinuclear lúcida). A presença da zona epinuclear lúcida é considerada uma sinapomorfia dos Squamata (Jamieson, 1995b). A ausência desse caracter, como no gekkonídeo *Heteronotia binoei* (Jamieson *et al.*, 1996) e no Scincídeo *Tiliquia* scincoides (Jamieson & Scheltinga, 1994) é presumivelmente uma reversão à condição ancestral.

Perforatório

O perforatório é um bastão existente entre o acrossoma e o núcleo, preenchido por um material fibroso e que exerce funções mecânicas. Sua origem é incerta e nenhuma atividade enzimática tem sido claramente detectada. O perforatório é uma estrutura citoesquelética formada por actina, e por isso tem uma grande capacidade de alongamento (Baccetti & Afzelius, 1976).

A ativação do alongamento durante a reação acrossomal é conhecida por processo acrossomal. Em filos aquáticos, o perforatório possui actina em dois estados diferentes, actina monomérica (actina-G) e actina filamentosa (actina-F). O processo acrossomal ocorre por um simples mecanismo de polimerização da actina-G, que se mantém no estado monomérico devido à presença da proteína prifilina. Sua polimerização é estimulada pela

espectrina. Em grupos terrestres, o perforatório é formado apenas por actina filamentosa (actina-F) e o processo acrossomal ocorre graças a um mecanismo de mudança de conformação dos filamentos. Os filamentos passam de um arranjo helicoidal para feixes paralelos, devido ao desligamento da α-actinina dos filamentos de actina (Baccetti, 1979; 1986; Shiroya *et al*, 1986).

A função do perforatório é ainda muito discutida. Em invertebrados, o perforatório tem um papel importante durante a reação acrossomal e a penetração no óvulo. Em aves, o perforatório não se alonga na reação acrossomal. A única função sugerida é a de sustentar o acrossoma. Em mamíferos, ao invés do típico perforatório, há apenas uma camada subacrossomal de actina nas espermátides (Campanella *et al.*, 1979) e que certamente exerce função no alongamento do núcleo e do acrossoma. Nesses dois últimos casos, a organela parece ser residual, o que sugere sua progressiva simplificação e desaparecimento ao longo da evolução (Baccetti *et al.* 1980).

A morfogênese do perforatório de vertebrados ainda é pouco conhecida, no entanto o perforatório de aves (Nagano, 1962; Humphreys, 1975) e répteis (Del Conte, 1976) parece ser originado a partir de um grânulo interposto entre o acrossoma e o núcleo e o de mamíferos, a partir de uma camada subacrossomal (Baccetti *et al.*, 1980). Del Conte (1976) sugeriu que em répteis o grânulo é um produto da interação entre o acrossoma e o núcleo. Clermont *et al.* (1955) insinuaram que a camada subacrossomal em mamíferos poderia ser uma extensão da membrana nuclear. No entanto, de acordo com Baccetti *et al.* (1980) a morfogênese do perforatório em vertebrados não é muito diferente da que ocorre nos invertebrados, iniciando-se a partir de um agrupamento de actina.

Os Squamata possuem apenas um perforatório, que se localiza sempre acima do topo do núcleo, por essa razão é denominado de perforatório prenuclear. Esta característica

é claramente uma apomorfia, isto é, a condição mais recente do perforatório, surgida por modificações de uma condição anterior. A condição plesiomórfica, ou seja, a condição anterior do perforatório que foi alterada resultando na condição recente é a presença do perforatório dentro de canais endonucleares. Esta condição ocorre em tuataras (Healy & Jamieson, 1992; 1994; Jamieson & Healy, 1992), crocodilianos (Saita *et al.*, 1987), tartarugas (Sprando & Russell, 1988), aves (Sprando & Russell, 1988), alguns peixes (Mattei *et al.*, 1988) e anfíbios (Jamieson *et al.*, 1993). Isto confirma que os canais endonucleares são simplesiomorfias dos tetrápodes (caracteres plesiomórficos compartilhados por este grupo) (Healy & Jamieson, 1992).

O topo do perforatório dos espermatozóides de Squamata tem dois estados possíveis: pontudo e quadrado. No ápice do cone subacrossomal ou na região basal do perforatório, uma placa elétron-densa, conhecida por placa da base perforatorial é presente em algumas famílias. No entanto, quando presente, pode aparecer como uma pequena saliência ou como uma estrutura ovóide ou esférica (Jamieson, 1995b; Jamieson *et al.*, 1996; Oliver *et al.*, 1996).

1.3.2 - NÚCLEO

O núcleo do espermatozóide é bem menor que o de uma célula somática. Isto devido a algumas razões: ao número haplóide de cromossomos; à ausência de síntese de DNA e RNA; à ausência de nucléolo; e ao meio desidratado (Baccetti & Afzelius, 1976). A característica principal do núcleo de um espermatozóide é a extrema compactação do material nuclear, o que resulta em uma forte elétron-densidade. A forma condensada da cromatina favorece uma melhor mobilidade e protege o genoma contra alterações físicas e químicas durante o armazenamento ou transporte em direção ao óvulo (Krause, 1996).

A estrutura da cromatina de um espermatozóide maduro é bastante diferente de uma célula somática típica, não apenas pela condensação, mas também pelo arranjo bem regular de seus componentes (MacInnes & Uretz, 1968; Walker, 1971). Além de diferenças estruturais, a cromatina do núcleo do espermatozóide também apresenta diferenças na composição química. Estudos feitos com vários filos têm demonstrado que as histonas somáticas presentes nas espermatogônias são substituídas por outras proteínas nas espermátides. A cromatina do espermatozóide é caracterizada pela presença de histonas ricas em arginina e proteínas básicas de baixo peso molecular, chamadas protaminas ao invés de histonas ricas em lisina (Das *et al.*, 1964; Chevaillier, 1970).

Estas modificações na composição química da cromatina são essenciais para a inativação temporária do genoma do espermatozóide. Após a penetração do espermatozóide no óvulo a cromatina volta a ficar ativa, pois as protaminas são imediatamente extraídas e substituídas pelas histonas (Courtens *et al.*, 1991).

O formato do núcleo é estabelecido durante o processo da espermiogênese. Nesse período aparece a "manchette", uma estrutura composta por microtúbulos associados lateralmente e que circunda o núcleo. A função da "manchette" ainda é muito discutida. Existem fortes evidências que os microtúbulos estão envolvidos no estabelecimento e na manutenção do formato do núcleo e na compactação da cromatina. No entanto, observações feitas em espermiogênese de mamíferos, répteis, aves, insetos e anelídeos sugerem que a forma do núcleo não é conseqüência exclusiva da modelagem externa exercida pelos microtúbulos e sim pelo padrão de agregação do DNA e das proteínas durante a compactação da cromatina (Fawcett *et al.*, 1971).

O núcleo do espermatozóide dos Squamata tem uma cromatina fortemente elétron-densa e homogênea, com lacunas elétron-lucentes em alguns casos. Possui um formato

cilíndrico, curvo e afilado na sua porção anterior, a qual se encontra inserida no cone subacrossomal. O núcleo alongado, condição plesiomófica dos amniotas (Jamieson, 1995a), aparece na maioria das famílias estudadas, com exceção dos lagartos *Carlia pectoralis* e *Lampropholis delicata* pertencentes ao grupo *Eugongylus* da família Scincidae (Jamieson & Scheltinga, 1994; Jamieson *et al.*, 1996), onde os espermatozóides apresentam núcleos com diâmetro maiores, denominados "corpulentos" por Jamieson (1995b). A região de transição entre a porção anterior afilada e a região cilíndrica é abrupta e marcante, denominada de "ombros" nucleares. Esta região determina o limite posterior do complexo acrossomal, assim como o ponto onde o núcleo começa a ficar afilado. São reconhecidos três estados para esse caractere: agudo, arredondado e ausente, sendo o arredondado o mais encontrado entre os Squamata. O pólo posterior do núcleo é marcado por uma depressão, a fossa nuclear, onde se encontra um material elétron-denso, o material pericentriolar (Jamieson, 1995b; Oliver *et al.*, 1996).

1.3.3 - REGIÃO DO PESCOÇO

É uma região de conexão da cabeça com o flagelo do espermatozóide, onde a extremidade posterior do núcleo une-se à extremidade anterior da peça intermediária. É constituída por dois centríolos (proximal e distal) ortogonalmente posicionados, sendo que o segundo deles se encontra no mesmo eixo do axonema (Baccetti & Afzelius, 1976).

Os centríolos funcionam como organizadores do flagelo. O centríolo distal está situado na base do axonema e funciona como um corpúsculo basal, estrutura essencial na formação do flagelo, servindo como molde para o padrão de microtúbulos do axonema (Alberts *et al.*, 1997).

Em muitos animais, a região do pescoço contém um acúmulo de proteínas básicas. Isto ocorre em insetos, salamandras e mamíferos, dentre outros. Parece que essas proteínas são histonas, as quais foram expelidas do núcleo durante o processo de condensação na espermiogênese e permanecem na região do pescoço, para permitir uma rápida descompactação do núcleo depois de sua penetração no óvulo (Werner, 1975).

A região do pescoço nos Squamata consiste de dois centríolos, um depósito de material pericentriolar e o primeiro anel de corpos densos da peça intermediária. O material pericentriolar circunda os centríolos e projeta-se para o interior da fossa nuclear. O centríolo proximal é paralelo à base do núcleo e está localizado centralmente ao eixo longitudinal do espermatozóide. Sua presença é uma condição plesiomórfica dos tetrápodes (Jamieson, 1995a). O centríolo distal em Squamata consiste de nove trincas de microtúbulos, nove fibras periféricas associadas às trincas e dois microtúbulos centrais do axonema. Esse centríolo estende-se em direção à cauda, alcançando aproximadamente 2/3 da peça intermediária, dando lugar ao axonema. O centríolo curto é uma sinapomorfia dos squamatas, pois em Chelonia e Sphenodontidae, o centríolo distal é bastante alongado, ocupando toda extensão da peça intermediária (Jamieson & Healy, 1992; Healy & Jamieson, 1994; Jamieson 1995a; Jamieson *et al.*, 1996; Oliver *et al.*, 1996). O centríolo distal não é observado no espermatozóide de mamíferos (Fawcett, 1970), mas além dos Squamata ele é presente em anuros (Furieri, 1975) e aves (Asa & Phillips, 1987; Asa *et al.*, 1986). Como em todos os amniotas (aves, mamíferos e répteis), uma estrutura laminar é claramente observada em algumas famílias, principalmente nas espécies do grupo *Sphenomorphus* da família Scincidae (Jamieson & Scheltinga, 1993; 1994). Esta estrutura é encontrada perto do centríolo proximal e do material pericentriolar (Jamieson, 1995a;

Jamieson *et al.*, 1997). A presença ou ausência da estrutura laminar pode ser determinada pelos diferentes modos de polimerização de subunidades moleculares, que constituem essa estrutura (Hamilton & Fawcett, 1968).

1.3.4 - PEÇA INTERMEDIÁRIA

O espermatozóide de quase todos os animais possui uma região distinta posterior ao núcleo, denominada de peça intermediária, onde as mitocôndrias estão localizadas. No espermatozóide de algumas espécies (ex., invertebrados com fertilização externa), a peça intermediária é bem pequena, ao contrário de outros grupos, onde a peça intermediária é bastante longa (Phillips & Asa, 1993).

A peça intermediária nos Squamata consiste da região do pescoço e do axonema, que é circundado por uma bainha fibrosa, mitocôndrias e corpos densos. Esta região é limitada posteriormente por uma anel, denominado de annulus, localizado na extremidade basal da bainha mitocondrial. O tamanho da peça intermediária nos espermatozóides de Squamata pode ser pequeno, moderadamente longo ou longo (Jamieson, 1995a, 1995b; Jamieson *et al.*, 1996; Oliver *et al.*, 1996).

O mecanismo de formação da peça intermediária nos squamatas é bem diferente dos outros animais. No lagarto *Sphaerodactylus cinereus* (Gekkonidae), durante a espermiogênese, o centríolo distal ocupa sua posição entre o núcleo e o plasmalema. O annulus se forma e a bainha fibrosa se põe posteriormente a ele. Neste ponto, as vinte mitocôndrias ao redor do centríolo são separadas umas das outras pelos corpos densos e se alongam. Enquanto isso, o annulus vai deslizando sobre a bainha fibrosa. Talvez, o comprimento da peça intermediária seja determinado pelo tamanho total das mitocôndrias (Phillips & Asa, 1993).

Axonema

O axonema é uma estrutura composta por microtúbulos e proteínas associadas. Os microtúbulos estão modificados e dispostos num padrão de nove microtúbulos duplos especiais dispostos formando um anel ao redor de um par de microtúbulos simples. Este arranjo 9+2 é característico de quase todos os flagelos eucarióticos, inclusive dos Squamata. Os microtúbulos se estendem de modo contínuo, ao longo do comprimento do axonema. Cada microtúbulo do par central é completo, e cada dupla de microtúbulos externos é composto por um microtúbulo completo, túbulo A (13 subunidades de tubulina em corte transversal) e outro parcial, túbulo B (11 subunidades). Os microtúbulos estão associados com numerosas proteínas. Algumas servem para manter os feixes unidos (nexinas), outras geram força para o movimento (dineínas) e outras formam um sistema de revezamento ativado mecanicamente que controla o movimento em ondas sinusóides (Alberts *et al.*, 1997).

Cada dupla de microtúbulos está ligada a uma fibra protéica periférica, ou fibra densa externa, que diminue em diâmetro ao longo do axonema, com exceção das fibras ligadas as duplas 3 e 8. Esta é uma característica típica de todos os répteis estudados (Jamieson *et al.*, 1997). Estas fibras aparecem mais grossas que as outras e são destacadas de suas duplas. A função dessas fibras parece estar relacionada com o fornecimento de uma força motora extra (Phillips, 1972; Phillips & Olson, 1975). Hamilton & Fawcett (1968) acreditam que a disposição das fibras é consistente com a idéia que elas contribuem para movimentos de curvatura. Anderson & Personne (1969) sugerem que as fibras estão relacionadas com a regulação da concentração de cátions bivalentes, como um mecanismo de controle do movimento do espermatozóide. As fibras densas externas originam-se com o depósito de material denso na superfície externa das duplas ao longo de seus comprimentos.

As fibras inicialmente estreitas vão se tornando espessas com o depósito contínuo de material em suas superfícies e se tornam separadas das duplas, nas quais foram depositadas (Hamilton & Fawcett, 1968).

Bainha fibrosa

A bainha fibrosa é uma estrutura presente em todos os amniotas (Jamieson, 1995a). Nos Squamata, a bainha fibrosa é formada por anéis regulares e separados, conectados longitudinalmente por duas colunas, uma ventral e outra dorsal, formadas pelas fibras densas externas 3 e 8. Austin (1965) propõe que nos répteis essas fibras 3 e 8 servem mais como estabilizadores longitudinais da bainha fibrosa do que agentes responsáveis pela mobilidade do espermatozóide. Cada anel é composto por filamentos intimamente associados, orientados ao redor do axonema e das fibras periféricas (Fawcett, 1970). A bainha fibrosa começa logo abaixo à parte basal do centríolo distal, se estendendo para dentro da peça intermediária. Esta característica é considerada uma sinapomorfia dos Squamata, pois com exceção destes a bainha fibrosa dos outros amniotas começa apenas no início da peça principal (Jamieson, 1995b). De acordo com Fawcett & Phillips (1970) e Soley (1994) a bainha fibrosa surge com o acúmulo de uma camada de material amorfo entre o complexo axonemal e a membrana plasmática durante a espermiogênese, que progressivamente vai se transformando em uma estrutura anelada típica da bainha. Para Fawcett (1970) a bainha fibrosa tem propriedades elásticas, o que sugere um papel na mobilidade do flagelo.

Mitocôndrias

Com exceção dos invertebrados, cestódeos (tênias), trematódeos (cercárias), coccídeos (insetos) e decápodes primitivos (crustáceo), todos espermatozóides de animais possuem mitocôndrias. Entre os diferentes táxons, de filos até família, há uma grande variedade morfológica de mitocôndrias. Alguns espermatozóides possuem mitocôndrias normais, que seguem o padrão geral; e outros possuem mitocôndrias transformadas em derivados mitocondriais por uma complexa metamorfose. No primeiro caso, as mitocôndrias apresentam diferenças apenas na localização celular. Em animais com espermatozóides aflagelados, as mitocôndrias se encontram dispersas no citoplasma. Em espermatozóides flagelados, as mitocôndrias podem estar localizadas na região do núcleo ou amontoadas na peça intermediária, como ocorre na maioria dos animais (incluindo os Squamata). Num ponto de vista fisiológico, existe também uma variedade, quanto à origem e ao substrato utilizado na oxidação (Favard & André, 1970).

Nos Squamata, como em todos outros tetrápodes (anfíbios e amniotas), os espermatozóides apresentam mitocôndrias com cristas lineares. Esta característica é uma condição plesiomórfica (característica ancestral dos tetrápodes). O arranjo concêntrico das cristas com a presença de um corpo central denso parece ser uma característica apomórfica adquirida no início da evolução dos amniotas (aves, mamíferos e répteis), ou seja, uma condição recente das cristas, que surgiu com a modificação da condição anterior (cristas lineares) na fase inicial da evolução dos amniotas e que permaneceu apenas nos répteis Shenodontidae, Crocodilia, e Chelonia (Yasuzumi & Yasuda, 1968; Furieri, 1970; Phillips, 1970; Saita *et al.*, 1987; Healy & Jamieson, 1992; 1994; Jamieson & Healy, 1992). Estudos feitos com espermátides de Shenodontidae (Healy & Jamieson, 1992; 1994; Jamieson & Healy, 1992) e Crocodilia (Saita *et al.*, 1987) revelaram cristas mitocondriais lineares,

indicando que a condição concêntrica é obtida apenas no final da espermiogênese. Isto levou Healy & Jamieson (1992) a concluir que os espermatozóides dos outros amniotas com cristas lineares (aves, mamíferos e squamatas) devem possuir uma forma de suprimir a transformação das cristas de lineares em concêntricas na fase final da espermiogênese. O significado exato desta mudança sub-estrutural das cristas mitocondriais de lineares para concêntricas nos amniotas e sua permanência nos Sphenodontidae, Crocodilia e Chelonia ainda não é muito claro. No entanto, Yasuzumi & Yasuda (1968) sugerem que as cristas concêntricas maximizam a superfície das cristas e, por isso, surgiram em resposta a condições de grande demanda por uma alta atividade mitocondrial.

Entre as famílias de Squamata, as mitocôndrias apresentam formas bem variadas como arredondadas, colunares, túbulos sinuosos e intermediários entre arredondado e colunar. Em cortes transversais da peça intermediária, as mitocôndrias podem formar anéis completos ao redor do axonema, anéis interrompidos por corpos densos, denominados neste caso de incompletos, ou anéis intermediários, onde as mitocôndrias formam um anel ao redor do axonema juntamente com mitocôndrias transformando-se em corpos densos.

Corpos densos

Em Squamata, os corpos densos são estruturas formadas por um material denso intermitocondrial. São considerados como mitocondriais em origem e homólogos aos corpos densos intramitocondriais (Carcupino *et al.*, 1989; Healy & Jamieson, 1992; Jamieson & Healy, 1992). Como tal, são reminiscências da existência de cristas concêntricas no ancestral de Squamata. A origem deste material intermitocondrial a partir das mitocôndrias tem sido confirmada ontogeneticamente no espermatozóide de alguns Squamata (Oliver *et al.*, 1996). A presença de corpos densos extramitocondriais é restrita

aos Squamata, com exceção dos espermatozóides de *Geopelia striata* (Columbiformes, Aves), no qual são encontrados, pobremente desenvolvidos. Apesar de ser uma homoplasia, ou seja, uma semelhança com os Squamata adquirida de forma independente na evolução das aves, a presença desses corpos densos sugere a persistência de sua base genética adquirida desde os primeiros amniotas (Jamieson, 1995a).

Nos Squamata estudados, os corpos densos têm sido observados em algumas famílias formando anéis completos ao redor da bainha fibrosa, fileiras ao longo da peça intermediária, dois grupos, uma estrutura em espiral, ou de forma dispersa (Jamieson, 1995b; Jamieson *et al.*, 1996; Oliver *et al.*, 1996).

Annulus

O annulus é um anel denso, presente na junção da peça intermediária com a peça principal na maioria dos tetrápodes e é também encontrado em muitos grupos de invertebrados (Baccetti & Afzelius, 1976). É composto por um conjunto de subunidades filamentosas intimamente associadas e se desenvolve sempre unido à membrana plasmática, permanecendo firmemente aderido a esta. Para Fawcett (1970), a função do annulus é impedir o deslocamento das mitocôndrias da peça intermediária para regiões posteriores do flagelo, durante o movimento.

1.3.5 - PEÇA PRINCIPAL

A peça principal é uma região posterior à peça intermediária e constitui a maior parte do flagelo do espermatozóide. Nos Squamata, a peça principal consiste do axonema rodeado pela bainha fibrosa, citoplasma e membrana plasmática. O axonema nesta região apresenta o padrão 9+2 e todas as nove fibras periféricas são vestigiais ou ausentes

(Jamieson & Scheltinga, 1993; Jamieson, 1995a, 1995b). Em algumas famílias, ocorre a presença de um material granuloso citoplasmático entre a bainha fibrosa e a membrana plasmática da peça intermediária.

1.3.6 - PEÇA TERMINAL

A peça terminal se refere à região mais estreita do flagelo do espermatozóide, posterior ao término da bainha fibrosa e das fibras densas externas. Consiste do axonema e da membrana plasmática e possui um comprimento indeterminado. Em algumas famílias, o padrão de microtúbulos do axonema se mantém, mas em outras aparece totalmente desorganizado, assim como em muitos grupos de animais (Jamieson *et al.*, 1996).

1.4 EVOLUÇÃO DA ULTRAESTRUTURA DO ESPERMATOZÓIDE NOS TETRÁPODES

O espermatozóide dos tetrápodes modernos (Anfíbios e Amniotas) possui as seguintes sinapomorfias: complexo acrossomal com um padrão repartido em três regiões (vesícula acrossomal, cone subacrossomal e o topo afilado do núcleo); ombros nucleares; fibras densas adjacentes às duplas de microtúbulos 3 e 8 do axonema; e annulus no final da peça intermediária. Nos anfíbios (Anura, Caldata e Gymnophiona), a única característica comum a todos é a presença de uma membrana ondulante adjacente à dupla de microtúbulos 3 do axonema. Nos amniotas (aves, mamíferos e répteis), as similaridades dos espermatozóides incluem: núcleo alongado; centríolo distal ocupando todo comprimento da peça intermediária; presença do par de microtúbulos centrais do axonema no interior do

centríolo distal; mitocôndrias subesferóides com cristas concêntricas e um corpo denso intramitocondrial; uma bainha fibrosa ao redor do axonema; nove fibras periféricas ao redor do axonema; projeções que ligam as fibras 3 e 8 à bainha fibrosa. Nas aves, ocorre a perda do material subacrossomal e a adesão de todas as nove fibras periféricas as duplas do axonema. Nos mamíferos, não há perforatório; os centríolos são bem reduzidos, e as fibras periféricas se destacam das duplas do axonema. Em Chelonia e Sphenodontidae (répteis), o espermatozóide mantém as mesmas sinapomorfias dos amniotas em geral. O espermatozóide dos crocodilianos apresenta uma bainha grossa e densa ao redor da dupla de microtúbulos centrais do axonema ou do centríolo distal. Nos Squamata, o espermatozóide pode ser reconhecido pelo único perforatório localizado acima do topo do núcleo; ausência de canais endonucleares (canais que armazenam material perforatorial); presença da zona epinuclear lúcida; cristas mitocondriais lineares; presença de corpos densos intermitocondriais; extensão da bainha fibrosa na peça intermediária; ombros nucleares arredondados; material subacrossomal paracristalino e centríolos curtos (Jamieson, 1995a).

1.5- A ULTRAESTRUTURA DE ESPERMATOZÓIDE E A ANÁLISE FILOGENÉTICA

Desde a descoberta do espermatozóide, esta célula altamente especializada tem atraído a atenção de muitos pesquisadores. As áreas de interesse compreendem desde os aspectos morfológicos aos funcionais, do nível celular para o molecular e do campo básico ao aplicado. O estudo ultraestrutural do espermatozóide é utilizado para abranger a área de conhecimento sobre o desenvolvimento e a importância funcional das diversas organelas

desta célula (Stanley, 1971), clareando as relações entre seus componentes e os mecanismos da reprodução (Da Cruz-Landim & Da Cruz-Höfling, 1977). Ademais, as análises da ultraestrutura de espermatozóide são também direcionadas para o estudo das organelas responsáveis pelo movimento celular e das adaptações do gameta para a fertilização e ativação do óvulo (Newton & Trauth, 1992). A análise da espermiogênese e da estrutura do espermatozóide de répteis têm uma importância especial por fornecer homologias dos componentes nos vários grupos de organismos. Isto porque os répteis representam um estágio interessante na evolução do espermatozóide, sendo este intermediário ao modelo aquático primitivo e ao encontrado nos vertebrados terrestres superiores (Baccetti & Afzelius, 1976).

O espermatozóide além de possuir uma grande importância no estudo dos mecanismos celulares de células reprodutivas e na biologia reprodutiva, pode ser utilizado como um marcador ou indicador das relações de parentesco entre os organismos, informando as relações evolutivas entre eles e os padrões das mudanças evolutivas (Franzén, 1970). A ultraestrutura de espermatozóide fornece uma fonte de dados não tradicionais, tendo seus caracteres uma natureza mais conservativa que os caracteres morfológicos usados tradicionalmente (morfologia da língua, musculatura dos membros, osteologia, escamas). Conseqüentemente, a ultraestrutura do espermatozóide fornece dados mais informativos para as análises filogenéticas (Jamieson *et al.*, 1996). Por isso, o estudo da ultraestrutura de espermatozóide oferece sustento à reconstrução das características do espermatozóide ancestral e o curso da evolução desta célula e do indivíduo, por relacionar a morfologia do espermatozóide com a posição sistemática do organismo (Jamieson, 1995b). Além de sua importância na filogenia, serve também de base para estudos taxonômicos (Baccetti, 1987).

Valor da Biodiversidade

A diversidade de espécies existentes no planeta contribui para a saúde e o bem-estar dos seres humanos. Milhões de espécies interagem umas com as outras e com o ambiente físico, formando um emaranhado ecológico que sustenta a vida na Terra. Estas interações garantem a limpeza do ar e da água, a fertilidade dos solos, e exercem um papel importante nos ciclos geoquímicos do planeta. A diversidade de espécies fornece alimentos, abrigos, saúde e outras comodidades, além de contribuir para a economia mundial, expandindo e diversificando a agricultura, a indústria farmacêutica, têxtil e outros setores (Norton, 1988).

Devido ao vasto potencial da biodiversidade e ao seu rápido declínio em conseqüência do aumento da demanda de recursos biológicos causada pela expansão das populações humanas e pelas mudanças tecnológicas (Norgaard, 1988), torna-se essencial um conhecimento mais aprofundado das espécies, capacitando as pessoas a viverem de forma sustentável com toda a biodiversidade (Hanemann, 1988).

Conhecer e documentar as espécies não se baseia apenas nos motivos ecológicos e econômicos. O interesse em conhecer a diversidade biológica parece ter surgido simultaneamente à própria consciência, quando começaram a ser "percebidos" os demais seres ao redor do indivíduo presente (Amorim, 1997).

Importância da Análise Filogenética

Conhecer e documentar a biodiversidade implica em compreender os padrões atuais de comportamento, da anatomia, da fisiologia, e da distribuição geográfica das espécies. No entanto, explicar os padrões atuais exige o conhecimento dos eventos (genéticos e

ecológicos) passados que levaram às mudanças evolutivas. É preciso determinar a verdadeira história da evolução (Futuyma, 1992).

A análise de todas as questões sobre a história da evolução requer a história filogenética dos grupos de espécies, ou seja, as relações entre eles precisam ser inferidas, através da análise filogenética (Futuyma, 1992).

A análise filogenética é um componente da Sistemática, ciência dedicada a descobrir, organizar e interpretar a diversidade biológica. Ela consiste das seguintes tarefas: descobrir, descrever e classificar as espécies ou os grupos de espécies (taxonomia); fazer comparações entre as espécies inferindo suas relações evolutivas (análise filogenética); e por fim, agrupar as espécies com base em suas relações de parentesco (Systematics Agenda 2000, 1994).

Através da análise filogenética, os sistematas inferem as relações de parentesco entre os organismos, informando as relações evolutivas entre eles e os padrões das mudanças evolutivas. A análise filogenética é uma excelente ferramenta para se determinar a história dos organismos, por facilitar o gerenciamento do conhecimento sobre as espécies e sua evolução. Isto acontece, porque as informações ficam organizadas ao redor de uma estrutura conceitual, tornando mais eficiente a recuperação e o uso do conhecimento pelos pesquisadores e pela sociedade. Enfim, a análise filogenética fornece um referencial evolutivo, que permite a compreensão dos mecanismos evolutivos como a especiação, extinção, adaptação e conseqüentemente leva a inferências das tendências evolutivas (Systematics Agenda 2000, 1994).

A compreensão dos princípios e processos evolutivos é essencial à apreciação da diversidade dos organismos, já que esta diversidade é o resultado direto da evolução (Pough *et al.*, 1993).

1.6 - CONSIDERAÇÕES GERAIS SOBRE OS SQUAMATA

A linhagem dos Diapsida (Tabela 1) inclui os Squamata, que representam o segundo maior grupo de tetrápodes viventes; existem duas vezes mais espécies de Squamata do que de mamíferos. Desde sua origem no Triássico, os Squamata diversificaram-se bastante (veja as linhagens de Squamata na Tabela 2). Os Sphenodontia, atualmente representados somente pelo tuatara da Nova Zelândia (Cree & Daugherty, 1990; Daugherty *et al*, 1990), provavelmente são o grupo-irmão dos Squamata (Fraser, 1986; Evans, 1988; Gauthier *et al.*, 1988). Dentro do grupo, os lagartos podem ser distinguidos em termos coloquiais, mas não filogeneticamente, pois as serpentes e as cobras-de-duas-cabeças provavelmente derivaram desse grupo. Dessa forma, os lagartos constituem um grupo parafilético (não inclui todos os descendentes). Todavia, os lagartos, as serpentes e as cobras-de-duas-cabeças são muito distintos em sua ecologia e comportamento.

Os lagartos variam, em tamanho, desde as diminutas largatixas, com apenas 3 centímetros de comprimento, até o dragão-de-Komodo, que atinge comprimento de 3 metros. Quatro linhagens principais de lagartos divergiram no Jurássico: os Iguania e os Gekkota são compostos, em grande parte por lagartos de corpo robusto e apresentam uma diversidade considerável de formas corporais. Os Scincomorpha e os Anguimorpha são alongados e exibem menor diversidade morfológica que os Iguania. Os lagartos são animais facilmente adaptáveis que ocupam habitat que variam de pântanos a desertos, e até mesmo acima da faixa de floresta, em algumas montanhas. Muitas espécies são arborícolas e as mais especializadas destas freqüentemente são achatadas lateralmente e, muitas vezes, possuem projeções peculiares do crânio e do dorso, que ajudam a obscurecer seu contorno,

TABELA 2 - Taxonomia filogenética dos Squamata.

SQUAMATA
- ⇨ Iguania
  - → Iguanidae
  - → Acrodonta
    - ➢ Chamaeleonidae
- ⇨ Scleroglossa
  - → Incertae sedis: Dibamidae, Amphisbaenia, Serpentes
  - → Gekkota
    - ➢ Gekkonidae
    - ➢ Pygopodidae
  - → Autarchoglossa
    - ➢ Scincomorpha
      - ✓ Lacertoidea
        - ⊛ Xantusiidae
        - ⊛ Lacertiformes
          - • Lacertidae
          - • Teiioidea
            - · Teiidae
            - · Gymnophthalmidae
      - ✓ Scincoidea
        - ⊛ Scincidae
        - ⊛ Cordylidae
  - → Anguimorpha
    - ➢ Anguidae
    - ➢ Xenosauridae
    - ➢ Varanoidea
      - ✓ Helodermatidae
      - ✓ Varanidae
        - ⊛ *Lanthanotus*
        - ⊛ *Varanus*

FONTE: Estes *et al.*, 1988.

como ocorre nos camaleões do Velho Mundo (Chamaeleonidae). A maioria dos lagartos de grande porte é herbívora, com exceção dos Varanidae. Os Varanidae são predadores ativos que se alimentam de uma variedade de vertebrados e invertebrados, incluindo aves e mamíferos. Poucos lagartos são capazes de capturar e subjugar aves e mamíferos, mas os

Varanidae apresentam características morfológicas que os tornam predadores eficientes. A redução apendicular evoluiu repetidamente entre os lagartos e cada continente apresenta uma ou mais famílias com espécies ápodes ou quase ápodes. Nos lagartos, a redução apendicular está geralmente associada à vida em estrato herbáceo, onde um corpo alongado e delgado pode ser manobrado mais facilmente que um corpo curto e dotado das patas funcionais (Pough *et al.*, 1993).

As cobras-de-duas-cabeças incluem aproximadamente 150 espécies de Squamata extremamente fossóreos (*fossor* = escavador) (Halliday & Adler, 1986; Zug, 1993). Esses animais foram considerados diferentes o suficiente dos lagartos e cobras e passaram a pertencer a uma sub-ordem de Squamata, chamada Amphisbaenia (Gans, 1978; Bellairs & Gans, 1983; Romer & Parsons, 1986). O nome "Amphisbaenia" deriva-se das raízes gregas *amphi* (duplo) e *baen* (caminhar), referindo-se à capacidade desses animais em movimentar-se para frente e para trás com a mesma facilidade. Esta capacidade ocorre graças à pele frouxamente conectada ao tronco, que permite o deslizamento para frente e para trás dentro do tubo da pele. A maioria das cobras-de-duas-cabeças é ápode; contudo, as três espécies do gênero mexicano *Bipes* possuem patas anteriores bem desenvolvidas, que auxiliam a penetração no solo, mas não a escavação subterrânea. O crânio é utilizado na construção de túneis e apresenta uma constituição bastante rígida. Muitas espécies possuem a cabeça arredondada, mas algumas têm focinhos quilhados verticalmente ou em forma de pá horizontal. A estrutura dentária torna esses animais predadores formidáveis, capazes de arrancar pequenos pedaços de presas grandes demais para serem ingeridas inteiras (Pough *et al.*, 1993).

Nas serpentes, a remodelação da massa do corpo em uma forma serpentina foi acompanhada por especializações dos mecanismos de locomoção (serpentina, retilínea,

sanfonada e ondulação lateral), captura de presas (constrição e uso de peçonha) e deglutição (um crânio altamente cinético) (Pough *et al.*, 1993).

1.7 - MOTIVAÇÃO

As inferências filogenéticas dos Squamata têm sido feitas na maioria dos casos a partir de dados morfológicos (Estes *et al.*, 1988; Russel, 1988; Scwenk, 1988; Maddison & Maddison, 1996). Contudo, as relações filogenéticas entre as famílias deste grupo continuam mal resolvidas. O desenvolvimento de estudos com caráter descritivo e a utilização de caracteres da ultraestrutura de espermatozóide nos estudos filogenéticos de insetos (Jamieson, 1987) e peixes (Jamieson, 1991) têm conduzido naturalmente a uma alternativa adicional de criar hipóteses filogenéticas de Squamata baseadas na ultraestrutura de espermatozóide (Jamieson, 1995a, 1995b; Jamieson *et al.*, 1996; Oliver *et al.*, 1996).

A perspectiva de contribuir para o aperfeiçoamento da resolução das hipóteses filogenética das famílias de Squamata, dando subsídio para a construção de uma árvore filogenética mais provável do grupo, através da utilização de um novo conjunto de dados não tradicionais fornecido pela ultraestrutura de espermatozóide motivou a escolha do tema objeto desta dissertação.

1.8 - OBJETIVOS

A proposta do presente trabalho é descrever detalhadamente pela primeira vez a ultraestrutura de espermatozóides retirados do epidídimo das seguintes espécies de Squamata: *Amphisbaena alba* (Amphisbaenidae), *Micrablepharus maximiliani* (Gymnophthalmidae), *Polychrus acutirostris* (Polychrotidae), *Tropidurus semitaeniatus* e *T. torquatus* (Tropiduridae); compará-la às características ultraestruturais de espermatozóides de outras famílias de Squamata já descritas; e conduzir uma análise filogenética do grupo.

Assim sendo, são dois os principais objetivos propostos:

(1) Adicionar mais famílias ao conjunto de dados estruturais, organizado por Jamieson (1995b).

(2) Avançar os conhecimentos sobre essa nova opção de caracteres:

- Compreendendo a variabilidade nos caracteres da ultraestrutura de espermatozóide a nível interfamilial;
- Verificando se os caracteres de ultraestrutura possuem conteúdo filogenético entre as famílias de Squamata;
- Interpretando os padrões evolutivos dos novos caracteres;
- Avaliando a vantagem do uso dos novos caracteres na reconstrução filogenética;
- Visualizando o grau de congruência entre as filogenias derivadas da ultraestrutura de espermatozóide e dos caracteres morfológicos;

E como conseqüência, esta dissertação busca uma melhor compreensão das relações de parentesco entre as famílias.

2. ARTIGOS PUBLICADOS

Durante a realização desta dissertação os seguintes trabalhos foram publicados:

1. **Teixeira, R. D.**, Colli, G. R. & Báo, S. N. The ultrastrucutre of the spermatozoa of the lizard *Micrablepharus maximiliani* (Squamata, Gymnophthalmidae), with considerations on the use of sperm ultrastructure characters in phylogenetic reconstruction. *Acta Zoologica* 78, 1999.

2. **Teixeira, R. D.**, Colli, G. R. & Báo, S. N. The ultrastructure of the spermatozoa of the worm-lizard *Amphisbaena alba* (Squamata, Amphisbaenidae), and the phylogenetic relationships of amphisbaenians. *Can. J. Zool.* No prelo.

3. **Teixeira, R. D.**, Colli, G. R. & Báo, S. N. Ultrastructural study of spermatozoon of the lizard *Polychrus acutirostris* (Squamata, Polychrotidae). *J. Submicrosc. Cytol. Pathol.* 31(3), 1999.

4. **Teixeira, R. D.**, Vieira, G. H. C.; Colli, G. R. & Báo, S. N. Ultrastructural study of spermatozoa of the Brazilian Tropidurid lizards, *Tropidurus semitaeneatus* and *Tropidurus torquatus* (Squamata, Tropiduridae). *Tissue Cell.* No prelo.

1. **Teixeira, R. D.**, Colli, G. R. & Báo, S. N. The ultrastrucutre of the spermatozoa of the lizard *Micrablepharus maximiliani* (Squamata, Gymnophthalmidae), with considerations on the use of sperm ultrastructure characters in phylogenetic reconstruction. *Acta Zoologica* 78, 1999.

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The ultrastructure of the spermatozoa of the lizard *Micrablepharus maximiliani* (Squamata, Gymnophthalmidae), with considerations on the use of sperm ultrastructure characters in phylogenetic reconstruction

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Abstract

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We describe, for the first time, the ultrastructure of the spermatozoa of a member of the family Gymnophthalmidae. Mature spermatozoa of *Micrablepharus maximiliani* are characterized by: acrosome circular in transverse section, absence of perforatorial base plate, perforatorial tip pointed, absence of epinuclear lucent zone, midpiece short, mitochondria in transverse section forming a circlet interrupted by dense bodies, trapezoid mitochondria, dense bodies solid and arranged in regular rings and linear series, linear mitochondrial cristae, rounded nuclear shoulders, elongate nuclear shape, absence of endonuclear canal, fibers 3 and 8 enlarged, absence of multilaminar membranes, and fibrous sheath in midpiece. Phylogenetic analysis of the Squamata after the addition of the Gymnophthalmidae to the ultrastructure data set previously published by Jamieson, resulted in 8733 equally parsimonious trees that conflicted with phylogenetic hypotheses derived from morphological data sets. An analysis of tree-length distribution skewness, however, indicated that the ultrastructure data set contains significant phylogenetic information. We suggest that rates of evolution for spermatozoa ultrastructure characters might be higher than currently thought, resulting in incongruent tree topologies derived from distinct data sets. Finally, we suggest that because only optimal trees were selected, the heterogeneity between the data sets might be apparent and more analyses are necessary to evaluate the nature and degree of the heterogeneity between them.

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Introduction

In recent years, the ultrastructure of spermatozoa has been described for various families of Squamata (reviewed in Jamieson 1995b; Jamieson *et al.* 1996; Oliver *et al.* 1996). The development of such an extensive data set, paralleled by the utilization of spermatozoal ultrastructure characters in phylogenetic

studies of insects (Jamieson, 1987) and fishes (Jamieson, 1991), has naturally led to phylogenetic analyses of squamates based on the ultrastructure of spermatozoa (Jamieson, 1995b; Jamieson *et al.* 1996; Oliver *et al.* 1996). These analyses revealed, on the one hand, strong support for the monophyly of Squamata and, on the other, major incongruencies between phylogenies derived from sperm ultrastructure and those derived from morphological characters (Estes *et al.* 1988; Russel 1988; Schwenk 1988; Maddison and Maddison 1996). For example, the Iguania, Scleroglossa, Gekkota, Scincomorpha, and Anguimorpha are not monophyletic in phylogenetic hypotheses derived from the ultrastructure of spermatozoa (Jamieson 1995b).

A common product of the increased number of data sets available for a group of taxa is the disagreement between phylogenies derived from each data set. This heterogeneity among phylogenetic reconstructions often results from sampling error, different stochastic processes acting on different data sets or portions of data sets, and different phylogenetic histories (e.g. Swofford 1991b; de Queiroz *et al.* 1995; Miyamoto and Fitch 1995). In the absence of studies that qualify the nature and degree of the heterogeneity between the morphological and sperm ultrastructure data sets from squamates, there is no reason to a priori regard either one as superior to the other in the ability to accurately reflect true phylogenetic relationships. However, data on the ultrastructure of spermatozoa of squamates still suffer from two deficiencies. First, the number of characters employed in phylogenetic analysis (Jamieson 1995b) is rather limited (17), what may lead, with the broadening of the taxonomic coverage, to the uncomfortable situation of having more taxa than character states in the study matrix. Second, the ultrastructure of spermatozoa of several families of squamates still awaits description (Jamieson 1995b).

The Gymnophthalmidae comprises an array of 30 genera that range from southern Mexico to northern Argentina (Presch 1980). The group was previously considered a subfamily of the Teiidae until Presch (1983) raised gymnophthalmines to the familial rank. Even though a closer relationship with the Lacertidae was once suggested (Presch 1983), the Gymnophthalmidae are currently believed to be the sister-taxon of the Teiidae (Estes *et al.* 1988; Schwenk 1988). Herein we describe, for the first time, the ultrastructure of the spermatozoa of a member of the family Gymnophthalmidae, *Micrablepharus maximiliani*, and make comparisons with other families of squamates. In addition, we conduct a phylogenetic analysis of the Squamata after the addition of a new family to the sperm ultrastructure data set of Jamieson (1995b), attempting to evaluate the usefulness of such characters in phylogeny reconstruction and to provide directions for future work.

Materials and Methods

Spermatozoa ultrastructure

We obtained sperm samples from an adult specimen of *Micrablepharus maximilini* collected at Minaçu, Goiás State, Brazil (13°38′ S, 48°15′ W) in February 1997. We killed the specimen with Tiopental®, removed the epididymis by dissection, placed it in a Petri dish with phosphate buffered saline (PBS) pH 7.2, and cut it into small pieces. We fixed spermatozoa and epididymal tissues overnight at 4 °C in a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, and 3% sucrose in 0.1 m sodium cacodylate buffer pH 7.2. Subsequently, we rinsed the samples in 0.1 m sodium cacodylate buffer pH 7.2 and postfixed them for 1 h in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5 mm $CaCl_2$ in 0.1 m sodium cacodylate buffer. We dehydrated the material in acetone and embedded it in Spurr's epoxy resin. We cut sections with diamond knives, on a Reichert ultramicrotome. After sectioning and staining with uranyl acetate and lead citrate, we examined and photographed sections with a Jeol® 100C transmission electron microscope at 80 kV. We made light microscopic observations of spermatozoa, from glutaraldehyde-paraformaldehyde fixed sperm smears, under Nomarski contrast using a Zeiss® Axiophot microscope.

Phylogenetic analyses

We scored *Micrablepharus maximiliani* for the 17 ultrastructural characters of squamate sperm described by Jamieson (1995b) (Table 1). Then, we built a taxon-character matrix (Table 2), by joining *M. maximiliani* to the matrix assembled by Jamieson (1995b). It should be noted that we assigned *Pogona barbata* to the Chamaeleonidae, following Frost and Etheridge (1989) in that Agaminae is a subfamily of Chamaeleonidae. We produced most parsimonious phylogenetic hypotheses using Paup vs. 3.0 s for the Macintosh (Swofford 1991a) and a branch-and-bound search, performed with the default options. We regarded all characters as unordered, and Chelonia and *Sphenodon punctatus* as a paraphyletic outgroup with respect to the ingroup. Reconstructions of character evolution were produced with MacClade v.3.06 (Maddison and Maddison 1992).

Results

Ultrastructure of spermatozoa

Spermatozoa of *Micrablepharus maximiliani* are filiform and ≈ 60 μm long (Figs 1, 2A). The head is short and curved, and ≈ 11 μm in total length, from light microscopy. The midpiece, a thick and short portion in the posterior

Table 1 Ultrastructure characters of the spermatozoa of squamates used in phylogenetic analyses. Data from Jamieson (1995b).

Character	States
1 Acrosome in transverse section	(0) circular (1) depressed
2 Perforatorial base plate	(0) absent or indistinct (1) knoblike (2) stopperlike
3 Perforatorial tip	(0) pointed (1) square ended
4 Perforatoria number	(0) two or more (1) one
5 Epinuclear lucent zone	(0) absent (1) poorly developed (2) well developed
6 Midpiece	(0) short (1) moderately long (2) very long
7 Mitochondria in transverse section	(0) regular circlet (1) not regular (2) intermediate
8 Mitochondria shape	(0) rounded (1) columnar (2) sinuous tubes (3) intermediate rounded-columnar (4) trapezoid
9 Dense bodies	(0) intramitochondrial (1) regular rings (2) scattered (3) linear series (4) stellate spiral (5) 2 groups
10 Dense bodies, if regular	(0) not applicable (1) solid (2) granular (3) single file granules
11 Mitochondrial cristae	(0) concentric (1) linear
12 Nuclear shoulders	(0) sharp (1) rounded (2) absent
13 Nuclear shape	(0) elongate (1) stout
14 Endonuclear canal	(0) present (1) absent
15 Fibers 3 and 8	(0) enlarged (1) grossly enlarged anteriorly
16 Multilaminar membranes	(0) absent (1) present
17 Fibrous sheath	(0) not in midpiece (1) in midpiece

Table 2 Comparative ultrastructure of Squamata sperm, *Sphenodon punctatus* and Chelonia. All data from Jamieson (1995b), except for *M. maximiliani*.

Taxon	Characters																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Chelonia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sphenodon punctatus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ctenotus robustus (Scincidae)	1	0	0	1	1	0	0	1	1	1	1	1	0	1	0	0	1
Chalcides ocellatus (Scincidae)	1	0	0	1	?	0	0	1	1	3	1	1	0	1	0	0	1
Lacertidae	1	0	0	1	1	0	0	1	5	0	1	1	0	1	0	0	1
Cnemidophorus sexlineatus (Teiidae)	1	0	0	1	2	0	0	1	1	1	1	1	0	1	0	0	1
Tiliqua scincoides (Scincidae)	1	0	0	1	0	0	0	1	1	1	1	1	0	1	0	0	1
Carlia pectoralis (Scincidae)	0	1	1	1	2	1	1	2	2	0	1	0	1	1	1	0	1
Lampropholis delicata (Scincidae)	0	1	1	1	2	1	1	2	2	0	1	0	1	1	1	0	1
Heteronotia binoei (Gekkonidae)	0	2	0	1	0	1	0	1	4	0	1	1	0	1	0	0	1
Lygodactylus picturatus (Gekkonidae)	0	2	0	1	2	1	0	1	4	0	1	1	0	1	0	0	1
Lialis burtonis (Pygopodidae)	0	?	1	1	2	1	1	2	3	0	1	2	1	1	0	1	1
Pogona barbata (Chamaeleonidae)	1	1	0	1	2	0	2	3	1&3	1	1	1	0	1	0	0	1
Varanus gouldii (Varanidae)	1	1	0	1	2	0	0	1	1	2	1	1	0	1	0	0	1
Boiga irregularis & Stegonotus cucullatus (Colubridae)	0	0	0	1	1	2	1	2	3	0	1	1	0	1	0	0	1
Oxyuranus microlepidotus (Elapidae)	0	0	0	1	?	2	1	2	3	0	1	1	0	1	0	1	1
Aspidites melanocephalus (Boidae)	0	0	0	1	?	2	1	2	3	0	1	1	0	1	0	1	1
Iguanidae	0	0	0	1	2	0	0	2	1&2	1	1	0&1	0	1	0	0	1
Bradypodion karroicum (Chamaeleonidae)	1	0	0	1	2	1	0	2	2	0	1	1	0	1	0	0	1
Micrablepharus maximiliani (Gymnophthalmidae)	0	0	0	1	0	0	2	4	1&3	1	1	1	0	1	0	0	1

segment of the head, is ≈ 2.5 μm long, from transmission electron microscopy. The tail (principal piece and endpiece) is ≈ 46.5 μm long from transmission electron microscopy.

Acrosome complex. The acrosome complex consists of an external cap, the acrosome vesicle, an internal cap, the paracrystalline subacrosomal cone, and the perforatorium (Fig. 2B). In cross-section, the acrosome complex appears circular (Fig. 2D,E). The acrosome vesicle is a homogeneous and electron-dense structure that ensheathes the subacrosomal cone (Fig. 2B). A narrow central canal, the perforatorium, extends from the anterior region of the subacrosomal cone and projects into the acrosome vesicle (Fig. 2B-D). The subacrosomal cone wraps the tapered anterior end of the nucleus (Fig. 2E,F), and is separated from the acrosome vesicle by an electron-lucent space

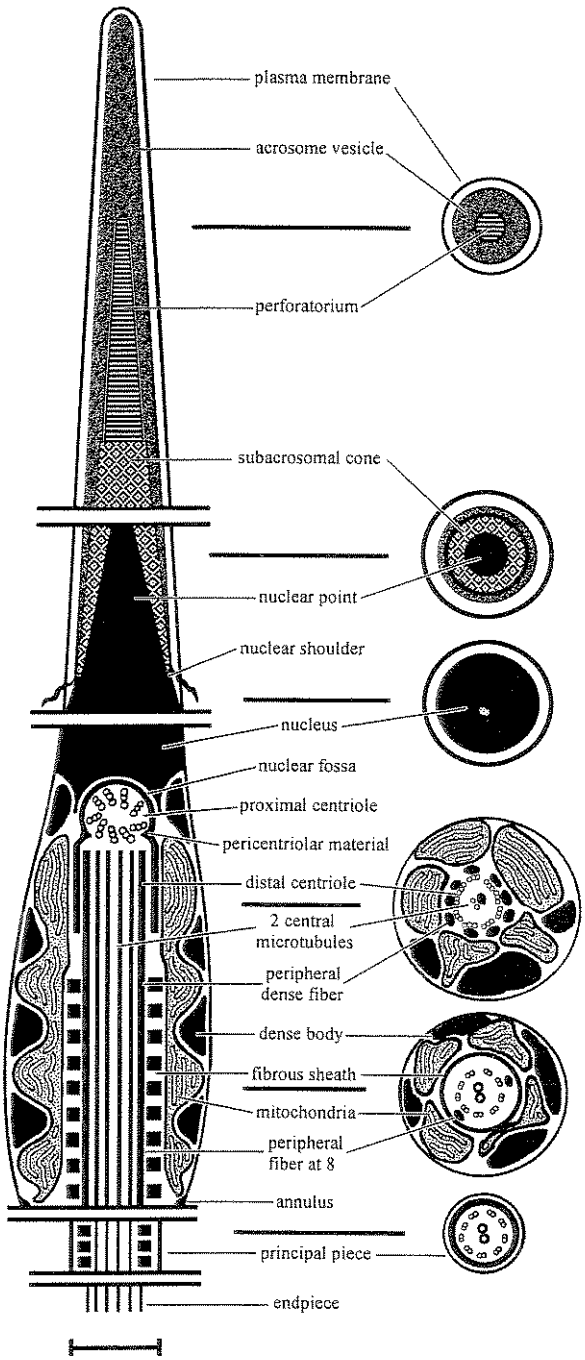

Fig. 1.—Diagram of the spermatozoa of *Micrablepharus maximiliani*, in longitudinal section and corresponding transverse sections. Scales of various components are only approximate. Scale bar 0.5 μm.

(Fig. 2C). At its posterior end, the acrosome complex lies on a postero-lateral membranous flange (Fig. 2F).

Nucleus. The nucleus is a curved cylinder, with the apical region tapering within the subacrosomal cone (Fig. 2B,F). In transverse section, the nucleus is circular (Fig. 2H,I). The chromatin is strongly electron-dense and condensed, with some electron-lucent lacunae (Fig. 2G,H). The transition from the tapered apical portion (nuclear rostrum) to the cylindrical region is abrupt and marked by small rounded 'shoulders' (Fig. 2F). At its base, the nucleus has a moderately deep nuclear fossa, that houses electron-dense material and partially covers the proximal centriole (Fig. 2I, J). The nuclear point is not capped by an electron-lucent space.

Neck region. The neck region is the transition between nucleus and midpiece. It contains the proximal and distal centrioles, the first ring of dense bodies (mitochondrial transformations) and mitochondria (Fig. 3A). The proximal centriole is transversely oriented in longitudinal section and is partially surrounded by dense pericentriolar material, that conforms in shape to the nuclear fossa and extends posteriorly between the two centrioles (Fig. 2J, 3A, B). An electron-lucent space separates the pericentriolar material from the nuclear fossa. An electron-dense laminar structure sits above the proximal centriole (Fig. 3C). The distal centriole is in the long axis of the flagellum (Fig. 3A,B) and consists of nine triplets of microtubules, nine peripheral fibers associated with the triplets, and the two central singlets of the axoneme, which extend into the posterior region of the distal centriole (Fig. 3D). One of the central microtubules is surrounded by dense material.

Midpiece. The midpiece consists of the neck region and the axoneme, surrounded by mitochondria, rings of dense bodies (mitochondria transformations) and a fibrous sheath (Fig. 3A,F). The midpiece ends posteriorly with the annulus (Fig. 3G). The axoneme is formed by a pair of central microtubules surrounded by nine doublets and nine peripheral fibers of dense material. The peripheral dense fibers associated with doublets three and eight are

Fig. 2.—Spermatozoa of *Micrablepharus maximiliani*.—**A,** Light micrograph showing whole spermatozoon with head, midpiece and flagellum.—**B-J,** Transmission electron micrographs of the head (acrosome complex and nucleus). (B) Longitudinal section of the acrosome complex surrounding the nuclear point. (C) Detail of the acrosome complex showing the perforatorium. Arrow head indicates electron-lucent space. (D-E) Transverse section of the acrosome complex at, respectively, the perforatorium and nuclear point levels. (F) Posterior region of acrosome complex with arrow heads indicating the nuclear shoulders. (G-H) Longitudinal and transverse sections, respectively, of nucleus showing the lacunae. (I-J) Basal region of nucleus and the nuclear fossa in transverse and longitudinal sections, respectively. Note the pericentriolar material. **Fig.** 2A: scale bar 10 m; Fig. 2B-J: scale bar 0.2 μm. Abbreviations: a = acrosome vesicle; an = annulus; ax = axoneme; db = dense bodies; dc = distal centriole; f = flagellum; fs = fibrous sheath; h = head; l = nuclear lacuna; ls = laminar structure; m = mitochondria; mp = midpiece; *n* = nucleus; nf = nuclear fossa; p = perforatorium; pc = proximal centriole; pf = peripheral dense fibre; pm = pericentriolar material; sc = subacrosomal cone.

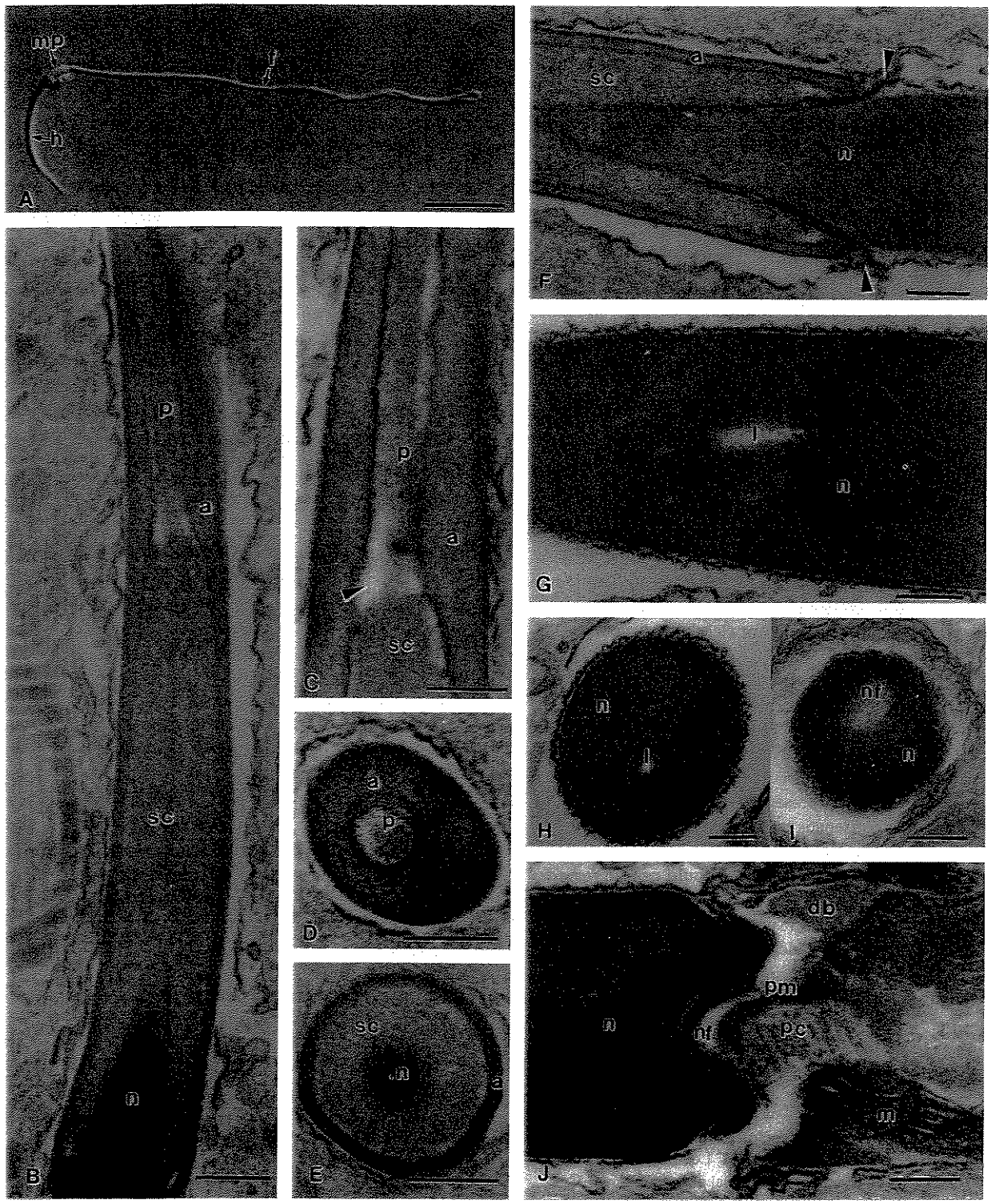

Fig. 2

thicker than the others and are separated from their corresponding doublets (Fig. 3F). The axoneme is enclosed in the fibrous sheath, formed by regularly spaced, approximately square dense blocks, connected by the longitudinal thicker peripheral fibers three and eight (Fig. 3E,F). The trapezoid mitochondria are of variable size, contain linear cristae and apparently surround the distal centriole and the fibrous sheath in a spiral fashion (Fig. 3A,B,D,F). Regularly spaced dense bodies lie in external depressions of the mitochondria, almost always separated from the centrioles and fibrous sheath by mitochondrial projections (Fig. 3A). In transverse sections of the midpiece, dense bodies are interrupted by mitochondria, hence they do not form continuous rings (Fig. 3F). At the posterior end of midpiece, the mitochondria become slender in cross section and are well separated from the fibrous sheath by cytoplasmatic material (Fig. 3G,H). The annulus appears like a very small dense ring (Fig. 3G).

Principal piece. This is the longest portion of the flagellum, consisting of the axoneme surrounded by the fibrous sheath, cytoplasm and plasma membrane (Fig. 3I). In this region, the axoneme presents a 9 + 2 microtubules pattern, and neither peripheral dense fibers can be observed (Fig. 3J). The diameter of the principal piece gradually diminishes, as a result of a decreasing cytoplasm between the fibrous sheath and the plasma membrane and a reduction in the width of the fibrous sheath.

Endpiece. The endpiece was not clearly observed.

Phylogenetic analyses

A total of 8733 alternative tree topologies were discovered (length = 47, C.I. = 0.729, R.C.I = 0.583). A strict consensus tree and a 50% majority-rule consensus tree (Miyamoto 1985) are presented in Fig. 4. It is worth emphasizing that consensus trees are often less parsimonious than the original trees from which they derive (Miyamoto 1985; Barret *et al.* 1991), thus they should not be regarded as phylogenies but rather as statements about areas of agreement among trees (Swofford 1991b). The strict consensus tree (Fig. 4A) clearly indicates a monophyletic Squamata, but it is not very informative regarding relationships among squamates, except for the monophyletic groups *Pogona barbata* + *Varanus gouldii*, another formed by an unresolved trichotomy comprising the Serpentes (*Oxyuranus microlepidotus*, *Aspidites melanocephalus*, Colubridae), skinks of the *Eugongylus* species-group (*Carlia pectoralis*, *Lampropholis delicata*), and the pygopodid *Lialis burtonis*, and a third monophyletic group comprising the Serpentes.

The 50% majority-rule tree indicates a monophyletic Squamata (Fig. 4B), supported by 9 characters (Table 3). Four clades emerge from a basal polytomy. Two of these clades contain just a single family: the Iguanidae and the Gymnophthalmidae. The third one appeared in 72% of the 8733 alternative trees and contains the Lacertidae, Scincidae (part), Teiidae, Varanidae, and Chamaeleonidae (part). This clade is basically unresolved, except for two sister-groups: Lacertidae and *Ctenotus robustus*, which is weakly supported (54%), and Varanidae and Chamaeleonidae (*Pogona barbata*), which was common to 100% of the alternative trees. The fourth clade is relatively well supported, having appeared in 81% of the most-parsimonious reconstructions. It is formed by the sequential addition of the Gekkonidae, Chamaeleonidae (*Bradypodion karroicum*), Serpentes, Pygopodidae (*Lialis burtonis*), and Scincidae of the *Eugongylus* species-group (*Carlia pectoralis*, *Lampropholis delicata*). Most of these groups were strongly supported, as indicated by the frequency of occurrence among the alternative trees, except for Chamaeleonidae (part) + Serpentes + Pygopodidae + Scincidae of the *Eugongylus* species-group (55%), and Pygopodidae + Scincidae of the *Eugongylus* species-group (56%).

Because of the presence of unresolved ('soft') polytomies (Maddison and Maddison 1992), few unambiguous character transformations could be identified (Table 3). These included the autapomorphic states in the Lacertidae (dense bodies not regular, dense bodies in 2 groups), *Varanus gouldii* (dense bodies granular), *Pogona barbata* (mitochondria intermediate in transverse section, mitochondria shape intermediate rounded-columnar), *Heteronotia binoei* (epinuclear lucent zone absent), and *Bradypodion karroicum* (acrosome depressed in transverse section), and the synapomorphic states of the Gekkonidae (stopperlike perforatorial base plate), Serpentes + Pygopodidae + Scincidae of the *Eugongylus* species-group (mitochondria not regular in transverse section), Pygopodidae + Scincidae of the *Eugongylus* species-group (perforatorial tip square-ended, nuclear shape elongate), and Scincidae of the *Eugongylus* species-group (fibers 3 and 8 grossly enlarged anteriorly).

Fig. 3.—Spermatozoa of *Micrablepharus maximiliani*. Transmission electron micrographs of the tail (midpiece and principal piece).—**A,** Longitudinal section of the midpiece.—**B,** Longitudinal section of the neck region with pericentriolar material and centrioles.—**C,** Detail of the neck region in longitudinal section, showing the laminar structure.—**D,** Transverse section of the neck region showing the distal centriole with the central singlets and peripheral fibers.—**E,** Longitudinal section of the midpiece, showing the transition between the distal centriole and the axoneme. Note the fibrous sheath surrounding the axoneme.—**F,** Transverse section of the midpiece showing the axoneme with peripheral fibers 3 and 8 (arrow heads) enlarged and detached from their doublets.—**G-H,** Transition region between midpiece and principal piece in longitudinal and transverse sections, respectively. Note the annulus in longitudinal section.—**I-J,** Longitudinal and transverse sections, respectively, of principal piece, showing fibrous sheath and axoneme without peripheral fibers. Fig. 3A-C,E-I: scale bar 0.5 μm; Fig. 3D and J: scale bar 0.2 μm. Abbreviations as in Fig. 2.

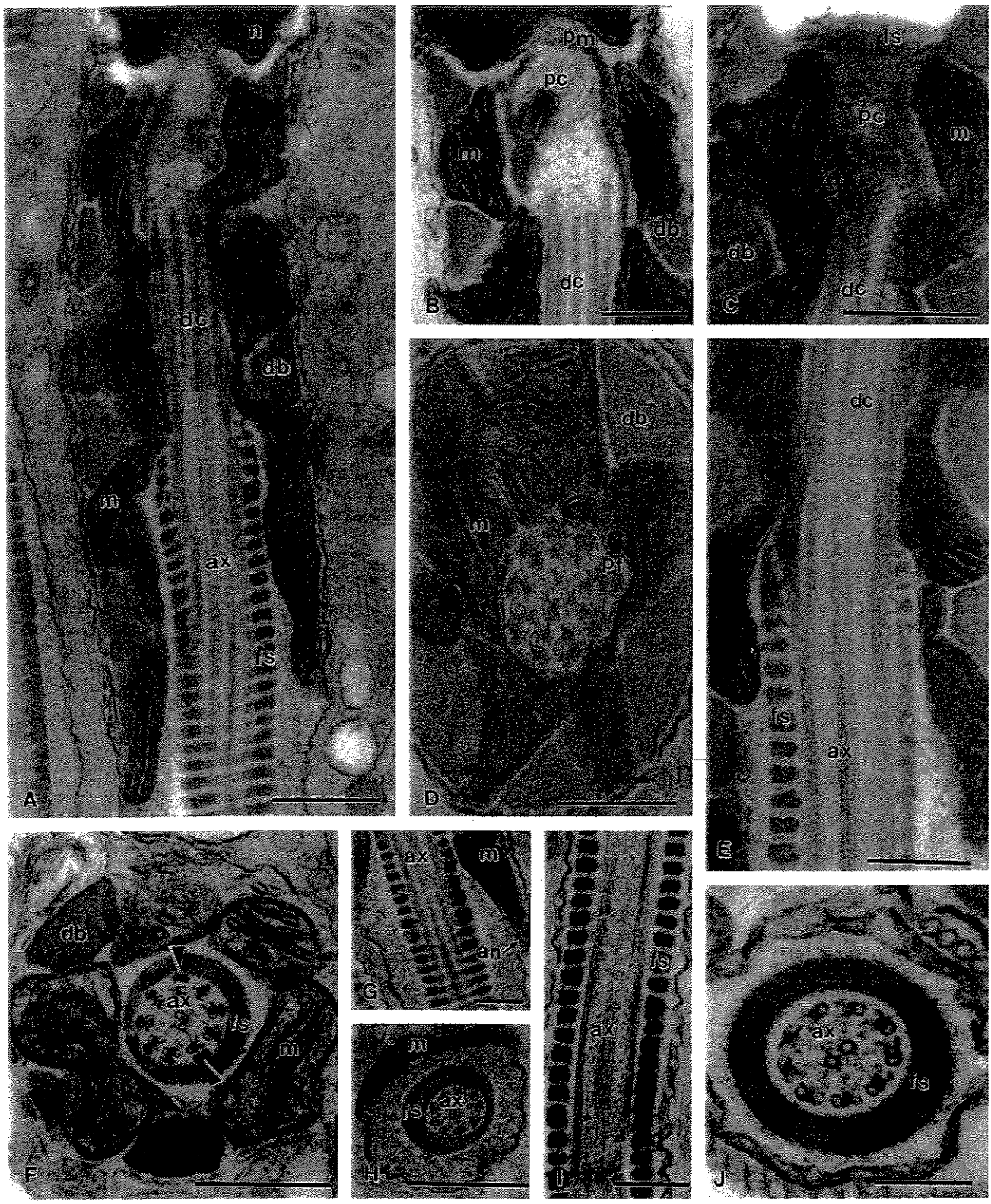

Fig. 3

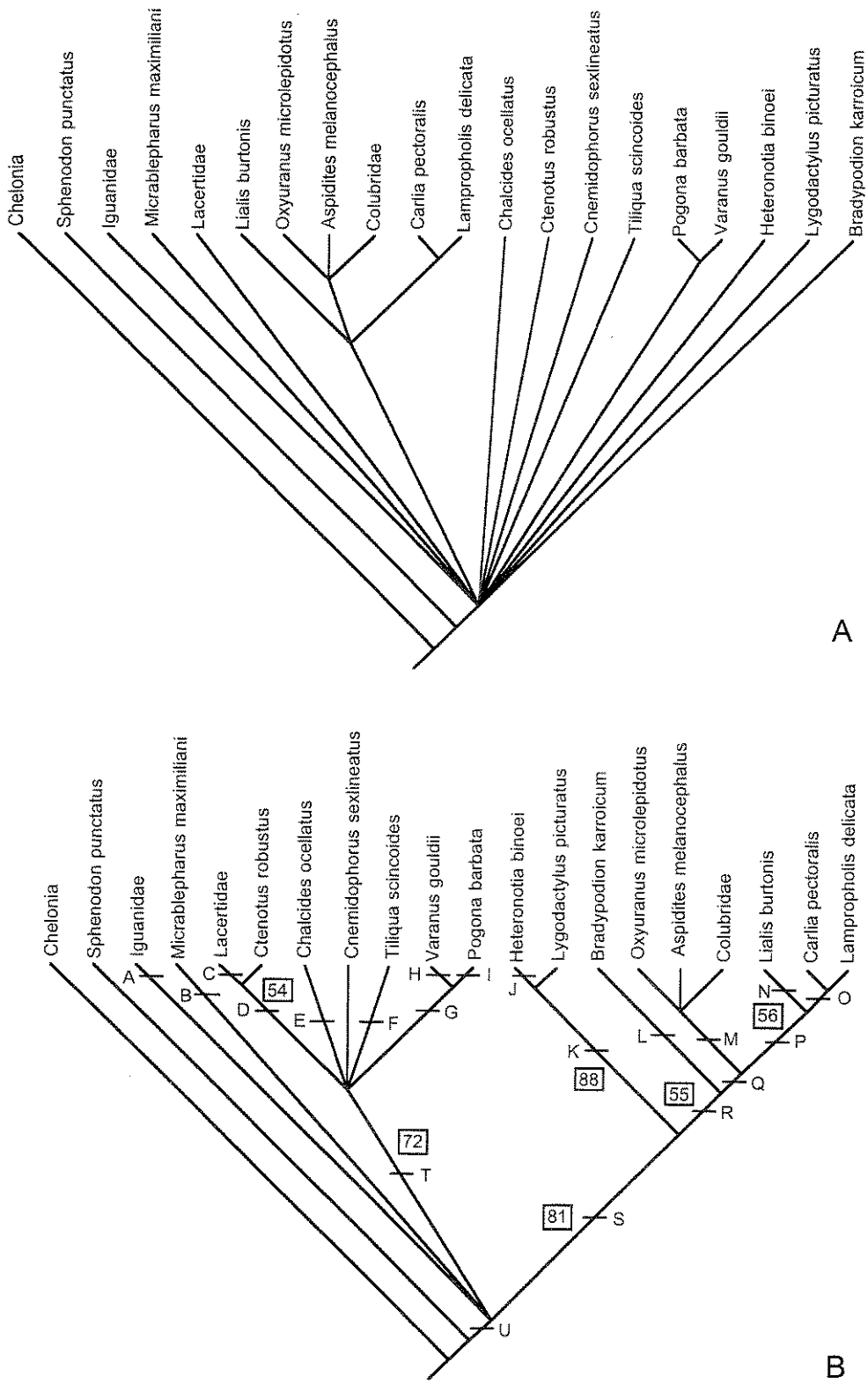

Fig. 4.—**A,** Strict consensus tree for 8733 equally parsimonious trees, derived from spermatozoa ultrastructure data listed in Tables 1 and 2.—**B,** 50% majority-rule consensus tree for the same 8733 trees. Numbers indicate the percentage of occurrence of clades among the equally parsimonious trees. Character-state changes in each labelled branch are listed in Table 3.

Node	Character	Change
A (Iguanidae)	8 (mitochondria shape)	1 → 2
B (*Micrablepharus maximiliani*)	5 (epinuclear lucent zone)	2 → 0
	7 (mitochondria in transverse section)	0 → 2
	8 (mitochondria shape)	1 → 4
C (Lacertidae)	9 (dense bodies)	1 → 5
	10 (dense bodies, if regular)	1 → 0
D	5 (epinuclear lucent zone)	2 → 1
E (*Chalcides ocellatus*)	10 (dense bodies, if regular)	1 → 3
F (*Tiliqua scincoides*)	5 (epinuclear lucent zone)	2 → 0
G	2	0 → 1
H (*Varanus gouldii*)	10 (dense bodies, if regular)	1 → 2
I (*Pogona barbata*)	7 (mitochondria in transverse section)	0 → 2
	8 (mitochondria shape)	1 → 3
J (*Heteronotia binoei*)	5 (epinuclear lucent zone)	2 → 0
K (Gekkonidae)	2 (perforatorial base plate)	0 → 2
	9 (dense bodies)	2 → 4
L (*Bradypodium karroicum*)	1 (acrosome in transverse section)	0 → 1
M (Serpentes)	5 (epinuclear lucent zone)	2 → 1
	6 (midpiece)	1 → 2
N (*Lialis burtonis*)	12 (nuclear shoulders)	0 → 2
O (*Eugongylus* species-group of	9 (dense bodies)	3 → 2
Scincidae)	15 (fibers 3 and 8)	0 → 1
	16 (multilaminar membranes)	1 → 0
P	2 (perforatorial base plate)	0 → 1
	3 (perforatorial tip)	0 → 1
	12 (nuclear shoulders)	1 → 0
	13 (nuclear shape)	0 → 1
Q	7 (mitochondria in transverse section)	0 → 1
	9 (dense bodies)	2 → 3
	16 (multilaminar membranes)	0 → 1
R	8 (mitochondria shape)	1 → 2
S	6 (midpiece)	0 → 1
	9 (dense bodies)	1 → 2
	10 (dense bodies, if regular)	1 → 0
T	1 (acrosome in transverse section)	0 → 1
U (Squamata)	4 (perforatoria number)	0 → 1
	5 (epinuclear lucent zone)	0 → 2
	8 (mitochondria shape)	0 → 1
	9 (dense bodies	0 → 1
	10 (dense bodies, if regular)	0 → 1
	11 (mitochondrial cristae)	0 → 1
	12 (nuclear shoulders)	0 → 1
	14 (endonuclear canal)	0 → 1
	17 (fibrous sheath)	0 → 1

Table 3 Character-state changes in the 50% majority-rule tree depicted in Figure 4B. Reconstruction of character evolution according to ACCTRAN tracing. Unambiguous changes are underlined.

Discussion

Ultrastructure of spermatozoa

The acrosome of *Micrablepharus maximiliani* exhibits the acrosome vesicle, the subacrosomal cone and the constricted nuclear tip, forming a tripartite pattern which is presumably plesiomorphic for amniotes and lissamphibians (Jamieson 1995a). The single perforatorium and the paracrystalline substructure of the subacrosomal cone of *M. maximiliani* are presumably synapomorphies of the Squamata (Jamieson 1995b). The acrosome is circular in transverse section throughout its length, as in the *Eugongylus* species-group (*Carlia* and *Lampropholis*) of the Scincidae (Jamieson and Scheltinga 1994; Jamieson *et al.* 1996), Gekkonidae (Furieri 1970; Jamieson *et al.* 1996), Pygopodidae (Jamieson *et al.* 1996), Iguanidae (Saita *et al.* 1988) and Serpentes (Oliver *et al.* 1996). The perforatorium of *M. maximiliani* is pointed at its anterior end and no basal plate was observed at its posterior end. In contrast, skinks of the *Eugongylus* species-group and the Pygopodidae display a square-ended perforatorial tip (Jamieson *et al.* 1996). As with *Micrablepharus*, the *Sphenomorphus* and *Egernia* species-groups of Scincidae (Furieri 1970; Jamieson *et al.* 1996), Lacertidae (Furieri 1970; Butler and Gabri 1984; Courtens and Depeiges, 1985),

Chamaeleonidae (Jamieson 1995b), Iguanidae (Saita *et al.* 1988) and Serpentes (Oliver *et al.* 1996) also lack the perforatorial basal plate.

Two nuclear traits are regarded as synapomorphic for the Squamata: the loss of the endonuclear canal and the presence of an epinuclear electron-lucent region (Jamieson 1995b). The spermatozoa of *Micrablepharus maximiliani* also lacks the endonuclear canal, but has no epinuclear electron-lucent space, like the skink *Tiliqua scincoides* (Jamieson and Scheltinga 1994) and the gecko *Heteronotia binoei* (Jamieson *et al.* 1996), what presumably is a reversal to the ancestral condition exhibited by *Sphenodon* and the Chelonia (Jamieson 1995b). The gently rounded nuclear shoulders, at the base of the tapered nuclear tip, in *M. maximiliani* sperm resembles the condition exhibited by most squamates (Jamieson 1995b). The basal nuclear fossa of *M. maximiliani* is dome-shaped, but it is not so well developed as in the sperm of the *Sphenomorphus* and *Egernia* species-group of skinks (Jamieson and Scheltinga 1993; Jamieson *et al.* 1996).

In the anterior portion of the midpiece, the proximal and distal centrioles are clearly observed in *Micrablepharus maximiliani*, a plesiomorphic condition in all tetrapods (Jamieson 1995a). As in other squamates, the sperm of *M. maximiliani* presents a short distal centriole, which forms the basal body of the axoneme and is penetrated by two central singlets from the axoneme (Jamieson 1995b; Jamieson *et al.* 1996; Oliver *et al.* 1996). *Micrablepharus maximiliani* presents dense material surrounding its distal centriole, a feature that has been reported in all amniotes (Jamieson *et al.* 1997). An electron-dense laminar structure was observed in *M. maximiliani*, extending from the pericentriolar apparatus, above the proximal centriole. Its presence has been clearly described in the *Sphenomorphus* species-group of skinks (Jamieson and Scheltinga 1993, 1994).

The short midpiece of *Micrablepharus maximiliani* sperm has been reported for the *Sphenomorphus* and *Egernia* species-group of Scincidae (Jamieson and Scheltinga 1993, 1994; Jamieson *et al.* 1996), Lacertidae (Furieri 1970; Butler and Gabri 1984; Courtens and Depeiges 1985), Teiidae (Newton and Trauth 1992), Agamidae (Oliver *et al.* 1996), Varanidae (Oliver *et al.* 1996) and Iguanidae (Saita *et al.* 1988). The mitochondria and dense bodies of *M. maximiliani* differ in shape and organization from those of other squamates studied to date. The mitochondria are trapezoid in longitudinal section, apparently arranged in spiral fashion around the fibrous sheath. Dense bodies lie in depressions of the mitochondrial cover, being in most sections separated from the fibrous sheath by mitochondria, and form regular rings that are not continuous in transverse sections. The arrangement of dense bodies in *M. maximiliani* most closely resembles that of *Pogona barbata*, where the ring structures are not continuous in transverse sections (Oliver *et al.* 1996).

The fibrous sheath in *Micrablepharus maximiliani* extends well into the midpiece, a synapomorphy of the Squamata (Jamieson 1995b). Nine peripheral dense fibers are associated with the nine doublets of the axoneme, and the peripheral fibers adjacent to doublets 3 and 8 are enlarged and detached from their respective doublets. These features are typical of all reptiles studied to date (Jamieson *et al,* 1997). Dense material is associated with one of the central microtubules of the distal centriole in *M. maximiliani*. This was also observed in snakes (Jamieson and Koehler 1994; Oliver *et al.* 1996), skinks (Jamieson and Scheltinga 1993, 1994), and geckos (Jamieson *et al.* 1996), and may be more widespread in squamates, depending upon appropriate sections of the distal centriole (Jamieson and Koehler 1994).

In *Micrablepharus maximiliani* the principal piece consists of the axoneme wrapped by the fibrous sheath, as in other amniotes (Jamieson 1995a), and the nine peripheral fibers are absent from the principal piece, as in other squamates (Jamieson 1995b).

Phylogeny of the Squamata

The addition of *Micrablepharus maximiliani* to the data set of Jamieson (1995b) basically produced two major effects: it significantly raised the number of optimal trees and lowered the resolution of both the strict and the 50% majority-rule consensus trees. Four groups, however, remained unaltered in the strict consensus tree after the addition of *M. maximiliani*: (1) *Pogona barbata* and *Varanus gouldii*; (2) Serpentes; (3) *Carlia pectoralis* and *Lampropholis delicata*; and (4) *Lialis burtonis*, Serpentes, and *C. pectoralis* and *L. delicata*.

Most-parsimonious trees depicting phylogenetic relationships among the Squamata, derived from spermatozoa ultrastructural characters, contain major areas of disagreement relative to trees produced from gross morphological characters (Estes *et al.* 1988), tongue morphology (Schwenk 1988), and limb musculature (Russel 1988). Major groups, such as the Iguania, Gekkota, Scincomorpha, and Anguimorpha, and families such as the Chamaeleonidae (*sensu* Frost and Etheridge 1989) and Scincidae, whose monophyly is supported by the three latter data sets, are not monophyletic in trees derived from the sperm ultrastructure data set.

Given the conflict between the trees supported by the sperm ultrastructure and the gross morphology data sets, it is reasonable to assume that at least one of the data sets is misleading, since there is just one tree of life. One could then conceive that, because the number of charaters and degree of support for the tree(s) derived from the morphology data sets surpass to a large extent those derived from the sperm ultrastructure data set, this latter data set is probably too noisy and contains little phylogenetic signal. Even though Jamieson (1995b) stated that the utility of spermatozoal ultrastructure as a source of characters for phylogenetic

analysis is well established', no one has explicitly tested his assertion.

A useful criterion for evaluating the phylogenetic content of characters is the analysis of tree-length skewness, as tree-length distributions with significant left skewness contain more phylogenetic signal than more symmetrical or right-skewed distributions (Hillis 1991; Huelsenbeck, 1991). The g_1 statistic is used to test for the skewness of frequency distributions: for a symmetrical distribution $g_1 = 0$, whereas a right-skewed distribution has a $g_1 > 0$ and a left-skewed distribution has a $g_1 < 0$ (Zar, 1984). Hillis (1991) pointed out that when testing for the skewness of tree-length distributions it is not appropriate to use the normal distribution as a null model, because the degree of expected departures from symmetry for a normal distribution decreases with increasing sample size, and sample size increases very rapidly with an increase in the number of taxa. Thus, he suggested that an empirically generated distribution of g_1 statistics provides a better means of testing for significant skewness, because no a priori assumptions about the shape of the distribution are made (Hillis, 1991). The skewness test has been severely criticized by Källersjö *et al.* (1992), who showed that under a particular set of circumstances (two hypothetical data sets) the skewness test can be misleading and that it can be more affected by the frequencies of states within characters than by congruence among characters. Further, they showed that the skewness test is insensitive to the number of characters. However, Källersjö *et al.* (1992) did not explore the issue of how likely the character-state distributions they presented can be observed in nature. This is still a largely unexplored issue but, apparently, the two hypothetical data sets presented by Källersjö *et al.* (1992) are so improbable that they will never be observed empirically under any tree model or under any model of character evolution and character sampling in which stochastic processes play a part (J. Lyons-Weiler, pers. comm.). The fact that one can, by intelligent design, create a set of conditions under which a given measure will fail, sheds little light on the general performance of that measure. For instance, Felsenstein (1978) has shown that under certain circumstances parsimony methods can be positively misleading. However, parsimony is still a dominant method in phylogenetic reconstruction. Every single method will have its assumptions violated at least occasionally or will perform poorly under some set of circumstances, but until it can be demonstrated how often this occurs, no arguments for or against it can be regarded as overly compelling (Maddison and Maddison 1992).

To test the null hypothesis that the squamate spermatozoa ultrastructure data set contains no phylogenetic signal, we constructed 100 data sets, each with 20 taxa and 17 characters (the same dimensions as the real data set), using the random data generator of MacClade v.3.06 (Maddison and Maddison 1992). Two states were allowed for each character, 0 and 1, each with a 0.5 probability of occurrence. Given the large number of possible unrooted, labelled trees for 20 taxa ($\approx 2.22 \times 10^{20}$), we generated 10 000 random trees from each of the 100 random data sets and calculated the g_1 value of each tree-length distribution using Paup vs. 3.0 s (Swofford 1991a) in order to produce an empirical distribution of g_1 statistics. We also generated a g_1 statistic from the squamate spermatozoa ultrastructure data, based on a random sample of 10 000 trees. The lower 5% and 1% of the empirical g_1 distribution were, respectively, –0.19 and –0.21 (Fig. 5A). This indicates that the g_1 statistic produced from the real data (–0.80) is highly significant and therefore sperm ultrastructure data does indeed contain significant phylogenetic signal (Fig. 5B).

Another possibility is that characters in each data set are affected by distinct stochastic processes, which lead to different rates of evolutionary change (de Queiroz *et al.* 1995). For instance, even though some ultrastructural characters seem rather conservative in terms of change, as the possession of a single perforatorium, linear mitochondrial cristae, absence of endonuclear canal, and a fibrous sheath that extends into the midpiece, members of the family with the highest number of species in the study matrix (Scincidae) are highly scattered in each of the best phylogenetic reconstructions, forming para-or polyphyletic groups (Fig. 4B). Among the two other families with more than one species in the study matrix, the chamaeleonids *Pogona barbata* and *Bradypodion karroicum* are also placed at very distant portions of the best trees (Fig. 4B). It is possible, as suggested by Jamieson (1995b), that some of these groups are not monophyletic, but we advance that the intrafamilial variability may be higher than currently thought. Therefore, spermatozoa ultrastructure characters might be more useful, as a source of phylogenetic information, at the generic or familial level and tree topology estimates derived from them might be rather incongruent with those derived from putatively more conservative morphological characters, because of heterogeneous rates of evolution. Additional studies are necessary to further our understanding on the levels of variability in spermatozoa ultrastructure characters across taxonomic categories.

Finally, we should also entertain the possibility that the heterogeneity between the spermatozoa ultrastructure and the morphological data sets is only apparent. Swofford (1991b) argued that the selection of trees for comparison is a critical issue in the evaluation of congruence. Selecting only the optimal trees for each data set results in the loss of all the uncertainty associated with the estimate of the tree. Hence, near-optimal trees should also be considered in the analysis of congruence. If the incongruence between the data sets is greater than that expected by chance, i.e. if the data sets can be regarded as independent, then individual data sets that are incompatible with others can be targeted for future studies concerning the sources of their significant heterogeneity (Miyamoto and Fitch 1995).

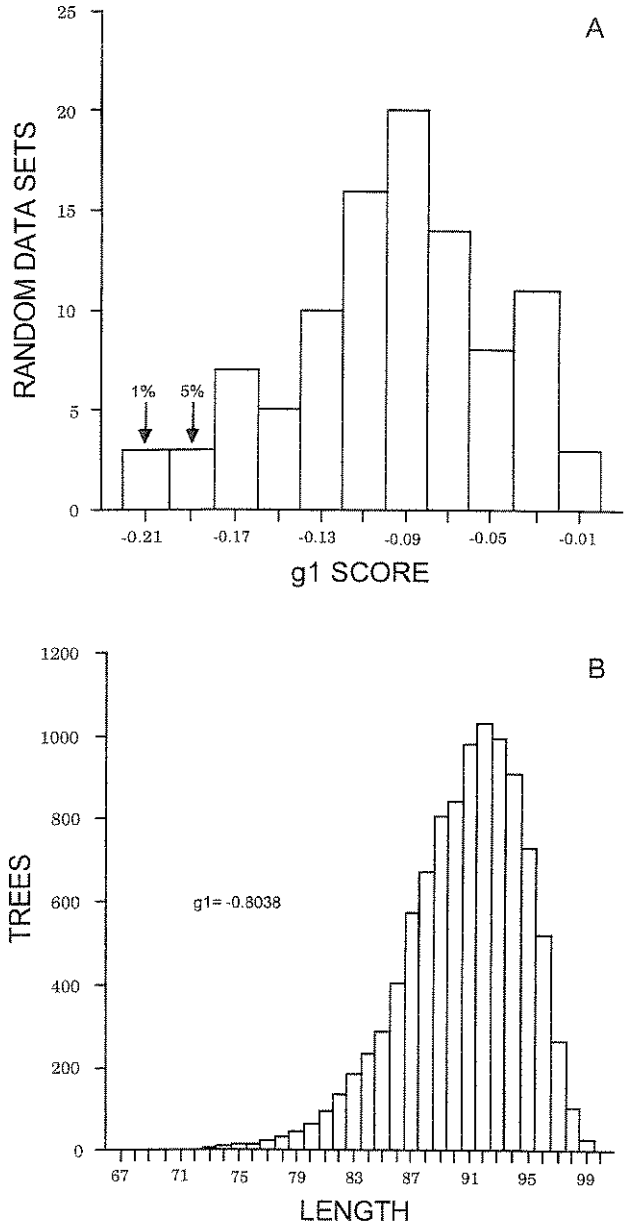

Fig. 5.—**A,** Distribution of skewness statistics (g_1) for tree-length distributions produced from 100 random data matrices. Arrows indicate the lower 1% and 5% critical values of the distribution. —**B,** Tree-length distribution of 10 000 random trees obtained from the spermatozoal ultrastructure data set of the Squamata.

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2. **Teixeira, R. D.**, Colli, G. R. & Báo, S. N. The ultrastructure of the spermatozoa of the worm-lizard *Amphisbaena alba* (Squamata, Amphisbaenidae), and the phylogenetic relationships of amphisbaenians. *Can. J. Zool.* No prelo.

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Dear Dr. Báo:

Subject: The ultrastructure of the spermatozoa of the worm-lizard Amphisbaena alba (Squamata, Amphisbaenidae), and the phylogenetic relationships of amphisbaenians

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The ultrastructure of the spermatozoa of the worm-lizard *Amphisbaena alba* (Squamata, Amphisbaenidae), and the phylogenetic relationships of amphisbaenians

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Key words: Sperm ultrastructure, *Amphisbaena alba*, phylogeny

Abstract

Teixeira, R. D., G. R. Colli, and S. N. Báo. The ultrastructure of the spermatozoa of the worm-lizard *Amphisbaena alba* (Squamata, Amphisbaenidae), and the phylogenetic relationships of Amphisbaenians.

We describe the ultrastructure of epididymal spermatozoa of *Amphisbaena alba* and make comparisons with other squamates. The mature spermatozoon of *A. alba* is filiform and characterized by the following features: acrosome in transverse section depressed; perforatorial base plate absent; perforatorial tip pointed; perforatorium single; epinuclear lucent zone well developed; midpiece moderately long; mitochondria columnar, forming regular circlets interrupted by dense bodies in transverse section; dense bodies solid, arranged in regular rings and linear series; nuclear shoulders rounded; nucleus elongate; endonuclear canal absent; fibers 3 and 8 enlarged; multilaminar membranes absent; and fibrous sheath in midpiece. A phylogenetic analysis of squamates using sperm ultrastructure characters resulted in 2415 equally-parsimonious, largely unresolved, trees. The use of a constraint tree derived from gross anatomical characters suggested that Amphisbaenia is the sister-group of Autarchoglossa. We conclude that, to improve the resolution of phylogenetic hypotheses derived from sperm ultrastructure characters, the number of characters ought to be increased and more investigations are necessary to ascertain the degree of intrafamilial variability.

Introduction

Amphisbaenians or worm-lizards comprise an array of approximately 133 species of highly specialized, fossorial squamates (Pough et al. 1998). Not long ago, the group was regarded as distinctive enough from lizards and snakes to constitute a separate suborder, called Amphisbaenia (Gans 1978; Bellairs and Gans 1983; Romer and Parsons 1986). This arrangement, however, was not grounded on explicit phylogenetic analyses, but solely on the perceived degree of evolutionary divergence. Based on the phylogenetic analysis of a large data set composed mainly of osteological characters, Estes et al. (1988) placed amphisbaenians as *incertae sedis* within Scleroglossa, a monophyletic group comprising snakes and all lizards with the exception of iguanians (Frost and Etheridge 1989). More recently, the discovery of an ancient fossil amphisbaenian (*Sineoamphisbaena hexatabularis*) led Wu et al. (1993; 1996) to suggest a closer relationship between Amphisbaenia and Polyglyphanodontinae, a group of fossil lizards presumably related to teiids (Presch 1983), on the basis of traits of the palate and temporal region (but see Gao and Hou 1996; Gao 1997; Wu et al. 1997). A close relationship between amphisbaenians and scincomorphs was also suggested by Camp (1923), based on similarities in the tongue, hemipenis, pelvis, and the cervicomandibularis; Bogert (1964), based on the shared loss of hindlimbs and similarities in tongue structure between amphisbaenians and gymnophthalmids; and Schwenk (1988; 1993), based on a cladistic analysis of tongue and chemosensory characters. Further, several recent studies suggest that Dibamidae, a group of 10 species of small, highly specialized fossorial lizards (Pough et al. 1998), is the sister-taxon of Amphisbaenia (Greer 1985; Hallermann 1998; Caldwell 1999).

The sperm ultrastructure of many squamate families is well known (reviews in Jamieson 1995b; Jamieson et al. 1996; Oliver et al. 1996) and can provide an independent and

phylogenetically structured source of characters for phylogenetic analysis (Teixeira et al. 1999). In groups such as the Amphisbaenia, where habitat specialization has produced a highly derived body plan (Gans 1975; Gans 1978), sperm ultrastructure characters may be evolutionarily more conservative than traditionally used morphological characters and, hence, provide more informative data for phylogenetic analyses. However, with the exception of a brief account on the kinetic apparatus of the sperm of *Amphisbaena darwini* (Sotello and Trujillo-Cenóz 1958), nothing is known on the sperm ultrastructure of amphisbaenians. Herein we provide, for the first time, a detailed description of the ultrastructure of the spermatozoa of an amphisbaenian, *Amphisbaena alba*. Moreover, we make comparisons with other families of squamates and carry out a phylogenetic analysis using sperm ultrastructure data, attempting to uncover the phylogenetic relationships of amphisbaenians.

Materials and Methods

Spermatozoa ultrastructure

We obtained two adult males of *Amphisbaena alba* from Minaçu, Goiás State, Brazil (13°38' S, 48°15' W) in February 1997 and sacrificed them with an injection of Tiopental®. After the removal of the epididymides, we fixed the specimens in 10% formalin and deposited them in the Coleção Herpetológica da Universidade de Brasília (CHUNB 00430, 00435). We placed the epididymides in a Petri dish with phosphate buffered saline (PBS) pH 7.2, and cut it into small pieces. We fixed epididymal tissues in 2.5% glutaraldehyde, 2% paraformaldehyde, and 3% sucrose in 0.1 M sodium cacodylate buffer pH 7.2 at 4 °C overnight, postfixed for 60 min in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5mM $CaCl_2$ in 0.1 M sodium cacodylate buffer, dehydrated with acetone, and embedded in Spurr. We stained thin sections in uranyl

acetate followed by lead citrate and examined them in a Jeol® 100 C transmission electron microscope at 80 kV. We also made light microscopic observations of spermatozoa from glutaraldehyde-paraformaldehyde fixed smears under Nomarski contrast using a Zeiss® Axiophot microscope.

Phylogenetic analysis

We scored *Amphisbaena alba* for the 17 ultrastructural characters of squamate sperm described by Jamieson (1995b) (Table 1). Next, we built a taxon-character matrix (Table 2), by joining *A. alba* (Amphisbaenidae) to the matrices presented by Jamieson (1995b) and Teixeira et al. (1999), but collapsed the number of taxa by entering families, and not species, as taxa. This modification is based on the fact that using species as taxa in the working matrix renders several well established families as para- or polyphyletic (Jamieson 1995b; Teixeira et al. 1999). It should be noted that we assigned *Pogona barbata* to Chamaeleonidae, following Frost and Etheridge (1989) in that Agaminae is a subfamily of Chamaeleonidae.

Whenever two or more species of a single family had more than one state for a given character, we considered the character as polymorphic for that family. We produced most parsimonious phylogenetic hypotheses using PAUP v. 3.0s for the Macintosh (Swofford 1991a) through a branch-and-bound search, performed with the default options. We regarded all characters as unordered, and Chelonia and *Sphenodon punctatus* (Sphenodontidae) as a paraphyletic outgroup with respect to the ingroup. Reconstructions of character evolution were produced with MacClade v.3.07 (Maddison and Maddison 1992).

Results

Ultrastructure of spermatozoa

Spermatozoa of *A. alba* are filiform and approximately 84.3 μm long (Figs. 1, 2A). The head is short and curved, and approximately 14.3 μm in total length, from light microscopy. The midpiece is thick and approximately 4.3 μm long, and the tail (principal piece and endpiece) is approximately 65.7 μm long from light microscopy.

Acrosome complex

The acrosome complex is an elongate cone that covers the anterior region of the nucleus. It consists of an external cap, the acrosome vesicle, an internal cap, the paracrystalline subacrosomal cone, and the perforatorium (Fig. 2B). In cross-section, the acrosome complex appears depressed and the subacrosomal cone presents a radial aspect (Fig. 2C). The acrosome vesicle ensheathes the subacrosomal cone and is uniformly electron-dense (Figs. 2B, 2C). The single perforatorium, in longitudinal section, extends from the anterior region of the subacrosomal cone and projects into the acrosome vesicle (Fig. 2D). This structure is filled with a less electron-dense material, presenting a pointed tip and no base plate (Fig. 2D). The subacrosomal cone wraps the tapered anterior end of the nucleus (Figs. 2B-2F) and is separated from the acrosome vesicle by an electron-lucent space (Figs. 2C, 2E, 2F). At its posterior end, the acrosome complex lies on a postero-lateral membranous flange (Fig. 2G).

Nucleus

The nucleus has a strongly electron-dense chromatin, with some electron-lucent lacunae (Fig. 2H). It is a long curved cylinder, tapered anteriorly within the subacrosomal cone (Fig. 2F). The

nuclear point is capped by a well developed epinuclear lucent zone (Fig. 2E, 2F). In transverse section, the nucleus is circular (Fig. 2H). The transition from the tapered apical portion (nuclear rostrum) to the cylindrical region is abrupt and marked by small rounded "shoulders" (Fig. 2G). At its base, the nuclear fossa houses an electron-dense pericentriolar material that partially covers the proximal centriole (Figs. 3B, 3C).

Neck region

The neck region lies at the junction between the sperm head and the flagellum, where the posterior end of the nucleus adjoins the anterior extremity of the midpiece. It contains the proximal and distal centrioles, including the first ring of dense bodies (mitochondrial transformations) (Fig. 3A). The proximal centriole lies anteriorly to the distal centriole at a right angle to the long axis of the flagellum. It is surrounded by dense pericentriolar material that extends into the nuclear fossa, conforms in shape to it, and joins the peripheral fibers longitudinally (Figs. 3B, 3C). An electron-lucent space separates the pericentriolar material from the nuclear fossa (Figs. 3B, 3C). The stratified laminar structure is seen within the pericentriolar material, projecting at one side of the proximal centriole (Fig. 3C). A central, electron-dense structure lies at the interior of the proximal centriole (Figs. 3A, 3B). The distal centriole consists of nine triplets of microtubules, nine peripheral fibers that partially cover the triplets, and the two central singlets of the axoneme, which extend into the posterior region of the distal centriole and are embedded in dense material (Fig. 3D).

Midpiece

The midpiece is a region at the anterior portion of the flagellum, that consists of the axoneme, surrounded by mitochondria, rings of dense bodies (mitochondrial transformations) and the fibrous sheath (Fig. 3A). The axoneme follows posteriorly the distal centriole (Fig. 3F) and has the 9+2 pattern: a pair of central microtubules surrounded by nine doublets and nine peripheral fibers of dense material. The peripheral dense fibers associated with doublets three and eight are thicker than the others, are separated from their corresponding doublets, and are closely applied to the fibrous sheath (Fig. 3E). Mitochondria are of regular size, have linear cristae, and surround the distal centriole and the fibrous sheath-like hollow elongate cylinders (Fig. 3A). In longitudinal section of the midpiece, the mitochondria exhibit a columnar shape and are sometimes conjoined (Fig. 3A). Irregularly spaced rings of dense bodies are distributed between the mitochondria (Fig. 3A). In transverse sections of the midpiece, the mitochondria are mostly trapezoid-shaped, forming a circlet, interrupted by dense bodies, around the axoneme (Fig. 3E). Therefore, the dense bodies do not form continuous rings. The midpiece ends posteriorly with the annulus, an electron dense body beyond the last mitochondrial ring that appears to be closely attached to the inner surface of the plasma membrane. In longitudinal section, the annulus appears like electron-dense triangles at each side of the posterior end of the midpiece (Fig. 3G).

Principal piece

This is the longest portion of the spermatozoon. It runs for most of the long axis of the flagellum and its origin is characterized by the abrupt association of the plasma membrane with the axoneme, immediately after the annulus (Fig. 3G). The principal piece consists of the axoneme surrounded by the fibrous sheath, cytoplasm and plasma membrane (Figs. 3G-3I). The peripheral

fibers 3 and 8 are seen at the beginning of this region (Fig. 3H), but they disappear posteriorly (Fig. 3I).

Phylogenetic analyses

The branch-and-bound search produced 2415 alternative tree topologies (length= 58, CI = 0.914, RCI= 0.779). The strict consensus tree (Fig. 4A) clearly indicates a monophyletic Squamata, but the relationships within the ingroup are poorly resolved, except for two clades: Serpentes (Boidae, Colubridae, Elapidae) and Pygopodidae-Serpentes. The 50% majority-rule tree places Gekkonidae as the sister-group to the Pygopodidae-Serpentes clade and Gymnophthalmidae in a basal position relative to the other squamates (Fig. 4B). It is worth stressing that consensus trees are often less parsimonious than the original trees from which they are derived (Miyamoto 1985; Barrett et al. 1991), therefore they should not be regarded as phylogenies but rather as statements about areas of agreement among trees (Swofford 1991b).

In the 50% majority-rule tree, a monophyletic Squamata, present in 100% of the most-parsimonious reconstructions, is unambiguously (sensu Maddison and Maddison 1992) supported by six apomorphies (character number, following Table 1, in parentheses): perforatoria number (4) one; dense bodies (9) arranged in regular rings; mitochondrial cristae (11) linear; nuclear shoulders (12) rounded; endonuclear canal (14) absent; fibrous sheath (17) in midpiece. The clade containing all squamates except Gymnophthalmidae, present in 57% of the most-parsimonious reconstructions, cannot be unambiguosly supported. The Gekkonidae-Pygopodidae-Serpentes clade, present in 88% of the most-parsimonious reconstructions, also cannot be unambiguously supported. The Pygopodidae-Serpentes clade, present in 100% of the most-parsimonious reconstructions, is unambiguosly supported by two apomorphies:

mitochondria in transverse section (7) not regular; and multilaminar membranes (16) present. Finally, no apomorphies unambiguously support the Serpentes clade, present in 100% of the most-parsimonious reconstructions.

These results are not enlightening in regard to the phylogenetic relationships of amphisbaenians. Apparently, the high levels of intrafamilial polymorphism (see Table 2) coupled with the relatively low number of traits, prevent sperm ultrastructure characters from fully resolving the relationships among squamates. Despite the uncertain relationships of amphisbaenians, several independent data sets (mostly based on gross anatomy) are highly congruent with respect to the phylogenetic relationships of squamates (e.g., Estes et al. 1988; Schwenk 1988; Schwenk 1993). To further investigate the relationships of the Amphisbaenia, we ran the same phylogenetic analysis described above but constrained the search using a phylogeny of the Squamata based on Estes et al. (1988) and Frost and Etheridge (1989). In this phylogeny, independently derived from gross anatomical characters and regarded as the best hypothesis of relationships for the Squamata (Maddison and Maddison 1996), Amphisbaenia and Serpentes are Scleroglossa *incertae sedis* (Fig. 5).

An exhaustive search using the constraint tree produced a single most-parsimonious tree (length= 63, CI= 0.841, RCI= 0.594), where Serpentes is the sister-group of Gekkota and Amphisbaenia is the sister-group of Autarchoglossa (Figure 6). Character-state transformations in this tree are depicted in Table 3. A close relationship between Serpentes and the Pygopodidae-Gekkonidae clade is unambiguously supported by the shared presence of dense bodies arranged in linear series, modified in Gekkonidae to stellate spiral (Table 3). The relationship between Amphisbaenia and Autarchoglossa is unambiguously supported by the following apomorphies: acrosome in transverse section depressed and mitochondria shape columnar (Table 3).

Discussion

Ultrastructure of spermatozoa

Acrosome complex

The head of the spermatozoon of *Amphisbaena alba* exhibits plesiomorphic features common to all amniotes and lissamphibians: the elongate acrosome vesicle encloses a similarly-shaped subacrosomal cone, located on the anterior region of the tapered nucleus (Jamieson 1995a). The acrosome appears depressed in transverse section, a derived condition also present in the Chamaeleonidae (Jamieson 1995b), Lacertidae (Furieri 1970; Butler and Gabri 1984; Courtens and Depeiges 1985), *Sphenomorphus* and *Egernia* species-groups of Scincidae (Furieri 1970; Jamieson et al. 1996), Teiidae (Newton and Trauth 1992), and Varanidae (Oliver et al. 1996). Like most squamates, *A. alba* presents the plesiomorphic condition of a single perforatorium, pointed perforatorium tip, and no perforatorial basal plate. The well developed epinuclear lucent zone of *A. alba* is a derived condition also seen in the chamaeleonids *Bradypodion karroicum* (Jamieson 1995b) and *Pogona barbata* (Oliver et al. 1996), the gekkonid *Lygodactylus picturatus* (Furieri 1970), the Iguanidae (Furieri 1974; Saita et al. 1988), the pygopodid *Lialis burtonis* (Jamieson et al. 1996), the teiid *Cnemidophorus sexlineatus* (Newton and Trauth 1992), the skinks *Carlia pectoralis* (Jamieson and Scheltinga 1994) and *Lampropholis delicata* (Jamieson et al. 1996), and the varanid *Varanus gouldii* (Oliver et al. 1996). *Amphisbaena alba* has a unique feature, not previously described in any other squamate: a well developed electron-lucent space between the subacrosomal cone and the acrosome vesicle.

Nucleus

The spermatozoon of *Amphisbaena alba*, like most squamates, has rounded nuclear shoulders at the base of the tapered nuclear tip (Jamieson 1995b). The basal nuclear fossa has a low dome shape, but it is not so deep as in the sperm of the *Sphenomorphus* and *Egernia* species-group of skinks (Jamieson and Scheltinga 1993; Jamieson et al. 1996). The plesiomorphic conditions of absence of endonuclear canal and elongate nuclear shape are also present in *A. alba*.

Neck region

The proximal centriole, clearly seen in *Amphisbaena alba* and in all amniote classes, is a plesiomorphic condition of the tetrapods (Jamieson 1995a). The short distal centriole forms the basal body of the axoneme and is penetrated by two central singlets from the axoneme as in other squamates, differing from the elongate distal centriole seen in the Chelonia and Sphenodontidae (Jamieson 1995a; Jamieson et al. 1996; Oliver et al. 1996). Like in all amniotes, dense material surrounds the distal centriole and a laminar structure, extending from the pericentriolar apparatus, is present at one side of the proximal centriole of *A. alba* (Jamieson 1995a; Jamieson et al. 1997).

Midpiece

The sperm of *Amphisbaena alba* has a moderately long midpiece, resembling the chamaeleonid *Bradypodium karroicum* (Jamieson 1995b), the gekkonids *Heteronotia binoei* (Jamieson et al. 1996) and *Lygodactylus picturatus* (Furieri 1970), the pygopodid *Lialis burtonis* (Jamieson et al. 1996), and skinks of the *Eugongylus*-group (Jamieson and Scheltinga 1994; Jamieson et al. 1996). The columnar mitochondria of *A. alba* resemble the condition exhibited by gekkonids, lacertids, skinks of the *Sphenomorphus* and *Egernia* species groups, teiids, and varanids. The dense bodies form regular rings that are not continuous in transverse section, as in

the chamaeleonid *Pogona barbata* (Oliver et al. 1996) and the gymnophthalmid *Micrablepharus maximiliani* (Teixeira et al. 1999). The enlarged peripheral fibers associated with doublets 3 and 8 are detached from their respective doublets. These features are typical of most reptiles studied to date (Jamieson et al. 1997). Dense material is associated with one of the central microtubules of the distal centriole in *A. alba*, as in snakes (Jamieson and Koehler 1994; Oliver et al. 1996), skinks (Jamieson and Scheltinga 1993; Jamieson and Scheltinga 1994), geckos (Jamieson et al. 1996), and gymnophthalmids (Teixeira et al. 1999), and may be more widespread in squamates (Jamieson and Koehler 1994).

Principal piece

In *Amphisbaena alba* the principal piece consists of the axoneme wrapped by the fibrous sheath and the nine peripheral fibers are absent from the posterior region of the principal piece, as in other squamates (Jamieson 1995b).

Phylogenetic relationships of the Amphisbaenia

Amphisbaenian relationships have always been enigmatic because, as a consequence of the derived fossorial life-style, several traditionally used morphological characters either are absent in amphisbaenians and cannot be scored (e.g., limb characters) or their similarities to other taxa result from convergent evolution (e.g., body elongation, cranial consolidation). This possibility was apparently overlooked by Greer (1985), who suggested that amphisbaenians are most likely related to dibamids, but limited his comparisons to fossorial squamates, regarded as "most plausibly" related to dibamids. On the other hand, Estes et al. (1988) took a more conservative stand by considering amphisbaenians as Scleroglossa *incertae sedis*, even though their best tree

pointed to an amphisbaenian-dibamid relationship. A number of recent studies corroborate a close relationship among amphisbaenians, dibamids, and snakes (Wu et al. 1996; Hallermann 1998; Lee 1998), but they all have relied in part on the same osteological characters assembled by Estes et al (1988) and the possibility of convergence associated with burrowing habits in the characters supporting this arrangement cannot be ruled out.

Therefore, using presumably more conservative characters of the sperm ultrastructure (Jamieson 1995b; Jamieson 1995a) we hoped to cast some light on the relationships of amphisbaenians. Our cladistic analysis of sperm ultrastructure characters, however, produced unsatisfactory results in terms of resolving the phylogenetic position of amphisbaenians. Apparently, as suggested by Teixeira et al. (1999), adding more taxa to the data matrix, as we expand the knowledge on the sperm ultrastructure of squamates, will probably not improve the resolution of phylogenetic hypotheses. This outcome seemingly derives from the low number of characters employed to date (17) and the high level of polymorphism within families of Squamata.

Nevertheless, interpreting the evolution of sperm ultrastructure characters in light of a well corroborated phylogeny of squamates, using a constraint tree based on Estes et al. (1988) and Frost and Etheridge (1989), suggests a close relationship between Amphisbaenia and Autarchoglossa. Amphisbaenians share with the autarchoglossans scored to date a depressed acrosome in transverse section and columnar mitochondria. This conclusion is in agreement with the suggestion of Estes et al (1988) that Amphisbaenia is closer to Autarchoglossa than to Gekkota, based on the shared presence of the m. rectus abdominis lateralis. However, contrary to the suggestion of Estes et al. (1988), we found no compelling evidence that amphisbaenians may

be nested within Autarchoglossa. Rather, our results are more supportive of a sister group relationship between these two groups.

The sperm ultrastructure database for Squamata is rapidly expanding, but critical taxa such as Dibamidae are still missing. Further, considerable effort is still necessary to increase the number of characters and investigate the intrafamilial stability of characters (e.g., Teixeira et al. submitted). These developments will surely provide significant contributions to our undertanding of the relationships among squamates.

Acknowledgements

We thank Nelson J. da Silva Jr. for support during our stay at the Serra da Mesa hydroelectric dam, Ayrton K. Péres Jr. for assistance during field work, and Gustavo H. C. Vieira for excellent technical assistance with lab work. Michael Lee and an anonymous reviewer provided insightful comments on an earlier version of the manuscript. The study was partially supported by CAPES, CNPq and FAPDF.

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Table 1. Ultrastructure characters of the spermatozoa of squamates used in phylogenetic analyses.

Character	States
1 Acrosome in transverse section	(0) circular (1) depressed
2 Perforatorial base plate	(0) absent or indistinct (1) knoblike (2) stopperlike
3 Perforatorial tip	(0) pointed (1) square ended
4 Perforatoria number	(0) two or more (1) one
5 Epinuclear lucent zone	(0) absent (1) poorly developed (2) well developed
6 Midpiece	(0) short (1) moderately long (2) very long
7 Mitochondria in transverse section	(0) regular circlet (1) not regular (2) intermediate
8 Mitochondria shape	(0) rounded (1) columnar (2) sinuous tubes (3) intermediate rounded-columnar (4) trapezoid
9 Dense bodies	(0) intramitochondrial (1) regular rings (2) scattered (3) linear series (4) stellate spiral (5) 2 groups
10 Dense bodies, if regular	(0) not applicable (1) solid (2) granular (3) single file granules
11 Mitochondrial cristae	(0) concentric (1) linear
12 Nuclear shoulders	(0) sharp (1) rounded (2) absent
13 Nuclear shape	(0) elongate (1) stout
14 Endonuclear canal	(0) present (1) absent
15 Fibers 3 and 8	(0) enlarged (1) grossly enlarged anteriorly
16 Multilaminar membranes	(0) absent (1) present

17 Fibrous sheath	(0) not in midpiece (1) in midpiece	

Table 2. Comparative ultrastructure of the Squamata sperm, Sphenodontidae and Chelonia. All data from Jamieson (1995), except for Gymnophthalmidae (Teixeira *et al*, 1999) and Amphisbaenidae (present study).

Taxon	Characters																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Chelonia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sphenodontidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amphisbaenidae	1	0	0	1	2	1	0	1	1&3	1	1	1	0	1	0	0	1
Boidae	0	0	0	1	?	2	1	2	3	0	1	1	0	1	0	1	1
Chamaeleonidae	1	0&1	0	1	2	0&1	0&2	2&3	1&2&3	0&1	1	1	0	1	0	0	1
Colubridae	0	0	0	1	1	2	1	2	3	0	1	1	0	1	0	1	1
Elapidae	0	0	0	1	?	2	1	2	3	0	1	1	0	1	0	1	1
Gekkonidae	0	2	0	1	0&2	1	0	1	4	0	1	1	0	1	0	0	1
Gymnophthalmidae	0	0	0	1	0	0	2	4	1&3	1	1	1	0	1	0	0	1
Iguanidae	0	0	0	1	2	0	0	2	1&2	1	1	0&1	0	1	0	0	1
Lacertidae	1	0	0	1	1	0	0	1	5	0	1	1	0	1	0	0	1
Pygopodidae	0	?	1	1	2	1	1	2	3	0	1	2	1	1	0	1	1
Scincidae	0&1	0&1	0&1	1	0&1&2	0&1	0&1	1&2	1&2	0&1&3	1	0&1	0&1	1	0&1	0	1
Teiidae	1	0	0	1	2	0	0	1	1	1	1	1	0	1	0	0	1
Varanidae	1	1	0	1	2	0	0	1	1	2	1	1	0	1	0	0	1

Table 3. Character-state transformations in the most-parsimonious tree depicted in Figure 6. Reconstruction of character evolution according to ACCTRAN optimization. Unambiguous changes are underlined.

Node	Character	Change
A (Squamata)	4 (perforatoria number)	<u>0 → 1</u>
	5 (epinuclear lucent zone)	<u>0 → 2</u>
	8 (mitochondria shape)	<u>0 → 2</u>
	9 (dense bodies)	<u>0 → 1</u>
	10 (dense bodies, if regular)	0 → 1
	11 (mitochondrial cristae)	<u>0 → 1</u>
	12 (nuclear shoulders)	<u>0 → 1</u>
	14 (endonuclear canal)	<u>0 → 1</u>
	17 (fibrous sheath)	<u>0 → 1</u>
B (Scleroglossa)	6 (midpiece)	0 → 1
C (Autarchoglossa +	1 (acrosome in transverse section)	<u>0 → 1</u>
Amphisbaenidae)	8 (mitochondria shape)	<u>2 → 1</u>
D	6 (midpiece)	1 → 0
E	7 (mitochondria in transverse section)	0 → 1
	9 (dense bodies)	<u>1 → 3</u>
	10 (dense bodies, if regular)	1 → 0
	16 (multilaminar membranes)	0 → 1
F (Varanidae)	2 (perforatorial base plate)	<u>0 → 1</u>
	10 (dense bodies, if regular)	<u>0 → 1</u>

G (Serpentes)	5 (epinuclear lucent zone)	2 → 1
	6 (midpiece)	0 → 1
H (Gekkota)	2 (perforatorial base plate)	0 → 2
I (Lacertidae)	5 (epinuclear lucent zone)	2 → 1
	9 (dense bodies)	1 → 5
	10 (dense bodies, if regular)	1 → 0
J (Gymnophthalmidae)	1 (acrosome in transverse section)	0 → 1
	5 (epinuclear lucent zone)	2 → 0
	7 (mitochondria in transverse)	0 → 2
	8 (mitochondria shape)	1 → 4
K (Gekkonidae)	7 (mitochondria in transverse)	1 → 0
	8 (mitochondria shape)	2 → 1
	9 (dense bodies)	3 → 4
	10 (dense bodies, if regular)	2 → 1
	16 (multilaminar membranes)	1 → 0
L (Pygopodidae)	3 (perforatorial tip)	0 → 1
	12 (nuclear shoulders)	1 → 2
	13 (nuclear shape)	0 → 1
M (Chamaeleonidae)	1 (acrosome in transverse section)	0 → 1

Figure Captions

Figure 1. Diagram of the spermatozoon of *Amphisbaena alba.* Scale bar 0.5μm.

Figure 2. *Amphisbaena alba.* (A) Light micrograph showing whole spermatozoon with head and tail (midpiece and flagellum). (B-H) Transmission electron micrographs of the head (acrosome complex and nucleus). Longitudinal section (B) and corresponding transverse section (C) of the acrosome complex and anterior nuclear region. Apical end of the acrosome in longitudinal section at perforatorium level (D) and in transverse section at epinuclear lucent zone level (E), showing the perforatorium. (F) Detail of the anterior region of nuclear point, showing the epinuclear lucent zone and the electron-lucent space (*) between the acrosome vesicle and the subacrosomal cone. (G) Detail of the posterior region of the nucleus with nuclear shoulders (right arrowhead) and membranous flange (left arrowhead). (H) Transverse section through the nucleus showing lacunae. Fig 2A: scale bar 10μm; Figs 2B-H: scale bar 0.2μm. *a,* acrosome vesicle; *et,* epinuclear lucent zone; *f,* flagellum; *h,* head; *l,* nuclear lacuna; *mp,* midpiece; *n,* nucleus; *p,* perforatorium; *sc,* subacrosomal cone.

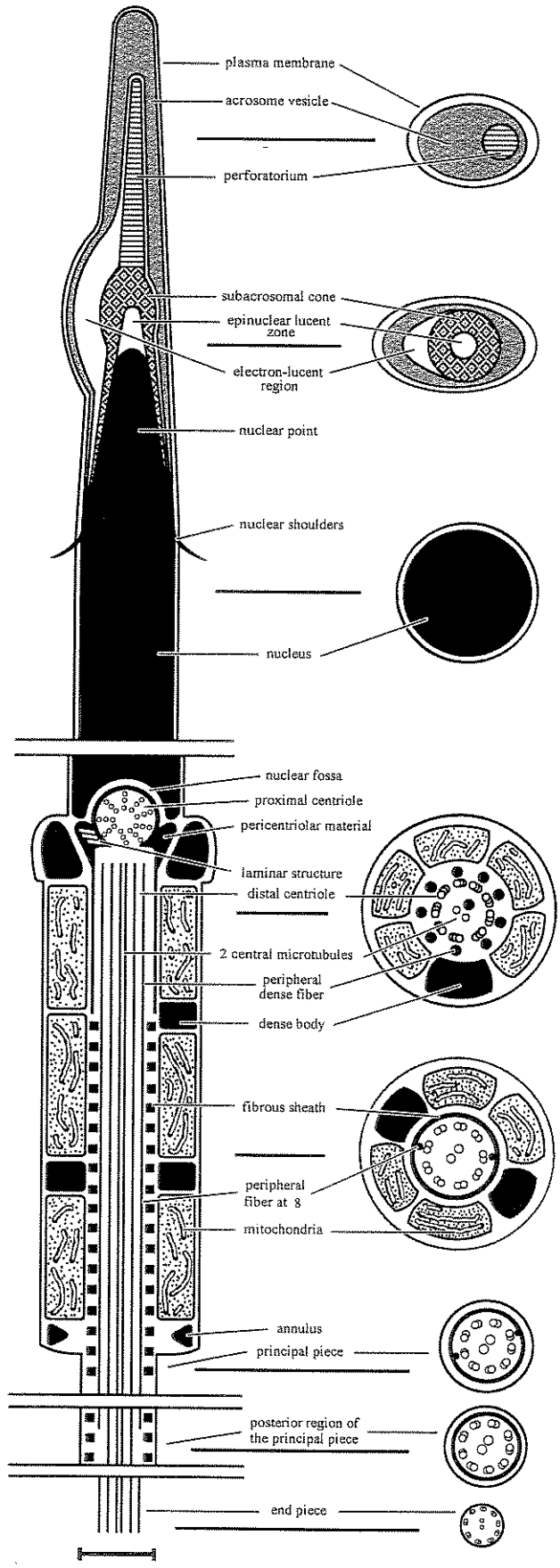
Figure 3. Transmission electron micrographs of the sperm tail of *Amphisbaena alba.* (A) Longitudinal section of the posterior end of the nucleus region and midpiece. (B-C) Detail of the neck region showing the pericentriolar material and the laminar structure, respectively. (D-E) Transverse section through the midpiece at, respectively, the distal centriole and axoneme levels. Note the central singlets within the distal centriole, the fibrous sheath surrounding the axoneme and its peripheral fibers 3 and 8 (arrow heads) thicker than the others and detached from their

doublets. (F) Longitudinal section of the midpiece showing the transition between the distal centriole and the axoneme. (G) Transition region between the midpiece and the principal piece. Note the closely association between plasma membrane and axoneme immediately behind the annulus. (H-I) Transverse section through the anterior and posterior regions, respectively, of the principal piece. Note that the posterior region of the principal piece lacks peripheral fibers. Figs 3A-E and H-I: scale bar 0.2μm; Figs 3F-G: scale bar 0.5μm. *an*, annulus; *ax*, axoneme; *db*, dense bodies; *dc*, distal centriole; *fs*, fibrous sheath; *ls*, laminar structure; *m*, mitochondria; *n*, nucleus; *nf*, nuclear fossa; *pc*, proximal centriole; *pf*, peripheral dense fiber; *pm*, pericentriolar material.

Figure 4. (A) Strict consensus tree of 2414 equally parsimonious trees, derived from spermatozoa ultrastructure data listed in Tables 1 and 2. (B) 50% majority-rule consensus tree of the 2414 most-parsimonious trees. Numbers indicate the percentage of occurrence of clades among the equally parsimonious trees. Character-stage changes in each labelled branch are listed in Table 3.

Figure 5. Phylogenetic relationships among the Squamata based on Estes et al. (1988) and Frost and Etheridge (1989), herein used as a constraint tree. Only taxa used in this study are included and Boidae, Colubridae, and Elapidae are grouped as Serpentes.

Figure 6. Cladogram depicting phylogenetic relationships among Squamata, Chelonia and Sphenodontidae.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electron-lucent region
nuclear point
nuclear shoulders
nucleus
nuclear fossa
proximal centriole
pericentriolar material
laminar structure
distal centriole
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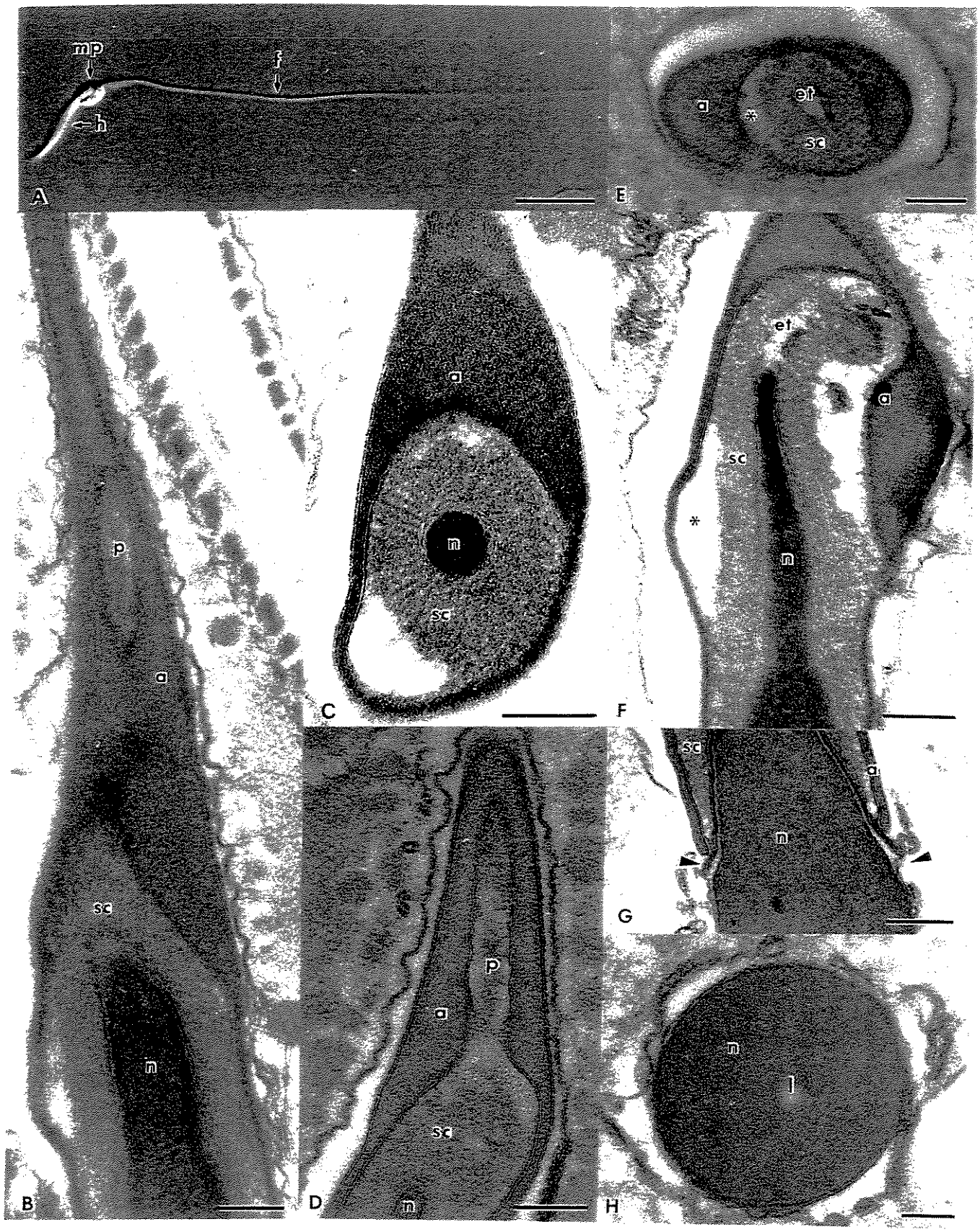
peripheral dense fiber
dense body
fibrous sheath
peripheral fiber at 8
mitochondria
annulus
principal piece
posterior region of the principal piece
end piece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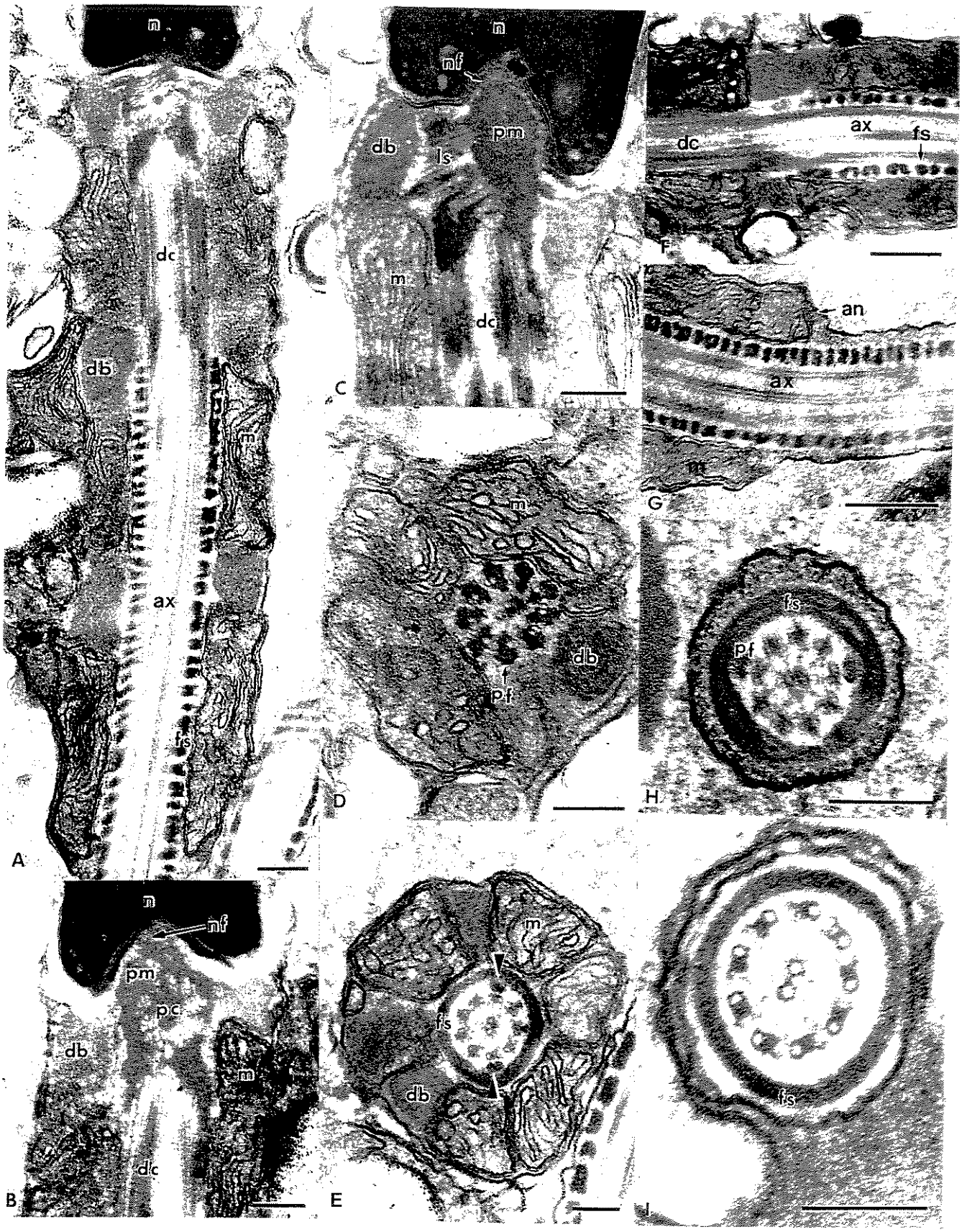
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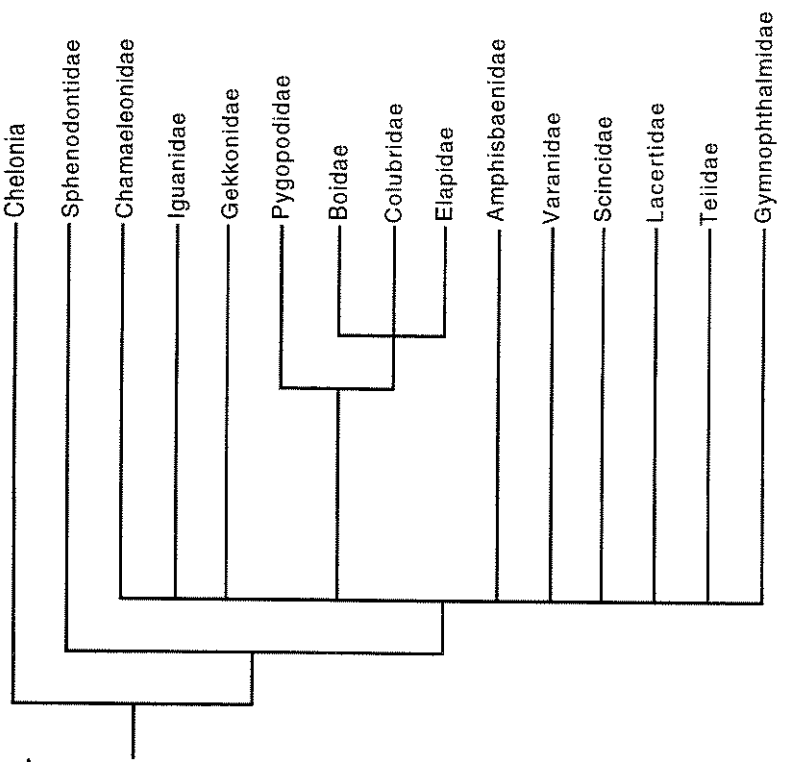
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Scincidae
Lacertidae
Teiidae
Gymnophthalmidae

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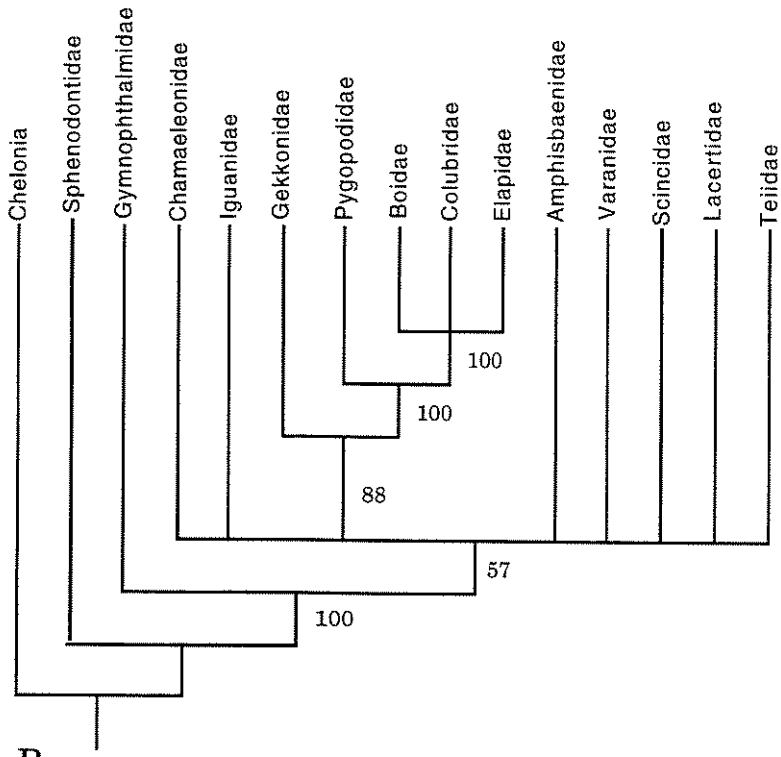
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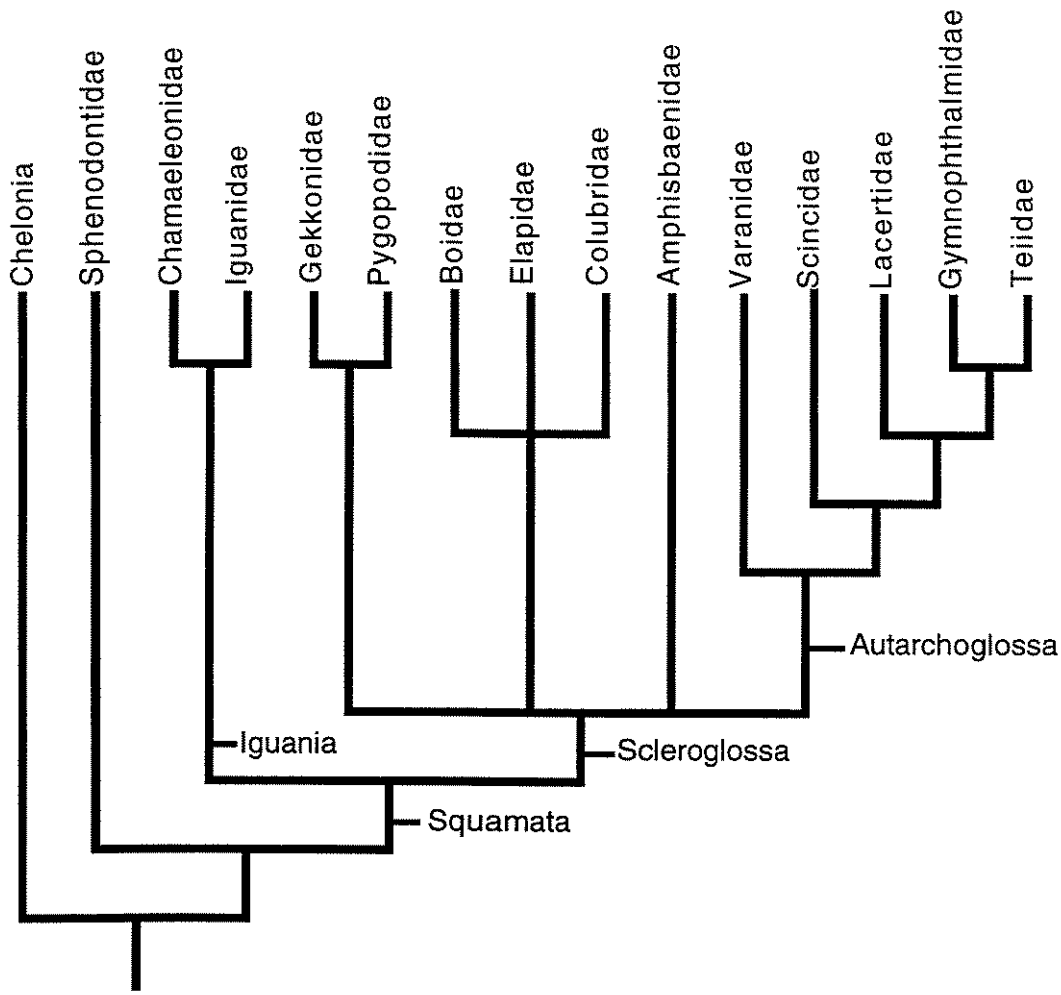
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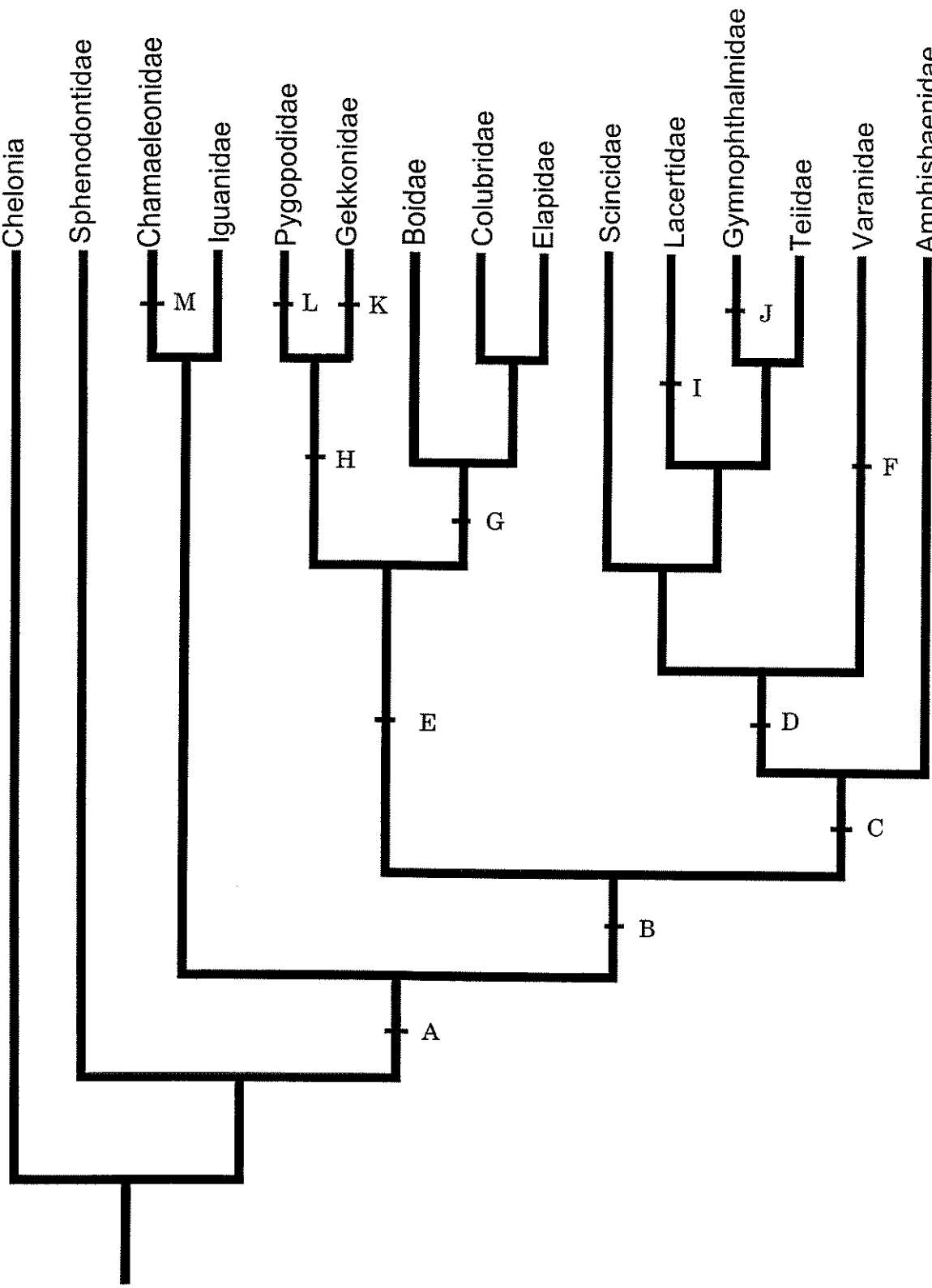
Gymnophthalmidae
Teiidae
Autarchoglossa
Iguania
Scleroglossa
Squamata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Gymnophthalmidae
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3. **Teixeira, R. D.**, Colli, G. R. & Báo, S. N. Ultrastructural study of spermatozoon of the lizard *Polychrus acutirostris* (Squamata, Polychrotidae). *J. Submicrosc. Cytol. Pathol.* 31(3), 1999.

Journal of
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Cytology
and
Pathology

Siena, may 21th 1999

Dr. Sônia Nair Báo
Departamento de Biologia Celular
Instituto de Biologia
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Brasile

Dear Dr. Báo,

we are pleased to inform you that your manuscript entitled "The ultrastructure of spermatozoa of the lizard *Polychrus acutirostris* (Squamata, Polychrotidae)", revised by the Editorial Board, has been accepted for publication in the Journal of Submicroscopic Cytology and Pathology in its present version.
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Sincerely

Laura Neri

The ultrastructure of spermatozoa of the lizard *Polychrus acutirostris* (Squamata, Polychrotidae)

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Running Head: Spermatozoa of *Polychrus acutirostris*

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SUMMARY – Epididymal spermatozoa of the lizard *Polychrus acutirostris* are similar to most vertebrate spermatozoa by possessing: a typical head, composed of acrosome and nucleus; a midpiece, wrapped with a set of mitochondria; and a flagellum, with the axoneme wrapped by a fibrous sheath, that terminates as an exposed axonemal tail piece. The spermatozoon is filiform; the acrosome vesicle is in the form of a hollow, that basally overlies the subacrosomal cone, which invests the tapered anterior end of the nucleus; the perforatorium is a slender rod extending anteriorly from the subacrosomal material with a subspheroidal base plate; the nucleus presents two tubular lacunae; the midpiece contains dense bodies and mitochondria and terminates with an annulus; the fibrous sheath extends anteriorly into the midpiece; fibers 3 and 8 are enlarged; and the endpiece lacks coarse fibers and fibrous sheath. Other features of the ultrastructure of *P. acutirostris* include: a spatulate, flattened acrosome complex; the subacrosomal cone is filled by an unevenly arranged matrix; a well developed epinuclear lucent zone; a layer of granular material between the condensed chromatin and the nuclear membrane; a spiral mitochondria arrangement; and granular sporadic dense bodies.

INTRODUCTION

Recently, the spermiogenesis and sperm ultrastructure of reptiles have received considerable attention (reviewed in Jamieson, 1995*b*; Jamieson *et al.*, 1996; Oliver *et al.*, 1996). On the one hand, these studies have furthered our understanding on organelles responsible for generating force for sperm motility and on adaptations responsible for fertilization and egg activation (*e.g.*, Newton and Trauth, 1992). On the other, they provided preliminary assessments of character-state transformations during the evolution of the spermatozoon, from the ancestral aquatic stage, to the derived condition found in higher terrestrial vertebrates (*e.g.*, Baccetti and Afzelius, 1976; Baccetti, 1979). Further, detailed studies on the sperm ultrastructure of reptiles have provided an alternate source of characters for phylogenetic analyses (Jamieson, 1995*a,b*; Teixeira *et al.*, 1999). Nevertheless, a number of reptile families still await adequate description.

The Iguania comprise nine lizard families whose phylogenetic relationships are poorly understood (Frost and Etheridge, 1989; Maddison and Maddison, 1996). Five families of iguanian lizards have been studied regarding the ultrastructure of spermatozoa. Among the Chamaeleonidae, mature spermatozoa of *Bradypodion karroicum* (Jamieson, 1995*b*) and *Pogona barbata* (Oliver *et al.*, 1996) have been described in detail, while accounts on the spermiogenesis are available for *Agama stellio* (Al-Hajj *et al.*, 1987), *A. adramitana* (Dehlawi *et al.*, 1992), and *Uromastyx philbyi* (Dehlawi and Ismail, 1990). A single study on the family Iguanidae deals with the spermiogenesis of *Iguana iguana*, misidentified as *I. delicatissima* by Saita *et al.* (1988). Very few details on the spermiogenesis of *Phrynosoma coronatum*, a member of the Phrynosomatidae, are described by Clark (1967). In the Polychrotidae, the mature spermatozoon has been described for *Pristidactylus* (=*Cupriguanus*) *scapulatus* (Furieri, 1974) and the spermiogenesis has been described for *Anolis carolinensis* (Clark, 1967). Among the

Tropiduridae, the mature spermatozoon of *Liolaemus austromendocinus* and *Phymaturus flagellifer* (=*palluma*) has been described (Furieri, 1974), while the spermiogenesis has been studied in *Tropidurus* cf. *torquatus* (Cruz-Landim and Cruz-Höfling, 1977; Cruz-Höfling and Cruz-Landim, 1978). Herein we describe in detail the sperm ultrastructure of the lizard *Polychrus acutirostris*, a member of the Polychrotidae family of iguanian lizards (Frost and Etheridge, 1989), and make comparisons with data obtained from the literature for other iguanians.

MATERIALS AND METHODS

We obtained mature spermatozoa from an adult specimen of *Polychrus acutirostris*, collected at Brasília, Distrito Federal, Brazil in February 1997. We killed the lizard with Tiopental®, removed the epididymides by dissection, placed them in a Petri dish with phosphate buffered saline (PBS) pH 7.2, and cut them into small pieces. We fixed spermatozoa and epididymal tissues overnight at 4 ^{0}C in a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, and 3% sucrose in 0.1 M sodium cacodylate buffer pH 7.2. Subsequently, we washed the specimens in 0.1 M sodium cacodylate buffer pH 7.2 and postfixed them for 1 h in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. We dehydrated the specimens in a series of ascending acetone (30% - 100%) and embedded them in Spurr's epoxy resin. We stained ultrathin setions with uranyl acetate and lead citrate, and observed them in a Jeol® 100C transmission electron microscope.

RESULTS

The spermatozoon of *Polychrus acutirostris* is filiform, consisting of a head (acrosome complex and nucleus), a midpiece, and a tail region (Fig. 1). Its dimensions were estimated from transmission electron microscopy as: length of the acrosome complex- 7 μm; length of the perforatorium- 1.8 μm; length of the tapered anterior portion of the nucleus- 2.3 μm; length of the midpiece- 7.5 μm. The head, and often the midpiece and flagellum, appear curved. As a result of this curvature, it was not possible to obtain a complete longitudinal section through the head.

Acrosome complex

The acrosome complex, which is located in the anteriormost region of the head, is composed of an external, elongate, and conical acrosome vesicle, an underlying subacrosomal cone, and a slender rod within the acrosome vesicle, the perforatorium (Fig. 2). In longitudinal section, the acrosome complex is long and appears flattened (Fig. 3). In cross section, the acrosome complex appears depressed (Figs. 4 to 6), becoming more circular at its base (Fig. 7). The acrosome vesicle has a homogeneous and moderately electron-dense matrix. Its anterior region extends beyond the tip of the nucleus, forming a thick-walled hollow cone that covers the perforatorium and continues posteriorly in a very thin layer, covering the subacrosomal material (Figs. 2 and 3). The perforatorium is a slender rod with a pointed tip (Figs. 2, 4, and 8). It is filled with a material less electron-dense than the other components of the acrosome complex, and contacts the subacrosomal material at its posterior end. The underlying subacrosomal cone (Fig. 2) caps the anterior end of the nucleus and is filled with a unevenly arranged matrix (Figs. 3, 8, 9, and 12). However, in cross section, this matrix has a slightly radial arrangement around the nuclear tip and the perforatorium (Fig. 5, 6, and 7). The matrix does not fill the entire

subacrosomal space and irregularly spaced gaps were observed next to the inner membrane of the subacrosomal cone (Fig. 9). A densification is present within the apical portion of the subacrosomal cone, that we regard as a subspheroid base plate (Figs. 8 and 9). The base of the acrosome complex rests on a widening region of the nucleus with a distinct shoulder-like shape, lying on a postero-lateral membranous flange of the subacrosomal cone (Fig. 10).

Nucleus

The nucleus is a curved and elongate cylinder that appears circular in cross section (Fig. 11). The anterior tapered portion of the nucleus is ensheathed by the posterior portion of the acrosome complex (Fig. 2). A narrow and elongate epinuclear lucent zone extends anteriorly, from the tip of the nucleus, into the subacrosomal cone (Figs. 5 and 9). The nuclear contents are strongly electron-dense, with two well-developed electron-lucent lacunae interspersed throughout it (Fig. 11), and are separated from the nuclear membrane by an overlying granular material, specially at the anterior region (Fig. 12). The nuclear shoulders are moderately developed and round-shaped (Fig. 10). The base of the nucleus is marked by a concave depression, the nuclear fossa, that surrounds the anterior half of the proximal centriole and enfolds the dense pericentriolar material (Fig. 13).

Neck Region

The neck region (Fig. 14) articulates the anterior extremity of the flagellum with the sperm head. It includes the proximal and distal centrioles and the first ring of mitochondria. The proximal centriole is short and parallels the base of the nucleus. The distal centriole forms the basal body of the axoneme. It is perpendicular to the proximal centriole, occupying a small

fraction of the midpiece, and does not project into the fibrous sheath. An extensive deposit of pericentriolar material extends from the nuclear fossa to cover the proximal centriole, posteriorly contacting the anterior portion of the distal centriole. In this region, the distal centriole presents a central dense structure (Fig. 15). The pericentriolar material provides a base for the nine large dense peripheral fibers (coarse fibers), which contact the distal centriole posteriorly (Fig. 14). In this region, the distal centriole is penetrated by a pair of central microtubules and a central dense structure connects triplet 3 with the central singlet (Fig. 16).

Midpiece

The midpiece consists of the neck region and the axoneme, surrounded by the fibrous sheath, mitochondria, and dense bodies (Fig. 17). It is relatively short and terminates posteriorly at a distinct annulus.

The axoneme has the usual 9+2 pattern and the large, dense peripheral fibers are applied to the outer surface of each of the nine microtubule doublets. In the anterior portion of the axoneme, peripheral fibers appear double and detached from doublets 3 and 8 (Fig. 18). More posteriorly along the axoneme, the dense peripheral fibers are reduced in diameter, but the coarse fibers at 3 and 8 remain prominent, separated from their corresponding doublets, and fused with the fibrous sheath (Fig. 19).

In longitudinal section, mitochondria appear as elongate sinuous tubules, forming a single layer around the axoneme, arranged in spiral fashion (Fig. 20). In transverse section mitochondria are irregularly shaped, with linear or slightly curved cristae, and are arranged in a circle around the fibrous sheath (Figs. 18, 19). Intermitochondrial dense bodies appear as

granular masses, sporadically interrupting the mitochondria in longitudinal and transverse sections, and apparently not forming complete rings (Figs. 18 and 19).

The fibrous sheath is an annulated structure, formed of regular rings that start just behind the base of the distal centriole and extend into the midpiece, surrounding the axoneme and the coarse fibers (Fig. 17). The annulus is an electron-dense ring at the end of midpiece, clearly marking the beginning of the principal piece (Fig. 21 and 22). In cross section, the fibrous sheath still shows the coarse fibers at 3 and 8 connected to it (Fig. 22).

Principal piece

The initial portion of the principal piece can be identified by a reduction in the diameter of the spermatozoon (Fig. 21). The fibrous sheath extends into the principal piece, surrounding the axoneme. Anteriorly, a wide ring with granular inclusions of cytoplasm separates the fibrous sheath from the plasma membrane, but posteriorly the plasma membrane approaches the fibrous sheath more closely. All nine peripheral fibers are absent in the principal piece (Fig. 23).

End piece

The end piece is referred to the very slender tail of the spermatozoon, posterior to the termination of the fibrous sheath and coarse fibers. It consists of the axoneme and the plasma membrane, with an undetermined length. The pattern of microtubules is maintained, although their diameter is very reduced (Fig. 24).

DISCUSSION

In *Polychrus acutirostris*, the acrosome complex forms a tripartite pattern (acrosome vesicle, subacrosomal cone and the constricted nuclear tip), a plesiomorphic condition of tetrapods (Jamieson, 1995*a*). The following features, regarded a plesiomorphic in amniotes (Jamieson, 1995*a*), are also present in *P. acutirostris*: nucleus elongate; distal centriole extending through midpiece, penetrated by two central singlets from the axoneme; several mitochondria in cross section of midpiece; annulus present; nine peripheral dense fibers associated with the nine doublets of the axoneme; peripheral fibers adjacent to doublets 3 and 8 enlarged, forming a double structure detached from their respective doublets. *Polychrus acutirostris* has a number of character-states considered synapomorphies of Squamata (Jamieson, 1995*b*): perforatorium single, wholly prenuclear; endonuclear canal absent; epinuclear lucent zone present; mitochondrial cristae linear; intermitochondrial dense bodies present; fibrous sheath extending into midpiece; and nuclear shoulders rounded.

The acrosome is depressed in cross section in *Polychrus acutirostris*, as in the chamaeleonids *Agama agama* (Charnier *et al.*, 1967), *A. adramitana* (Dehlawi *et al.*, 1992), *A. stellio* (Al-Hajj *et al.*, 1987), *Bradypodium karroicum* (Jamieson, 1995*b*) and *Pogona barbata* (Oliver *et al.*, 1996). On the contrary, the acrosome seems to be circular in cross section in the tropidurids *Liolaemus austromendocinus*, *Phymaturus flagellifer* (=*palluma*) (Furieri, 1974), and *Tropiduridus* cf. *torquatus* (Cruz-Landim and Cruz-Höfling, 1977; Cruz-Höfling and Cruz-Landim, 1978), but unambiguous sections of the anterior region of the acrosome are not presented in these works. Likewise, the acrosome appears to be circular in cross section in the polychrotid *Pristidactylus* (=*Cupriguanus*) *scapulatus* (Furieri, 1974), suggesting that the shape of the acrosome in cross section might be variable within Polychrotidae and Iguania. Nevertheless, as put above, unambiguous sections across the terminal portion of the acrosome

were not presented by Furieri (1974). His sections of *P. scapulatus* cut through the basal third of the acrosome and, in species with a depressed acrosome, it becomes more circular as one moves from the tip to the basal part of the acrosome complex. Therefore, more detailed studies are necessary to ascertain the degree of variability of this character.

In *Polychrus acutirostris*, the acrosome vesicle is a homogeneously electron-dense structure, as in the tropidurids *Liolaemus austromendocinus*, *Phymaturus flagellifer* (=*palluma*) (Furieri, 1974), and *Tropiduridus* cf. *torquatus* (Cruz-Landim and Cruz-Höfling, 1977; Cruz-Höfling and Cruz-Landim, 1978) and the polychrotid *Pristidactylus* (=*Cupriguanus*) *scapulatus* (Furieri, 1974). In chamaeleonids, however, the acrosome can be divided into a narrow, electron-lucent cortex and a wide, electron-dense medulla (Al-Hajj *et al.*, 1987; Dehlawi *et al.*, 1992; Jamieson, 1995*b*; Oliver *et al.*, 1996).

A paracrystalline substructure of the subacrosomal cone was regarded as a likely synapomorphy of the Squamata (Jamieson and Scheltinga, 1993). In *Polychrus acutirostris*, although the subacrosomal cone does not present a paracrystalline substructure in longitudinal section, its matrix has a slightly radial arrangement around the nuclear tip and the perforatorium, in transverse section.

Jamieson (1995*b*) regarded the perforatorial base plate as absent in *Bradypodion karroicum* (Chamaeleonidae) and the "iguanids" studied by Furieri (1974), but questionably present in *Pogona barbata* (Chamaeleonidae). In this latter species, the presumed base plate is poorly defined, appearing as a slightly widened basal modification of the perforatorium, arguably regarded as ovoid, and is not embedded in the subacrosomal material (Oliver *et al.*, 1996). Other studies on iguanians are not enlightening regarding the presence of the perforatorial base plate. The perforatorial base plate of *Polychrus acutirostris* is subspheroidal, appearing as a

densification in the apex of the subacrosomal cone. A similar condition is also present in *Pristidactylus* (=*Cupriguanus*) *scapulatus* (Polychrotidae) (see fig. 6 in Furieri, 1974), *Phymaturus flagellifer* (=*palluma*) (Tropiduridae) (see fig. 8 in Furieri, 1974), *Heteronotia binoei* (Gekkonidae) (Jamieson *et al.*, 1996) and in the snakes *Aspidites melanocephalus* (Boidae), *Boiga irregularis* and *Stegonotus cucullatus* (Colubridae), and *Oxyuranus microlepidotus* (Elapidae) (Oliver *et al.*, 1996). This densification has been alternatively regarded as a perforatorial base plate in Gekkonidae, but not in Serpentes by Jamieson (1995*b*). We advance that the apical densification within the subacrosomal cone should be regarded as a base plate.

The epinuclear lucent zone is clearly defined and well developed in *Polychrus acutirostris*, as in the chamaeleonids *Bradypodion karroicum* (Jamieson, 1995*b*) and *Pogona barbata* (Oliver *et al.*, 1996), and *Iguana iguana* (Saita *et al.*, 1988). Jamieson (1995*b*) considered the epinuclear lucent zone as present in the "iguanids" studied by Furieri (1974), but we regard Furieri's account inconclusive since no unequivocal evidence was presented. Other studies on iguanians are also not enlightening regarding the presence of the epinuclear lucent zone.

Two unusual features are present in the nuclear region of mature spermatozoa in *Polychrus acutirostris*. First, the nuclear contents do not fill the entire nuclear space, the condensed chromatin being separated from the nuclear membrane by a finely granular, evenly dispersed material. This material appears to be the nucleoplasm in a granular state, a condition observed in intermediate spermatids of several lizard species (*e.g.*, Clark, 1967), or, alternatively, an electron-lucent space formed by the condensation of the chromatin and the elongation of the nucleus. Second, the nucleus in *P. acutirostris* contains two large, central lacunae. Electron

lucent areas within the nucleus are not considered functionally significant, but to have arisen as an accident during chromatin condensation (Jones and Butler, 1988). However, in *P. acutirostris* these lacunae are well developed and elongated, resembling reminiscent endonuclear canals. This feature has not been observed in any other squamate.

Mature spermatozoa of *Polychrus acutirostris* have rounded nuclear shoulders, at the base of the tapered nuclear tip, supporting the posterior end of the acrosome. The same condition is observed in the chamaeleonids *Bradypodion karroicum* (Jamieson, 1995*b*) and *Pogona barbata* (Oliver *et al.*, 1996).

The midpiece in *P. acutirostris* sperm is relatively short, as in the polychrotid *Pristidactylus* (=*Cupriguanus*) *scapulatus* (Furieri, 1974), the tropidurids *Tropidurus torquatus* (Cruz-Landim and Cruz-Höfling, 1977; Cruz-Höfling and Cruz-Landim, 1978), *Liolaemus austromendocinus* and *Phymaturus flagellifer* (=*palluma*) (Furieri, 1974), the chamaeleonid *Pogona barbata* (Oliver *et al.*, 1996). The mitochondria are elongate sinuous tubules spirally arranged around the fibrous sheath. Small dense bodies sporadically appear between mitochondria, and form regular rings that are not continuous in transverse sections. This arrangement closely resembles that of other iguanians, except that in *P. acutirostris* the dense bodies are granular, whereas in other iguanians they form solid, condensed structures (Furieri, 1974; Jamieson, 1995*b*; Oliver *et al.*, 1996).

As usual in squamates, the nine peripheral fibers are associated with the nine doublets of the axoneme, each coarse fiber adjacent to double 3 and 8 is enlarged, closely associated with the fibrous sheath, and only these fibers are closely recognizable at the annulus (Oliver *et al.*, 1996). The relationship of the dense fibers and fibrous sheath to the 9+2 tubule pattern of the flagellum

may be a specific consequence of its mode of formation and \ or an important prerequisite for successful fertilization. (Phillips, 1970).

Detailed studies of the sperm ultrastructure in squamates have revealed a multitude of characters that can profitably be used in phylogenetic analyses where other datasets are uninformative (Teixeira *et al.*, 1999). Future works describing the sperm ultrastructure of members of the families Corytophanidae, Crotaphytidae, Hoplocercidae, Iguanidae, Opluridae, and Phrynosomatidae (Frost and Etheridge, 1989) are warranted and may cast light on the phylogenetic relationships of iguanians.

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FIGURE LEGENDS

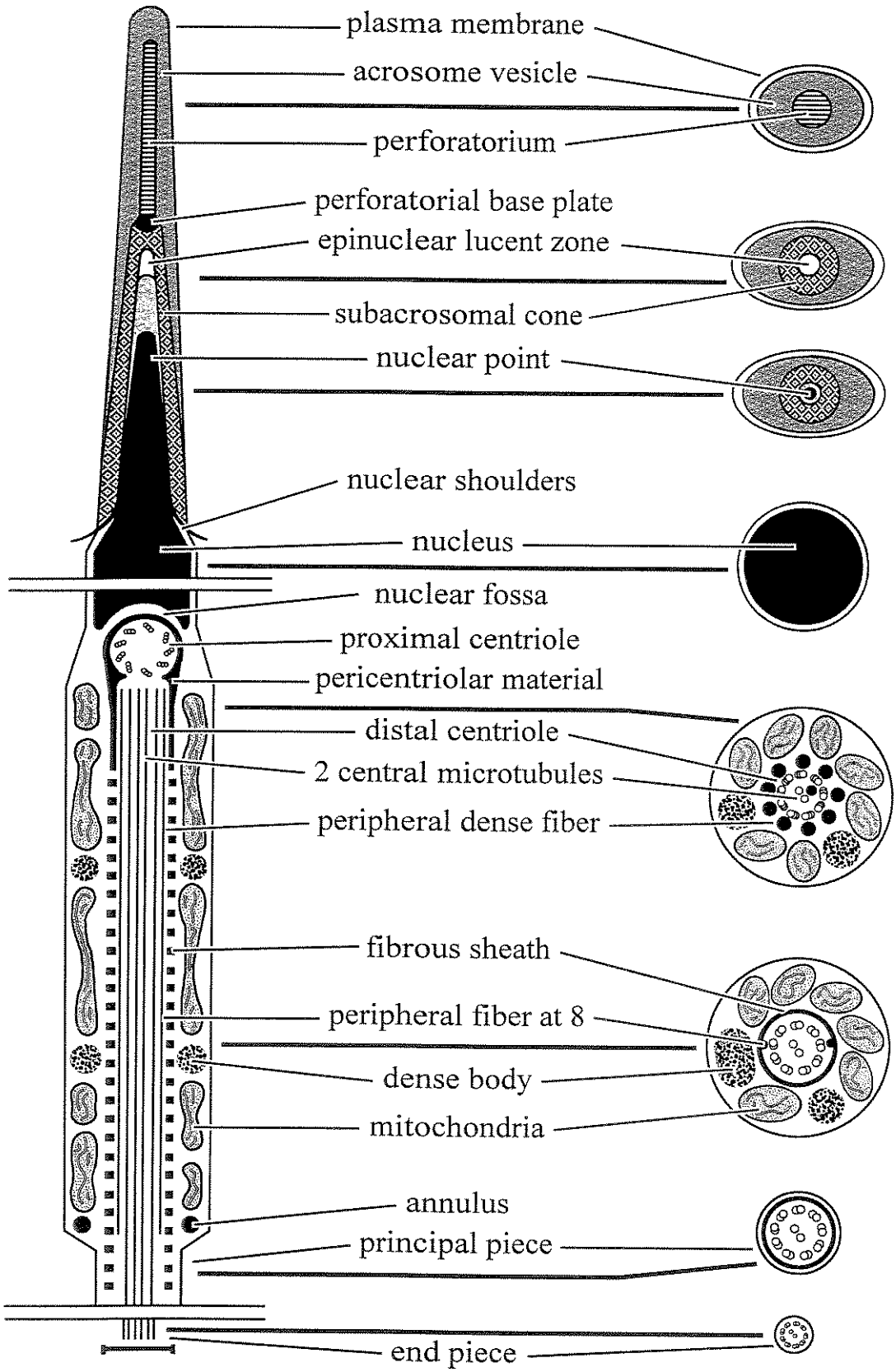
FIG. 1 *Polychrus acutirostris*. Diagram of the spermatozoon, and corresponding transverse section. Scales of various components are only approximate. Scale bar 0.5 μm.

FIG. 2 Longitudinal section of the acrosome complex and the anterior region of nucleus. FIG. 3 Anterior region of the acrosome complex. Note the flattening acrosome. FIG. 4 Transverse section of the acrosome complex at the perforatorium level. FIG. 5 Transverse section of the acrosome complex at the epinuclear lucent zone level. FIG. 6 Transverse section of the acrosome complex at the nuclear point level. FIG. 7 Transverse section of the posterior region of the acrosome complex. FIG. 8 Detail of the acrosome complex showing the perforatorium. Note the subspheroidal base plate (*) of the perforatorium at the posterior region of the subacrosomal cone. a: acrosome vesicle; et: epinuclear lucent zone; n: nucleus; p: perforatorium; sc: subacrosomal cone. Figs. 2 and 3: scale bar 0.5 μm; Figs. 4 to 8: scale bar 0.2 μm.

FIG. 9 Longitudinal section through the anterior region of the nucleus showing the epinuclear lucent zone and the base plate (*) of the perforatorium. FIG. 10 Posterior region of the acrosome complex showing the nuclear shoulders (arrowhead). Note the postero-lateral membranous flange (fl). FIG. 11 Transverse section of the nucleus showing the two lacunae (l). FIG. 12 Anterior nuclear region in longitudinal section showing the space (*) between the condensed chromatin and the nuclear membrane, occupied by a granular material. FIG. 13 Basal region of nucleus and the nuclear fossa. FIG. 14 Longitudinal section of the neck region with pericentriolar material and centrioles. FIG. 15 Transverse section of the neck region showing the

anterior region of the distal centriole. FIG. 16 Transverse section through the posterior region of the distal centriole. Note the central singlets and the peripheral fibers (pf). a: acrosome vesicle; db: dense bodies; dc: distal centriole; et: epinuclear lucent zone; fs: fibrous sheath; m: mitochondria; n: nucleus; nf: nuclear fossa; p: perforatorium; pc: proximal centriole; pm: pericentriolar material; sc: subacrosomal cone. Figs. 9 to 13, 15 and 16: scale bar 0.2 μm; Fig. 14: scale bar 0.5 μm.

FIG. 17 Longitudinal section of the midpiece. FIG. 18 Transverse section through the anterior region of the axoneme. FIG. 19 Transverse section through the posterior region of the axoneme. Note the peripheral fibers 3 and 8 thicker than the others and detached from their doublets (arrows). FIG. 20 Longitudinal section of the midpiece showing the mitochondria spirally arranged around the fibrous sheath. FIG. 21 Transition region between the midpiece and the principal piece. FIG. 22 Transverse section through the annulus. Note the reminiscent peripheral fibers at 3 and 8 (arrows). FIG. 23 Transverse section of the principal piece. FIG. 24 Transverse section of the end piece. an: annulus; ax: axoneme; db: dense bodies; dc: distal centriole; fs: fibrous sheath; m: mitochondria; n: nucleus; nf: nuclear fossa; pc: proximal centriole; pf: peripheral fibers; pm: pericentriolar material. Scale bar 0.2 μm.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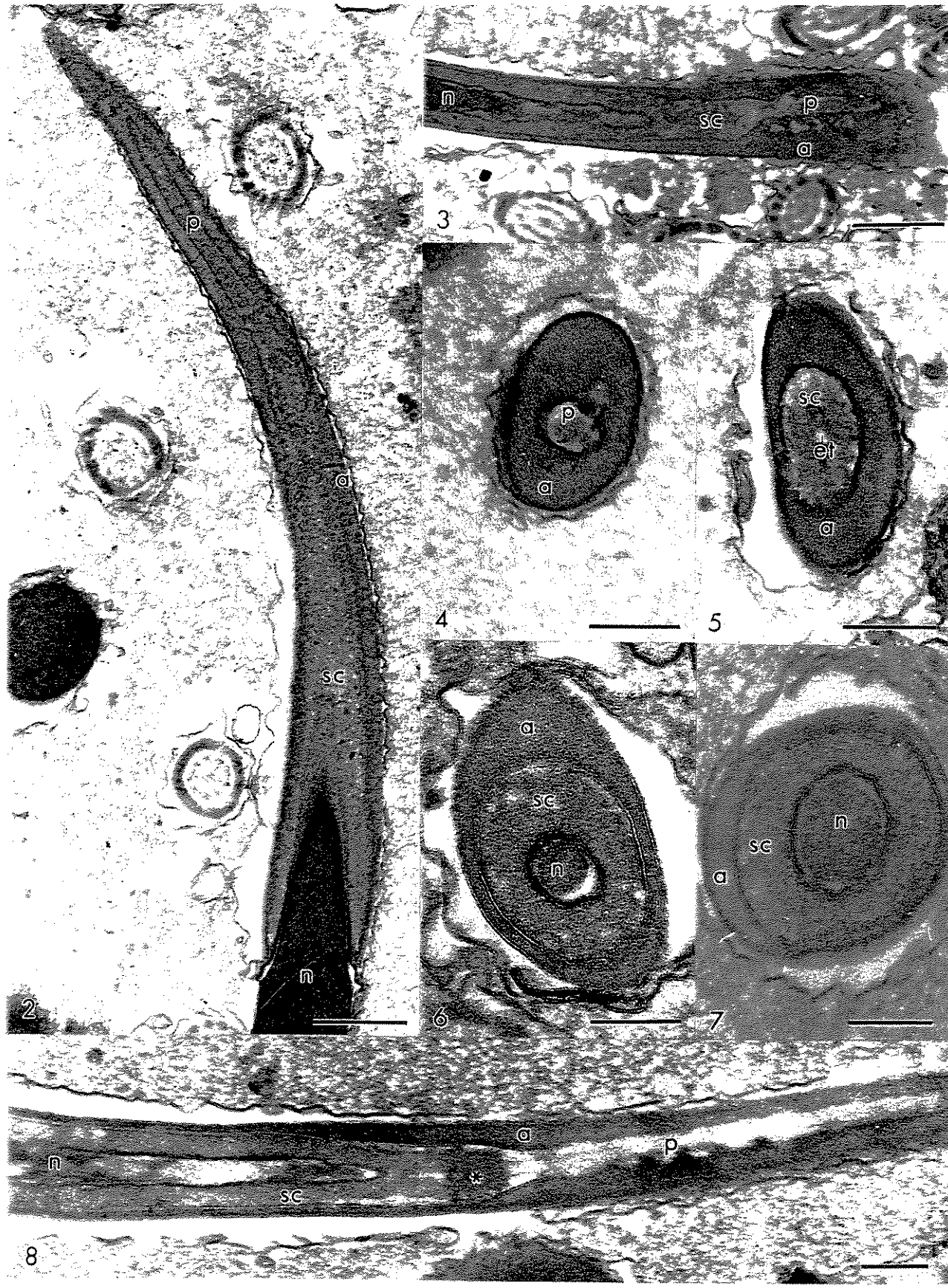
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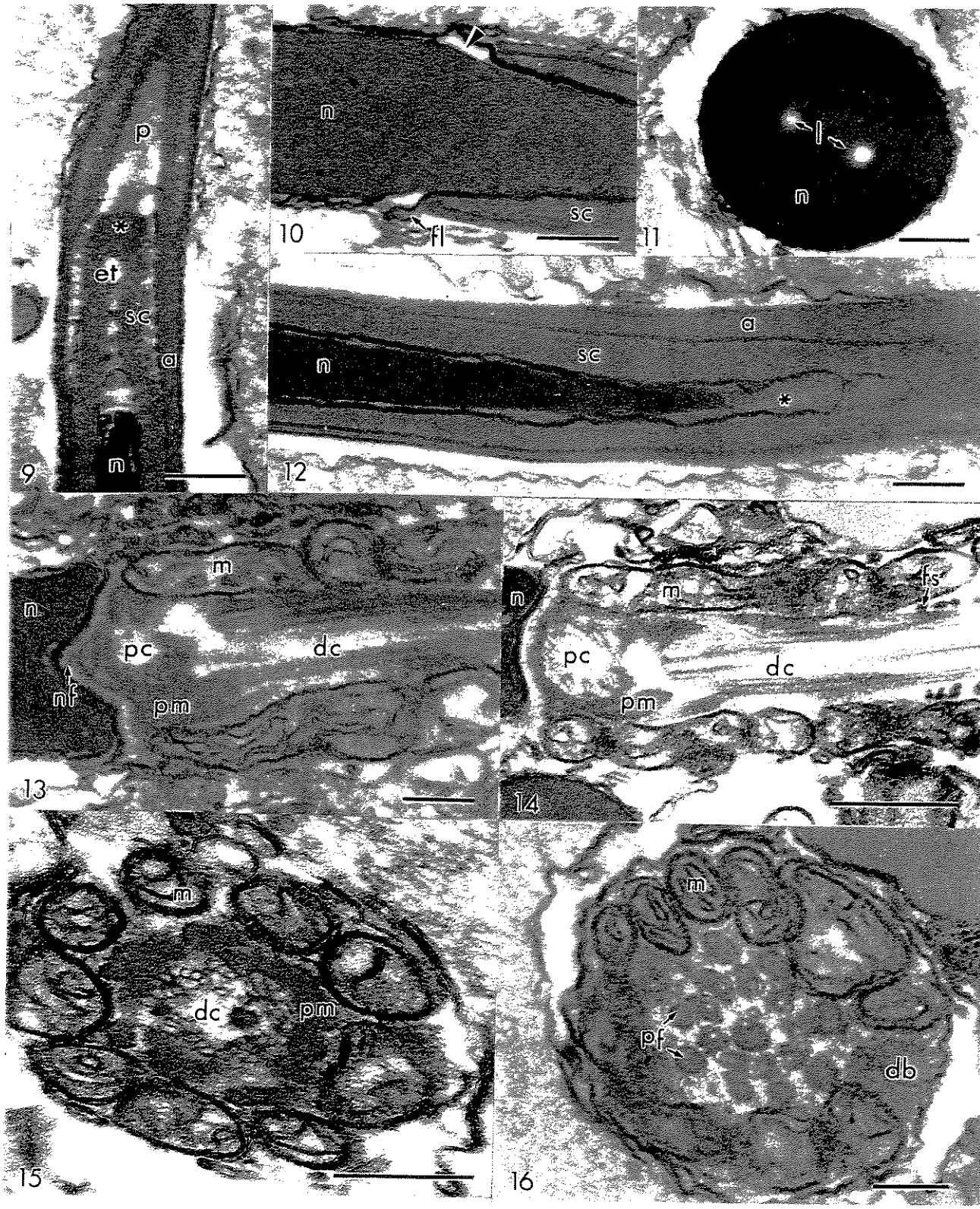
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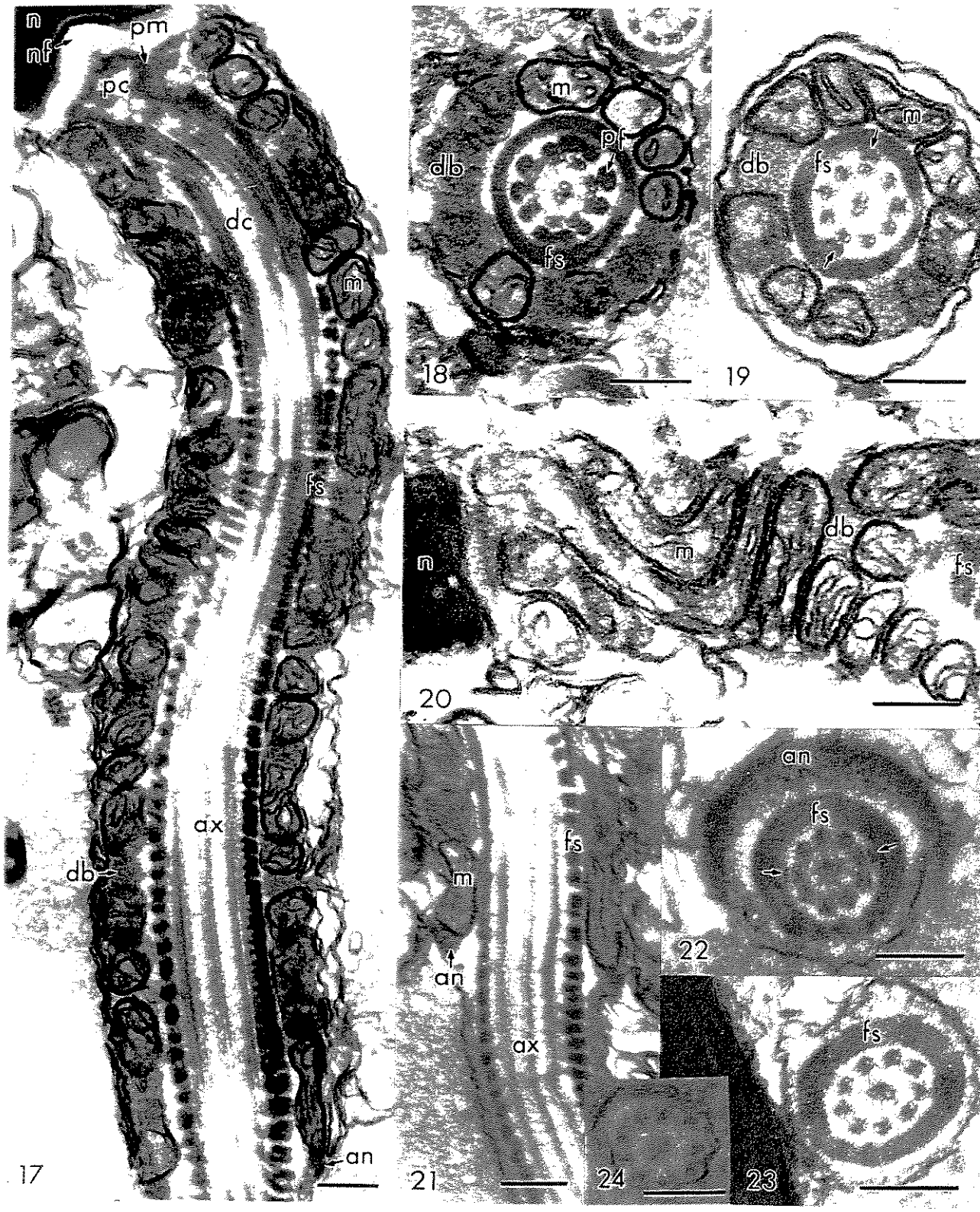
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4. **Teixeira, R. D.**, Vieira, G. H. C.; Colli, G. R. & Báo, S. N. Ultrastructural study of spermatozoa of the Brazilian Tropidurid lizards, *Tropidurus semitaeneatus* and *Tropidurus torquatus* (Squamata, Tropiduridae). *Tissue Cell.* No prelo.

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May 14,1999

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Ultrastructural study of spermatozoa of the neotropical lizards, *Tropidurus semitaeniatus* and *Tropidurus torquatus* (Squamata, Tropiduridae)

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Running title: Spermatozoa of two tropidurid lizards

Keywords: ultrastructure, spermatozoon, Reptilia, Squamata, Tropiduridae, lizard

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ABSTRACT. A detailed description of the sperm ultrastructure of *Tropidurus semitaeniatus* and *T. torquatus* is provided. Mature spermatozoa of *T. semitaeniatus* and *T. torquatus* are filiform and characterized by: apical portion of acrosome depressed; perforatorium single; epinuclear lucent zone well developed; midpiece short; mitochondria columnar; midpiece with three sets of alternating ring structures and mitochondria (rs1/m1, rs2/m2, rs3/m3); nuclear shoulders rounded; nucleus elongate; fibers 3 and 8 enlarged; and fibrous sheath in midpiece. Spermatozoa of *Tropidurus* are unusual in possessing a unilateral electron-lucent ridge at the surface of the acrosome and an epinuclear electron-lucent zone. The two species are very similar, differing in details such as: degree of acrosome flattening, presence of bulging at nuclear base, and arrangement of microtubules in the endpiece. Comparisons between Tropiduridae and other families of iguanian lizards are made.

Introduction

Studies on the ultrastructure of spermatozoa have fostered knowledge on the development and functional significance of many spermatozoal organelles (Stanley, 1971), elucidating the relationship among these components and reproduction itself (Cruz-Landim & Cruz-Höfling, 1977). Detailed accounts on the ultrastructure of spermatozoa also supply an independent source of characters for phylogenetic analyses, which are especially useful when other character sets are not enlightening (Jamieson, 1995a, 1995b; Teixeira et al., 1999). Moreover, phylogenetic studies may provide a framework for reconstructing the features of ancestral spermatozoa and the course of spermatozoal evolution, establishing the relationships between sperm morphology and systematic position.

Iguania is a monophyletic assemblage of nine families of squamate reptiles whose phylogenetics relationships are poorly understood (Frost & Etheridge, 1989). Studies on the sperm ultrastructure have been carried out in five families of iguanians: Chamaeleonidae, Iguanidae, Phrynosomatidae, Polychrotidae, and Tropiduridae. In Chamaeleonidae, the mature spermatozoon has been described in detail for *Bradypodion karrooicum* (Jamieson, 1995b) and *Pogona barbata* (Oliver et al., 1996), whereas the spermiogenesis has been examined in *Agama stellio* (Al-Hajj et al., 1987), *A. adramitana* (Dehlawi et al., 1992; Ismail et al., 1995), and *Uromastyx philbyi* (Dehlawi & Ismail, 1990; Dehlawi et al., 1990). A description of the spermiogenesis is available for *Iguana iguana* (Iguanidae), misidentified as *I. delicatissima*, by Saita et al. (1988). A few details of the spermiogenesis are known for a member of Phrynosomatidae, *Phrynosoma coronatum* (Clark, 1967). In Polychrotidae, Teixeira et al. (submitted) gave a detailed account on the ultrastructure of mature spermatozoa of *Polychrus acutirostris*, Furieri (1974) described the mature spermatozoon of *Pristidactylus (=Cupriguanus)*

scapulatus, and Clark (1967) described the spermiogenesis of *Anolis carolinensis*. Among Tropiduridae, the mature spermatozoon of *Liolaemus austromendocinus* and *Phymaturus flagellifer* (=*palluma*) have been described (Furieri, 1974), and an account on the spermiogenesis of *Tropidurus* cf. *torquatus* is available (Cruz-Landim &Cruz-Höfling, 1977; Cruz-Höfling & Cruz-Landim, 1978).

Herein we provide, for the first time, a detailed description of the ultrastructure of mature spermatozoa of Tropiduridae. We also make comparisons between tropidurids and other iguanian families. Finally, by examining two congeneric species, we attempt to ascertain the degree of intrafamilial variability in sperm ultrastructure characters, a problem that may plague phylogenetic analysis when supraspecific taxa are used, but has received little attention (Wiens, 1998).

Material and Methods

We obtained epididymal, mature spermatozoa from 2 adults specimens of *Tropidurus semitaeniatus* and 2 adult specimens of *T. torquatus*, collected at Lençóis, Bahia, Brazil and Brasília, Distrito Federal, Brazil, respectively. Specimens were deposited at the Coleção Herpetológica da Universidade de Brasília (*T. semitaeniatus* CHUNB 00571, 00572; *T. torquatus* CHUNB 09676, 10087).

We killed lizards with Tiopental®, removed the epididymides by dissection, placed them in a Petri dish with phosphate buffered saline (PBS) pH 7.2, and cut them into small pieces. We fixed epididymal tissues overnight at 4 ^{0}C in a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, and 3% sucrose in 0.1 M sodium cacodylate buffer pH 7.2. Subsequently, we washed specimens in 0.1 M sodium cacodylate buffer, pH 7.2, with 3% sucrose, and postfixed

them for 1 h in 1% osmium tetroxide, 0.8% potassium ferricyanide, and 5mM $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2. We dehydrated the material in a series of ascending acetone (30% - 100%) and embedded it in Spurr's epoxy resin. We stained ultrathin setions with uranyl acetate and lead citrate, and made observations in a Jeol® 100C transmission electron microscope.

Results

Spermatozoa of *Tropidurus semitaeniatus* and *T. torquatus* are filiform, consisting of a head region containing the nucleus and the acrosomal structures, a midpiece, and a tail region subdivided into principal piece and endpiece. A generalized spermatozoon based on both species is represented diagrammatically in Fig. 1.

Acrosome complex

In the acrosome, an external and an internal cap, the acrosome vesicle and the subacrosomal cone, respectively, are recognized (Figs. 2A-B, H; 4A-B, H). The acrosome vesicle has a homogeneous and moderately opaque matrix, bounded both outside and inside by a membrane (Figs. 2A-F, H; 4D-F, H). The acrosome vesicle is thicker in its apical half, whereas the basal half is hollowed to surround the subacrosomal cone. The apical portion of the acrosome vesicle is not completely solid, having a narrow central canal 1.2 μm long, the perforatorium (Figs. 2A, C, H; 4A-B, C). This structure is a slender rod with a pointed tip, that extends anteriorly from the subacrosomal cone. The subacrosomal cone fits into the basal half of the acrosome vesicle, being shorter and thicker than the latter, and lacks a membrane of its own (Figs. 2A-B, D-F, H; 4D-F, H). It consists of a material having an unevenly aspect, that tapers the anterior end of the nucleus. The

subacrosomal cone surface is closely associated with the inner membrane of the acrosome vesicle, except at its apical and anterolateral surface, where an electron-lucent layer separates these two caps, forming a unilateral ridge (Figs. 2B; 4H). At its base, the acrosome complex rests on a widening region of the nucleus with a distinct shoulder-like shape, lying on a postero-lateral membranous flange (Figs. 2H; 4H). The acrosome of the two species is circular at its base, develops an unilateral electron-lucent ridge anteriorly, and becomes increasingly depressed in transverse section near the apical tip (Figs. 2C-F; 4C-F). Apically, the acrosome vesicle of *T. torquatus* is more depressed than that of *T. semitaeniatus* (Figs. 4C; 2C; respectively).

Nucleus

The nucleus is elongate, slightly curved, and circular in cross-section. At its anterior extremity, it forms a point deeply wedged in the acrosome (Figs. 2H; 4H). The length of this nuclear projection, which is surrounded by the subacrosomal cone, is approximately 2.5 μm. Anterior to the nuclear tip and within the subacrosomal cone there is an elongate, narrow chamber with a membrane of its own, the epinuclear lucent-zone, filled with an electron-lucent substance (Figs. 2D, H; 4D, H). Small rounded shoulders (Figs. 2B, H; 4H) mark the transition from the tapered apical portion to the cylindrical region. The posterior pole of the nucleus is marked by a shallow conical depression, the nuclear fossa, for receiving the centriolar apparatus (Figs. 3A, H, I; 5A, I). The main body of the nucleus appear homogeneous and completely electron-dense, but in some instances there are small, electron-lucent channels penetrating it, the lacunae (Figs. 2G; 4G). Along the nuclear point the boundary between the nuclear contents and the nuclear envelope is irregular, containing evenly dispersed, fine granular material (Figs. 2B, E-F, H; 4B, E-F, H). In *T.*

semitaeniatus, the nuclear base abruptally increases in width, producing a conspicuous, rounded bulge (Fig. 3A), which is absent in *T. torquatus* (Fig. 4H; 5I).

Neck region

The neck region connects the tail with the sperm head. This region consists of two centrioles, an extensive deposit of pericentriolar material, and the first ring of dense bodies (Figs. 3A, H-I; 5A, I). The proximal centriole is positioned centrally, parallel to the base of the nucleus, and presents a central, electron-dense structure in its interior (Figs. 3H; 5A, I). The distal centriole constitutes the basal body of the flagellum. It lies perpendicular to the proximal centriole and extends caudally through approximately two-thirds of the midpiece, continuing with the axoneme. The distal centriole consists of nine triplets of microtubules. A peripheral dense fiber (coarse fiber) is associated with each of the nine triplets of the distal centriole (Figs. 3C; 5C). In both species, a pair of central microtubules extends into the transitional region between the distal centriole and the axoneme and a dense structure, presumably a fiber, appears connecting triplet 3 with one of the central microtubules. Both centrioles are enclosed within a homogeneously dense material, the pericentriolar material, that conforms in shape to the nuclear fossa. This material extends posteriorly between the two centrioles and contacts the anterior portion of the distal centriole, continuing as the dense peripheral fibers longitudinally (Figs. 3A, H; 5A, I).

Midpiece

The midpiece is about 2.8 μm long and is much shorter than the head. It consists of a single layer of mitochondria, interposed between rings of dense bodies, which encircles the anterior portion of the flagellum, including the neck region. The flagellum is formed by the axoneme, extending

throughout the remaining length of the spermatozoon. It is organized in the usual 9+2 microtubules pattern, surrounded by nine peripheral fibers. These peripheral fibers rapidly decrease in diameter along the axoneme, with the exception of fibers at the doublets 3 and 8, which are thicker than the others, double, and detached from their doublets (Figs. 3D-F; 5D-F). A fibrous sheath which surrounds the axoneme, extends into the midpiece to just posterior of the distal centriole (Figs. 3A; 5A, J). In longitudinal section it appears as regularly spaced, approximately square dense blocks connected by the longitudinal peripheral fibers at doublets 3 and 8. The mitochondria are elongate columnar structures, with longitudinal cristae, and surround the distal centriole and fibrous sheath (Figs. 3A; 5A, J). In cross section, they appear irregularly shaped, and usually 5-6 are seen around the axoneme (Figs. 3D; 5D-E). In the two species, the mitochondrial sheath is interspersed by dense bodies, which are not limited by a membrane, and are composed of a granular dense material identical to the pericentriolar material. However, they appear less electron-dense than the mitochondrial sheath (Figs. 3A, C, E, I; 5A, E, J). Dense bodies are arranged in three ring structures, separated from each other by portions of mitochondrial columns (Figs. 3A, I; 5A, J) symbolized as rs1/m1, rs2/m2, rs3/m3. The first ring of dense bodies adjoins the base of the nucleus. The second ring structure often appears only on one side in longitudinal section (Figs. 3A, I; 5A, J) and is often interrupted by mitochondria in transverse section (Figs. 3C; 5E). This suggests that this structure forms a semicircular ring. The third ring structure forms a closed ring (Figs. 3E; 5F), appearing in longitudinal section as kidney-shaped structures on each side of the fibrous sheath (Figs. 3A; 5A, J). Occasionally, mitochondria are present lateral to this ring structure (Fig. 3E). At the distal extremity of the midpiece, a small dense ring, the annulus, with an irregular, oval cross section, defines the terminus of the midpiece (Figs. 3A-B; 5A-B, J).

Principal piece

The principal piece constitutes the tail of the spermatozoon. It extends posteriorly from the annulus and is formed by the axoneme surrounded by the fibrous sheath and the plasma membrane. In its anterior portion, there is a short region in which a thin zone of finely granular cytoplasm is observed between the fibrous sheath and the plasma membrane (Figs. 3B, F; 5B, G). Posteriorly, the plasma membrane becomes closely attached to the fibrous sheath (Figs. 3G; 5G).

End piece

The very slender tail of the spermatozoon, posterior to the termination of the fibrous sheath, is referred to as the endpiece. In *Tropidurus torquatus*, the 9+2 pattern in this region becomes disarrayed and the doublets break apart into singlets (Fig. 5H), whereas in *T. semitaeniatus* the pattern of microtubules is apparently maintained, although its diameter becomes reduced (Fig. 3G).

Discussion

In *Tropidurus semitaeniatus* and *T. torquatus*, the acrosome complex forms a tripartite pattern (acrosome vesicle, subacrosomal cone and the constricted nuclear tip), a plesiomorphic condition of tetrapods (Jamieson, 1995a). The following features, regarded a plesiomorphic in amniotes (Jamieson, 1995a), are also present in the two species: nucleus elongate; distal centriole extending through midpiece, penetrated by two central singlets from the axoneme; several mitochondria in cross section of midpiece; annulus present; nine peripheral fibers associated with the nine doublets of the axoneme; peripheral fibers adjacent to doublets 3 and 8 enlarged, forming

a double structure detached from their respective doublets. *Tropidurus semitaeniatus* and *T. torquatus* have a number of character-states considered synapomorphies of Squamata (Jamieson, 1995b): perforatorium single, wholly prenuclear; endonuclear canal absent; epinuclear lucent zone present; mitochondrial cristae linear; intermitochondrial dense bodies present; fibrous sheath extending into midpiece; and nuclear shoulders rounded.

A suite of characters states present in mature spermatozoa of tropidurids is also seen in the chamaeleonids *Bradypodion karrooicum* (Jamieson, 1995b) and *Pogona barbata* (Oliver et al., 1996): apical portion of acrosome in transverse section depressed; perforatorial base plate absent (questionably present in *P. barbata*); epinuclear lucent zone clearly defined and well developed; midpiece short (moderately long in *B. karrooicum*); dense bodies forming regular rings, regularly separated from each other by mitochondria (dense bodies more scattered in *B. karrooicum*).

Tropidurus semitaeniatus and *T. torquatus* share with the polychrotid *Polychrus acutirostris* (Teixeira et al., submitted) the following features: apical portion of acrosome in transverse section depressed; acrosome vesicle homogeneous and electron-dense; subacrosomal cone filled with an unevenly arranged matrix, instead of a paracrystalline structure; nucleus with interspersed lacunae; epinuclear lucent zone well developed; a finely granular material dispersed between the condensed chromatin and the nuclear envelope; midpiece short; dense bodies without their own membrane; mitochondrial matrix more electron-dense than dense bodies. The account by Furieri (1974) on the polychrotid *Pristidactylus* (=*Cupriguanus*) *scapulatus* permits only limited comparisons with our results: the midpiece is short as in tropidurids.

Likewise, only limited comparisons are possible with the tropidurids studied by Furieri (1974): in *Liolaemus austromendocinus* and *Phymaturus flagellifer* (=*palluma*), the midpiece is

short as in *Tropidurus*, and only *P. flagellifer* has the arrangement of ring structures and mitochondria like *Tropidurus*.

This account reveals two unusual features of tropidurids relative to other iguanians: the presence of an electron-lucent, unilateral ridge at the acrosome surface, and an epinuclear lucent zone bounded by a membrane. The subacrosomal material is not closely attached to the acrosomal vesicle, being separated on one side by an electron-lucent layer. The lack of adherence can be explained by an uneven distribution of ionized groups on the surface of the inner membrane of the acrosome vesicle. This feature has been observed only in *Amphisbaena alba* (Teixeira et al., in press). The narrow chamber within the anterior limit of the subacrosomal cone, at the anterior end of the nucleus, is bounded by a membrane, suggesting that it can be a nuclear extension, with a small diameter. To the best of our knowledge, this feature has not been observed in any other squamate. Despite the presence of a membrane, we considered the chamber as an epinuclear lucent zone in this account.

The only published accounts on the sperm ultrastructure of *Tropidurus* are the papers by Cruz-Landim and Cruz-Höfling (1977) and Cruz-Höfling and Cruz-Landim (1978) on spermiogenesis. These works indicate that, in late spermatids of *T.* cf. *torquatus*, the fibrous sheath occurs posteriorly to the midpiece, the sperm head is not depressed, and dense bodies or ring structures are absent. In the mature spermatozoon of *T. semitaeniatus* and *T. torquatus*, however, the fibrous sheath penetrates into the midpiece to the level of m2, the acrosome is depressed, and ring structures are present, separating sets of mitochondria. In 1987, the *T. torquatus* species complex was split into 11 taxa (Rodrigues, 1987) and, without precise locality data and/or the re-examination of specimens, it is impossible to determine the current taxonomic status of the material studied by Cruz-Landim and Cruz-Höfling (1977) and Cruz-Höfling and

Cruz-Landim (1978). It is possible that the differences reported above between *T. semitaeniatus* and *T. torquatus* and the species studied by those authors reflect taxonomic differences, i.e. the *Tropidurus* studied by Cruz-Landim and Cruz-Höfling (1977) and Cruz-Höfling and Cruz-Landim (1978) was not *T. torquatus*, but a related species within the same species group. A phylogenetic analysis of *Tropidurus* based on osteology, squamation, color, and hemipenes suggests that *T. semitaeniatus* is a closer relative of *T. torquatus* than other members of the *T. torquatus* group (Frost, 1992).

Alternatively, the differences between *T.* cf *torquatus* and *T. torquatus* and *T. semitaeniatus* might reflect structural changes resulting from the maturation process during epididymal transit. Spermatozoa functionally immature undergo structural, biochemical, and functional changes during epididymal transit (Bedford & Nicander, 1971; Bedford, 1975; Orgebin-Crist, 1981; Carcupino et al., 1989), often completing maturation in the female reproductive tract as an adaptation to prevent premature acrosomal reactions during storage (Newton & Trauth, 1992). In this way, epididymides act not only as sperm storage organs, but also as sites of maturation (Healy & Jamieson, 1994). We strongly suggest that specimens should be deposited in accessible scientific collections and collection identification numbers should be provided in publications, enabling the re-examination of specimens in case of taxonomic changes. Further, mature, epididymal spermatozoa should preferentially be described.

The congeneric lizards *Tropidurus semitaeniatus* and *T. torquatus* are practically identical with respect to the ultrastructure of mature sperm cells. They differ in details such as: degree of acrosome flattening, presence of bulging at nuclear base, and arrangement of microtubules in the endpiece. Differences in sperm ultrastructure characters of reptiles have been reported at the level of orders and families (e.g., Jamieson, 1995a, 1995b), suggesting that they can be useful in

phylogenetic analyses. Nevertheless, the existence of major areas of disagreement between phylogenetic hypotheses derived from spermatozoa ultrastructural characters and gross morphological characters, led Teixeira et al. (1999) to suggest that the intra-familial variability in these characters may be higher than currently thought. That seems to be the case within Chamaeleonidae (e.g., Jamieson, 1995b; Oliver et al., 1996), Scincidae (e.g., Jamieson & Scheltinga, 1994; Jamieson, 1995b; Jamieson et al., 1996), and Tropiduridae. Further studies investigating the degree of variability in sperm ultrastructure characters across taxonomic categories might aid in clarifying at which taxonomic level they can be most profitably used in phylogenetic analysis.

A variety of characters derived from the sperm ultrastructure of squamates can profitably be used in phylogenetic analyses, where other datasets are uninformative (Teixeira et al., 1999). Additional work describing the sperm ultrastructure of the families Corytophanidae, Crotaphytidae, Hoplocercidae, Iguanidae, Opluridae, and Phrynosomatidae (Frost & Etheridge, 1989) are warranted and may cast light on the phylogenetic relationships of iguanians.

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Figure Captions

Fig. 1 Generalized diagram of the spermatozoon of *Tropidurus semitaeniatus* and *T. torquatus*, in longitudinal section and corresponding transverse section. Scales of various components are only approximate. Scale bar 0.5 μm.

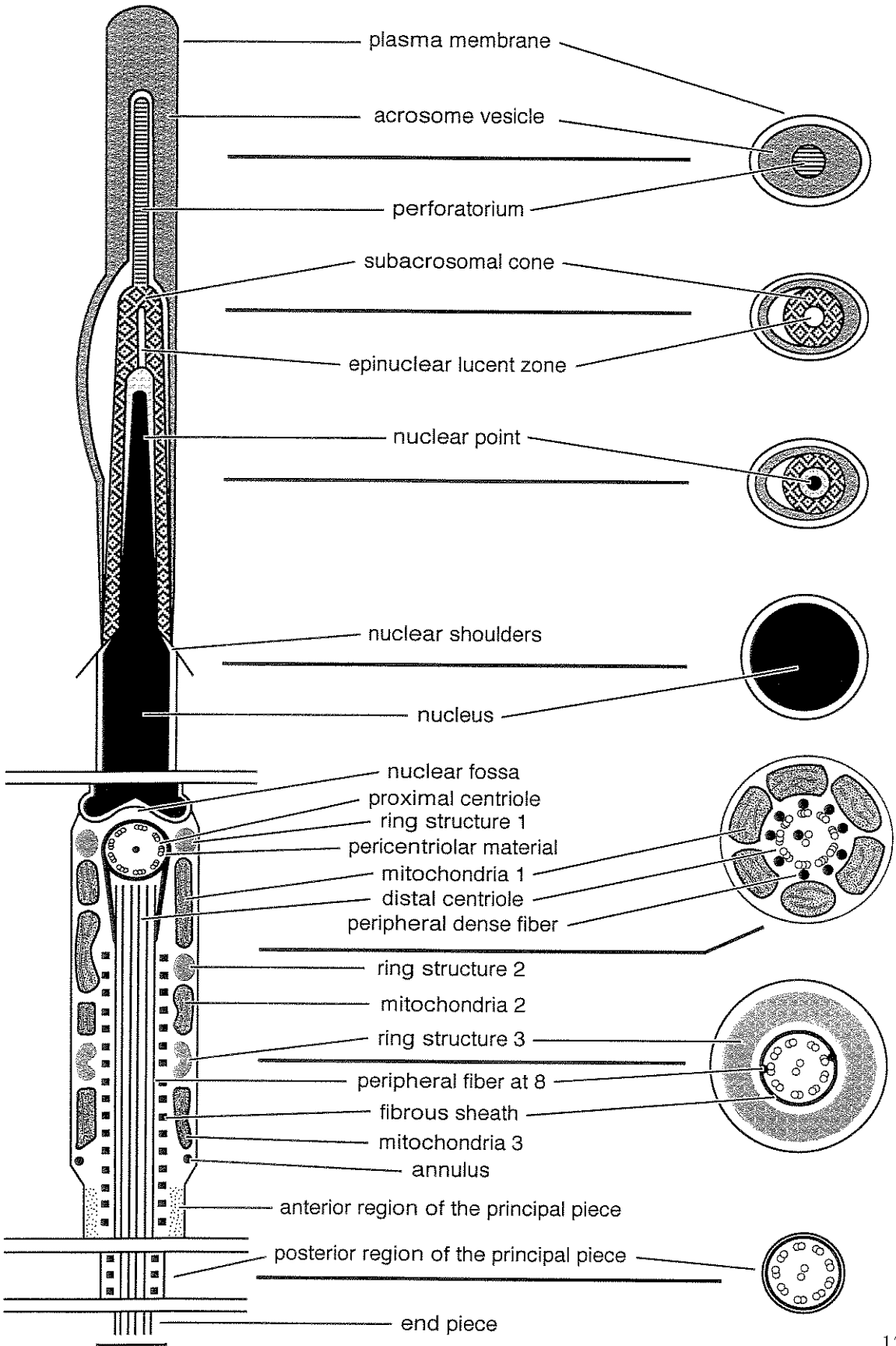
Fig. 2 *Tropidurus semitaeniatus*. (A) Longitudinal section through the apical end of the acrosome showing the perforatorium. (B) Longitudinal section through the basal portion of the acrosome showing the unilateral ridge, formed by an electron-lucent layer (*) between the acrosome vesicle and the subacrosomal cone. (C-F) Successive transverse sections through the acrosome. Note that anteriorly, in C, the acrosome appears depressed, while posteriorly, in D-F, it is more circular. An electron-lucent layer (*) between the acrosome vesicle and the subacrosome cone may be observed in D-F. (G) Transverse section through the nucleus showing lacuna. (H) Longitudinal section through the acrosome showing its flattened apical region and the well developed epinuclear lucent zone. Note the space between the condensed chromatin and the nuclear membrane, occupied by a granular material. An arrowhead and an arrow indicate the nuclear shoulders and the membranous flange, respectively. a: acrosome vesicle; et: epinuclear lucent zone; l, nuclear lacuna; n: nucleus; p: perforatorium; sc: subacrosomal cone. Scale bar 0.2 μm.

Fig. 3 *Tropidurus semitaeniatus*. (A). Longitudinal section of the posterior end of the nucleus and the midpiece. (B) Transition region between the midpiece and the principal piece. (C) Transverse section through the distal centriole showing the pair of microtubules and the dense material within it. (D) Transverse section through the axoneme showing the fibrous sheath and

the nine peripheral fibers associated with the doublets. Note that the peripheral fibers at 3 and 8 (arrows) are double and detached from their doublets. (E) Transverse section through the third ring structure at the posterior portion of the midpiece showing the peripheral fibers at 3 and 8 (arrows) thicker than the others and detached from their doublets. (F) Transverse section through the anterior portion of the principal piece. (G) Transverse section of the posterior portion of the principal piece and the end piece (arrowhead). (H) Detail of the neck region. (I) Longitudinal section through the midpiece showing three ring structures separated by three sets of columnar mitochondria. an: annulus; ax: axoneme; dc: distal centriole; fs: fibrous sheath; m: mitochondria; n: nucleus; nf: nuclear fossa; pc: proximal centriole; pf: peripheral fibers; pm: pericentriolar material; rs: ring structure. Scale bar 0.2 μm.

Fig. 4 *Tropidurus torquatus*. (A) Longitudinal section through the apical end of the acrosome showing its flattened portion and the perforatorium. (B) Longitudinal section through the acrosome showing the nuclear tip, the epinuclear lucent zone and the perforatorium. Note the space between the condensed chromatin and the nuclear membrane, occupied by a granular material, at the nuclear tip. (C-F) A series of transverse section through the acrosome. Note that anteriorly, in C-E, the acrosome appears very depressed, while posteriorly, in F, it is more circular. An electron-lucent layer (*) between the acrosome vesicle and the subacrosome cone may be observed in D-F. (G) Transverse section through the circular nucleus showing lacuna. (H) Longitudinal section through the basal region of the acrosome showing the electron-lucent ridge (*) between the acrosome vesicle and the subacrosomal cone. Note the nuclear shoulders (arrowhead) and the membranous flange (arrow). a: acrosome vesicle; et: epinuclear lucent zone; l, nuclear lacuna; n: nucleus; p: perforatorium; sc: subacrosomal cone. Scale bar 0.2 μm.

Fig. 5 *Tropidurus torquatus*. (A). Longitudinal section of the posterior end of the nucleus and the midpiece. (B) Detail of the transition region between the midpiece and the principal piece. (C) Transverse section through the distal centriole showing the pair of microtubules and the dense material within it. (D) Transverse section through the axoneme showing the fibrous sheath and the nine peripheral fibers associated with the doublets. Note that the peripheral fibers at 3 and 8 (arrows) are double and detached from their doublets. (E) Transverse section through the incomplete ring structure showing that the peripheral fibers at 3 and 8 (arrows) are thicker than the others and are detached from their doublets. (F) Transverse section through posterior portion of the midpiece showing the third ring structure. (G) Transverse section through the anterior (arrow) and posterior (arrowhead) portions of the principal piece and (H) the end piece. (I) Detail of the neck region in longitudinal section. Note the pericentriolar material and the proximal centriole. (J) Longitudinal section of the full length of the midpiece showing three ring structures separated by three sets of columnar mitochondria. an: annulus; ax: axoneme; dc: distal centriole; fs: fibrous sheath; m: mitochondria; n: nucleus; nf: nuclear fossa; pc: proximal centriole; pf: peripheral fibers; pm: pericentriolar material; rs: ring structure. Scale bar 0.2 μm.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mitochondria 1
distal centriole
peripheral dense fiber
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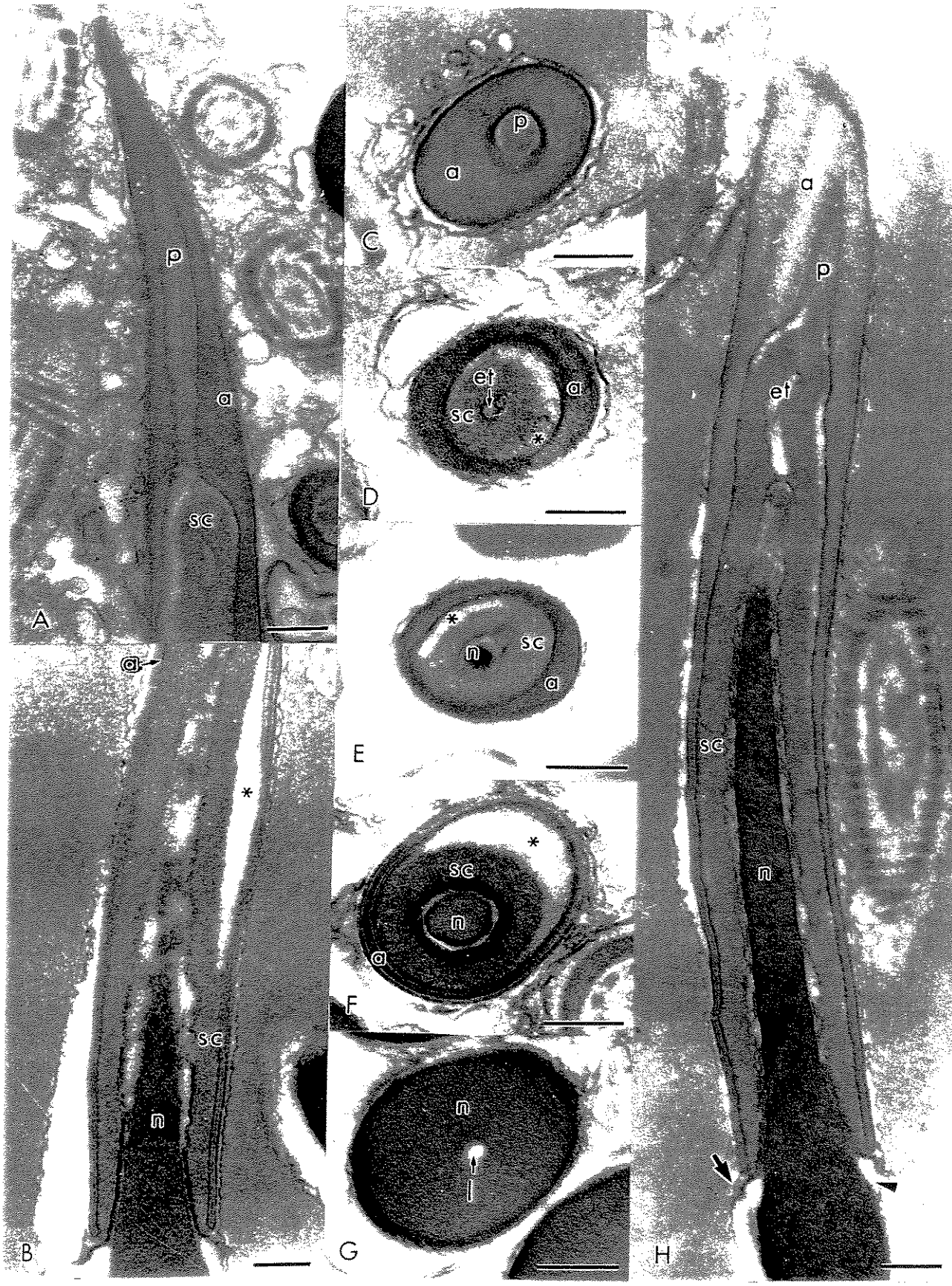
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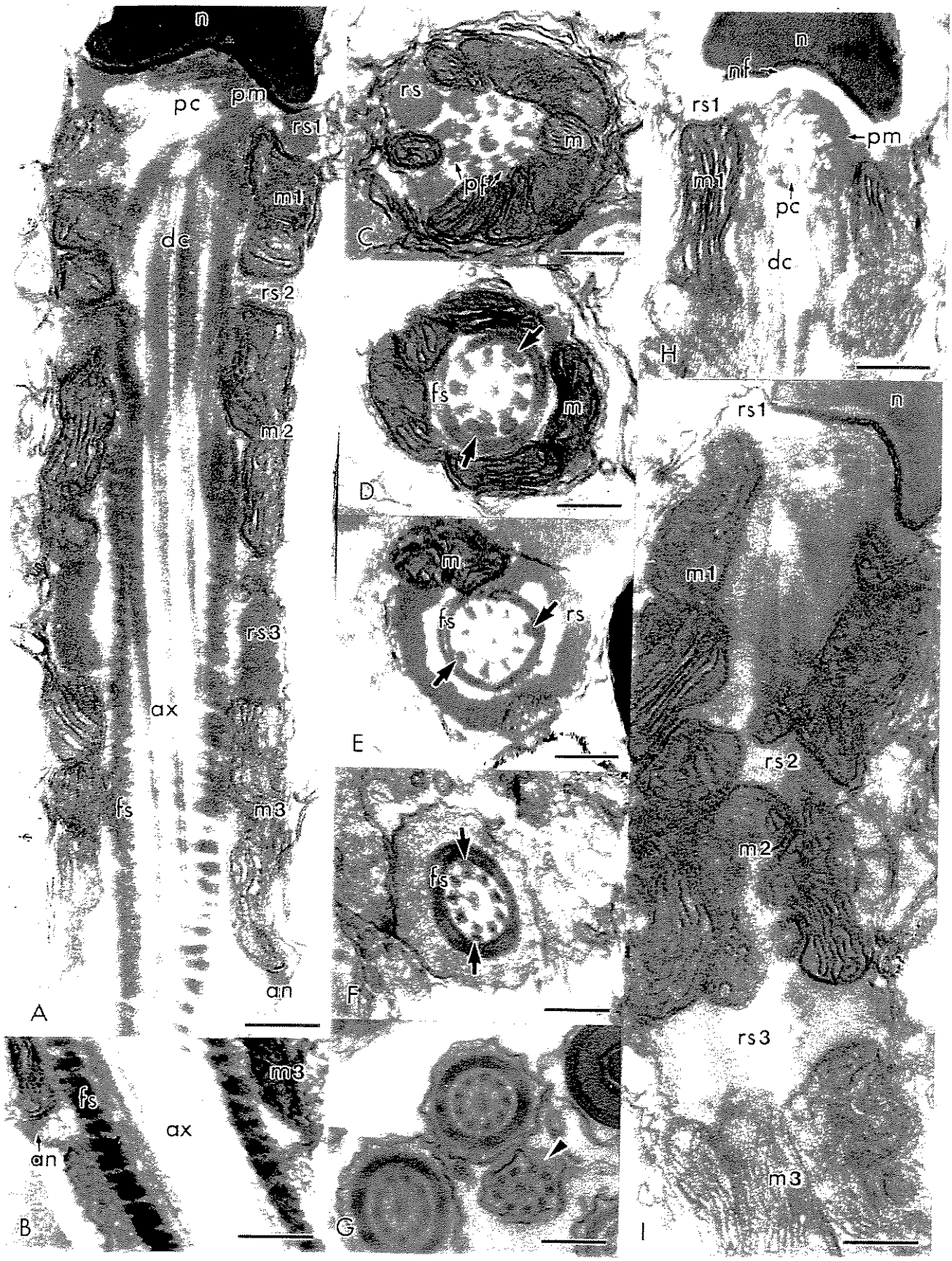
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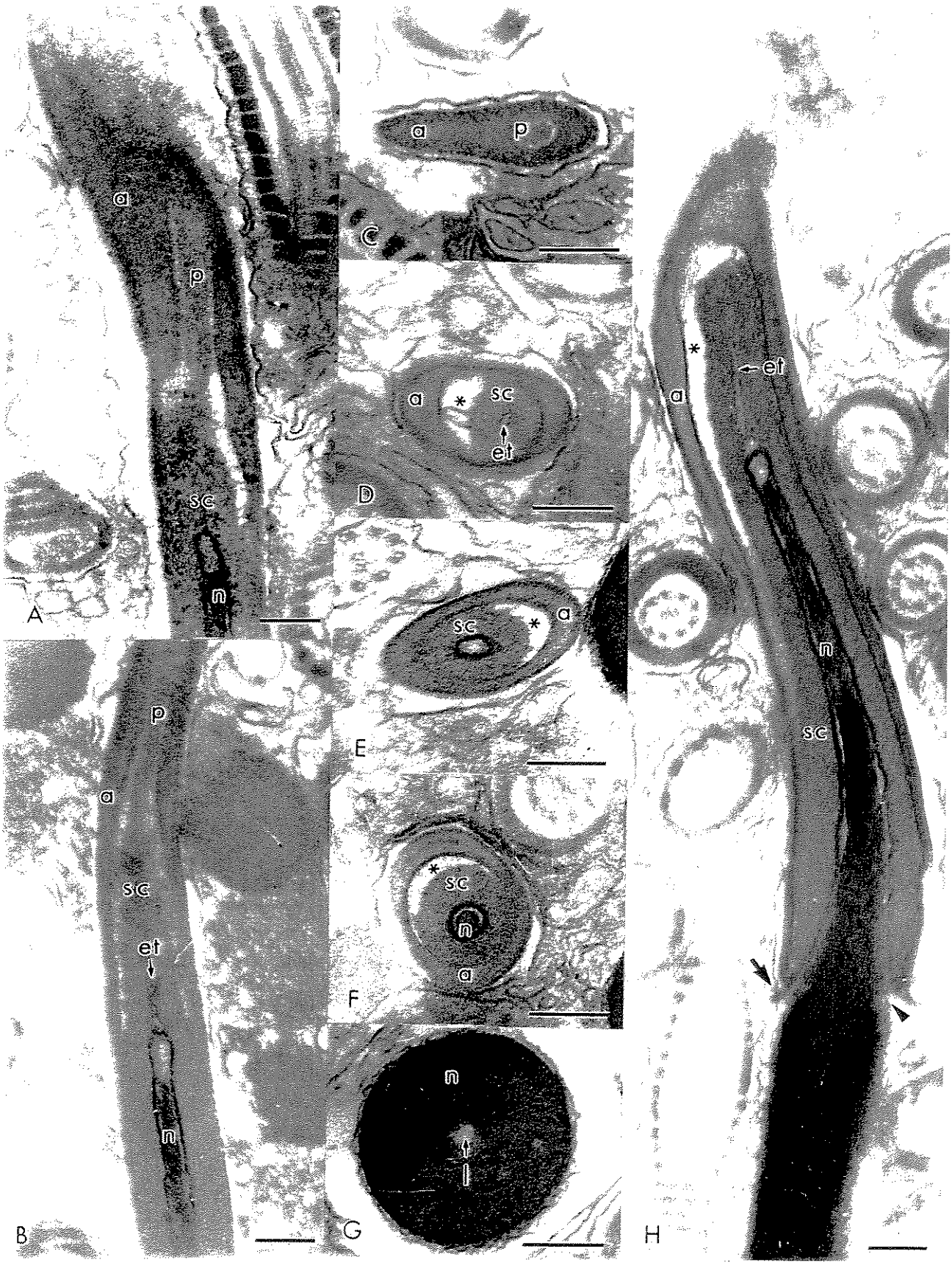
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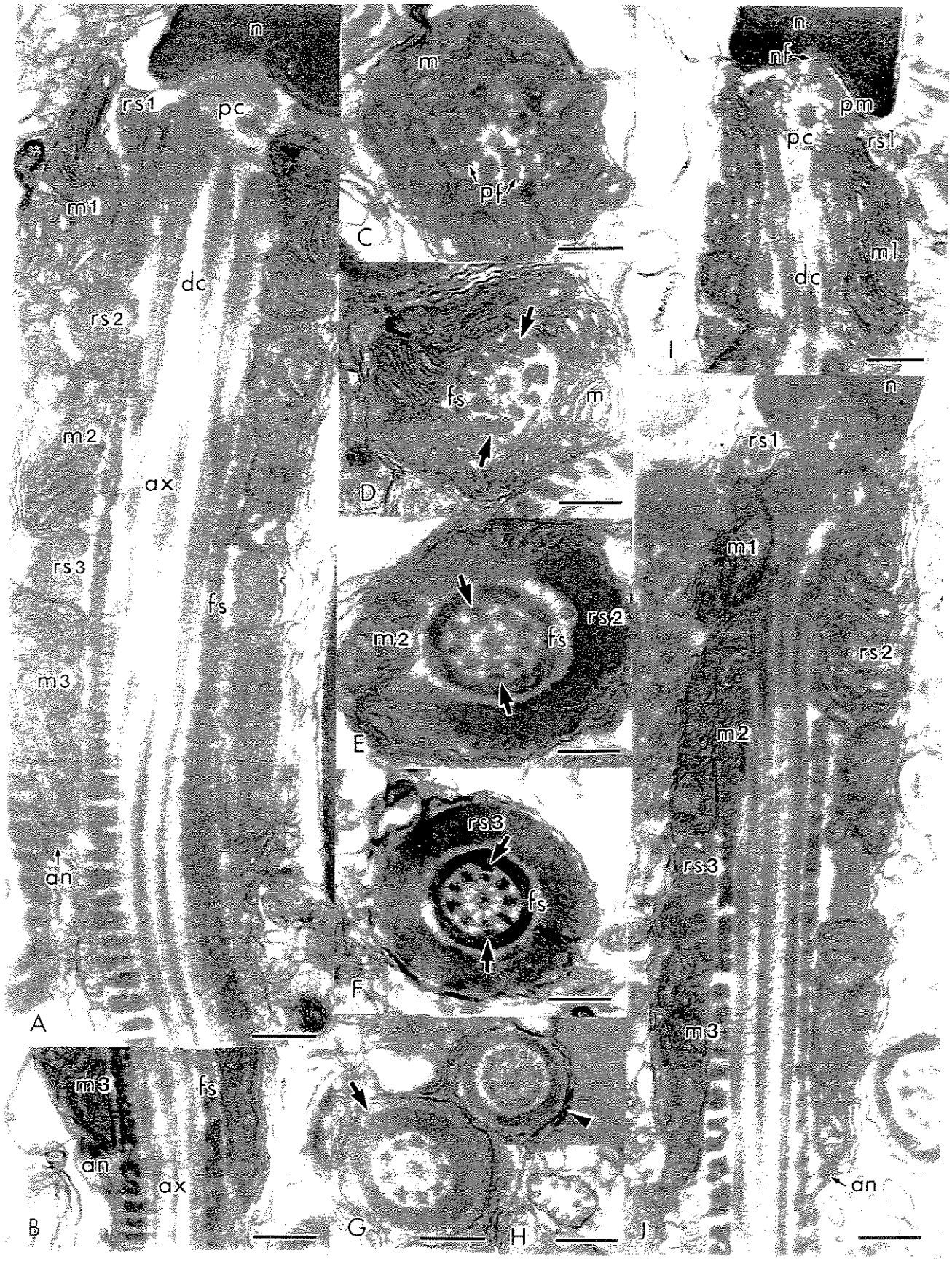
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3. CONCLUSÕES

3.1 - ULTRAESTRUTURA DE ESPERMATOZÓIDE

O estudo ultraestrutural do espermatozóide dos lagartos *Micrablepharus maximiliani* (Gymnphthalmidae), *Polychrus acutirostris* (Polychrotidae), *Tropidurus semitaeniatus*, *T. torquatus* (Tropiduridae) e da cobra-de-duas-cabeças, *Amphisbaena alba* (Amphisbaenidae) confirmou as sinapomorfias dos Squamata descritas por Jamieson (1995a; 1995b). Essas sinapomorfias são as seguintes: um único perforatório prenuclear, perda de canal endonuclear, a presença de uma região epinuclear electron-lucente (com exceção de *M. maximiliani*), corpos densos intermitocondriais, mitocôndrias com formato intermediário entre colunar e arredondado, cristas lineares, ombros nucleares arredondados. De acordo com Jamieson (1995a; 1995b), a extensão da bainha fibrosa na peça intermediária é a sinapomorfia e a autapomorfia mais significante e convincente dos Squamata. Além disso, os Squamata podem ser diagnosticados pela presença de um material paracristalino no cone subacrossomal

A descrição da ultraestrutura do espermatozóide de *T. semitaeniatus* e *T. torquatus* mostrou que as duas espécies congenéricas possuem espermatozóides praticamente idênticos, assegurando que a variabilidade intragenérica não é muito grande e que as diferenças ocorrem mais ao nível de ordem e família (Jamieson, 1995a; 1995b).

As similaridades entre os caracteres ultraestruturais dos espermatozóides de *Tropidurus* (Tropiduridae) e *Polychrus acutirostris* (Polychrotidae) indicam que estes dois grupos parecem ser mais aparentados entre si do que com os outros Iguania estudados, *Bradypodium karroicum* (Jamieson, 1995b), *Pogona barbata* (Oliver *et al.*, 1996), *Agama stellio* (Al-Hajj *et al.*, 1987), *A. adramitana* (Dehlawi *et al.*, 1992), *Uromastyx philbyi*

(Dehlawi & Ismail, 1990), *Iguana delicatissima* (Saita *et al.*, 1988), *Phrynosoma coronatum* (Clark, 1967), *Pristidactylus (=Cupriguanus) scapulatus* (Furieri, 1974), *Anolis carolinensis* (Clark, 1967), *Liolaemus austromendocinus*, e *Phymaturus flagellifer* (=*palluma*) (Furieri, 1974).

Um grande problema enfrentado durante o processo de descrição dos caracteres e de seus respectivos estados foi não encontrar definição exata das estruturas, assim como uma padronização das características ultraestruturais das estruturas nos trabalhos feitos previamente. A dificuldade de descrição e caracterização ocorreu principalmente com as seguintes estruturas: zona epinuclear lúcida, placa da base perforatorial e peça intermediária. A zona epinuclear lúcida não foi claramente demonstrada nas micrografias dos trabalhos realizados com as famílias de Squamata. Jamieson (1995b) considerou em suas análise, que os "Iguanidae" estudados por Furieri (1974), apresentavam a zona epinuclear lúcida. No entanto, a presença da estrutura nas micrografias aparentemente não ocorria. A própria definição da estrutura e de seus estados não ficou clara. Entre as espécies estudadas neste trabalho, a zona epinuclear lúcida não foi observada no espermatozóide de *Micrablepharus maximiliani*. Os espermatozóides de *Amphisbaena alba* e *Polychrus acutirostris* apresentaram a zona epinuclear lúcida, como sendo um canal bastante estreito e com delimitações indefinidas, ao contrário dos *Tropidurus*, onde foi observada como uma estrutura com membrana própria. Em relação à placa da base perforatorial, Jamieson (1995b) considerou a densificação no ápice do cone subacrossomal no Gekkonidae *Heteronotia binoei* (Jamieson *et al.*, 1996) como sendo a placa da base perforatorial, mas não considerou a mesma condição na serpente *Aspidites melanocephalus* (Oliver *et al.*, 1996). Entre as espécies de Squamata estudadas neste trabalho, o lagarto *Polychrus acutirostris* foi o único cujo espermatozóide apresentou esta densificação, que neste caso

foi considerada uma placa da base perforatorial. Quanto à peça intermediária, a determinação do seu tamanho não foi objetivamente estipulada para cada estado (curta, moderadamente longa e longa) presente na matriz construída por Jamieson (1995b). Devido a este problema, a peça intermediária do espermatozóide de *Amphisbaena alba*, um dos primeiros a serem descritos neste trabalho, foi considerada moderadamente longa com apenas 4.3 μm de comprimento, sendo na verdade curta em relação aos demais Squamata estudados. Este erro ocorreu, pois a análise do comprimento foi feita a partir de observações comparativas das micrografias dos outros trabalhos. Já as peças intermediárias de *Micrablepharus maximiliani* (2.5 μm de comprimento), *Tropidurus* (2.8 μm) e até de *Polychrus acutirostris* (7.5 μm) foram consideradas como curtas. Nestes casos, a determinação do tamanho foi estabelecida após as medições da peça intermediária de todos os outros espermatozóides de Squamata estudados.

3.2 - ANÁLISE FILOGENÉTICA

Os principais grupos de Squamata, tais como Iguania, Gekkota, Scincomorpha e Anguimorpha, e as famílias Chamaeleonidae e Scincidae, cuja monofilia (ancestralidade comum exclusiva) é suportada pelos conjuntos de dados morfológicos (Estes *et al.*, 1988), não são monofiléticos nas árvores derivadas dos dados ultraestruturais de espermatozóide.

Nas cobras-de-duas-cabeças (anfisbenas), suas especializações morfológicas para a escavação dificultam o uso de dados morfológicos tradicionais, devido às perdas de algumas características morfológicas e às similaridades com outros táxons resultantes da evolução convergente. Conseqüentemente a interpretação de suas relações filogenéticas se torna complicada. Por essa razão, o uso de caracteres mais conservativos (Jamieson, 1995a; 1995b), como os fornecidos pela ultraestrutura de espermatozóide, torna mais provável o

esclarecimento das relações de parentesco entre as anfisbenas. No entanto, as análises cladísticas feitas pelos caracteres ultraestruturais de espermatozóide produziram resultados conflitantes com as hipóteses feitas a partir dos dados morfológicos. De acordo com Estes *et al.* (1988), as anfisbenas poderiam ser membros do grupo Autarchoglossa, enquanto que os resultados deste trabalho sugerem que as anfisbenas seriam um grupo irmão de Autarchoglossa.

Como se pode observar, as inferências filogenéticas feitas a partir da ultraestrutura de espermatozóide, tanto entre os Squamata quanto entre as anfisbenas, não condizem com as hipóteses obtidas pelos conjuntos de dados morfológicos (Estes *et al.*, 1988; Russel, 1988; Schwenk, 1988; Maddison & Maddison, 1996). Essa heterogeneidade entre os conjuntos de dados ocorre, pois os caracteres evoluem em taxas diferentes e geralmente resultam histórias filogenéticas diferentes (Swofford, 1991; de Queiroz *et al.*, 1995; Miyamoto & Fitch, 1995), sendo que alguns evoluem mais rapidamente que outros. Na ausência de estudos que qualifiquem a natureza e o grau de heterogeneidade entre os dados morfológicos e os de ultraestrutura de espermatozóide, não é possível determinar a superioridade de um conjunto sobre o outro, em termos de qual fornece as verdadeiras relações filogenéticas. No entanto, os dados obtidos pela ultraestrutura de espermatozóide possuem três deficiências. Primeiro, o número de caracteres ultraestruturais de espermatozóide utilizado para a análise filogenética é bastante limitado em relação ao número de táxons empregados (Jamieson, 1995b), o que provoca um aumento no número de árvores e diminui a resolução das árvores obtidas. Segundo pode-se observar altos níveis de polimorfismo dentro das famílias de Squamata, ou seja, muitas mudanças nos caracteres dentro dos táxons. Isto acontece, porque as taxas de evolução dos caracteres da ultraestrutura de espermatozóides entre as famílias são muito mais altas do que as previstas,

resultando em árvores incongruentes. Terceiro, a ultraestrutura de espermatozóide de muitas famílias de Squamata ainda não foram descritas (Jamieson, 1995b). Essas deficiências tornam a ultraestrutura de espermatozóide um conjunto de dados com menos sinal filogenético entre as famílias.

Devido a essas deficiências, a análise filogenética feita para resolver tanto as relações enigmáticas das anfisbenas quanto dos Squamata em geral, a partir de dados ultraestruturais de espermatozóide, produziram resultados insatisfatórios.

3.3 - CONCLUSÕES FINAIS

Para tornar a ultraestrutura de espermatozóide, um conjunto de caracteres ideal para a obtenção de melhores resultados nas hipóteses filogenéticas de Squamata, estudos adicionais são necessários para:

- Reavaliar os caracteres utilizados;
- Aumentar a quantidade de caracteres a serem utilizados na análise;
- Investigar o grau de variabilidade nos caracteres da ultraestrutura de espermatozóide entre as categorias taxonômicas, com intuito de clarear qual nível taxonômico pode ser mais vantajoso nas análises.

Conclui-se que, apesar das deficiências, a ultraestrutura de espermatozóide fornece uma fonte de caracteres independente e filogeneticamente estruturada. Além disso, os estudos detalhados da ultraestrutura de espermatozóides apresentados nesta dissertação, revelaram uma variedade de caracteres que podem ser muito proveitosos nas análises filogenéticas, onde outros conjuntos de caracteres não podem ser tão informativos.

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