UNIVERSIDADE ESTADUAL DE CAMPINAS

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ESTUDO CITOGENÉTICO E DA ULTRA-ESTRUTURA DOS ESPERMATOZÓIDES DE ESPÉCIES DA SUBFAMÍLIA HYLODINAE (LEPTODACTYLIDAE) E DA FAMÍLIA DENDROBATIDAE (AMPHIBIA, ANURA)

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e aprovada pela Comissão Julgadora.

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SUMÁRIO

ABSTRACT

The Dendrobatidae is a large neotropical anuran family for which the evolutionary relationships are still unclear. Affinities with frogs belonging to the Ranoidea neobatrachian lineage, specifically with ranid frogs related to the genus *Petropedetes*, have been proposed. Alternatively, relationships with hylodine leptodactylids (Bufonoidea) have also been considered. The aim of this work was to examine the relationships between the Hylodinae and Dendrobatidae based on cytogenetic and ultrastructural studies. The karyotypes of four dendrobatid species of the genus *Epipedobates* (*E. flavopictus*, *E. trivittatus*, *E. femoralis* and *E. hahneli*), as well as seven Hylodinae species (*Hylodes phyllodes*, *H. asper*, *Crossodactylus* sp. n., *C.* cf. *caramaschi*, *Megaelosia massarti*, *M. boticariana* and *M. lutzae*), were analyzed using conventional Giemsa staining, C-banding and Ag-NOR techniques. An ultrastructural analysis of spermatozoa was done for the four species of *Epipedobates* (listed above) and for *Hylodes phyllodes*, *Crossodactylus* sp. n. and *Megaelosia massarti*. The cytogenetic results showed a conserved chromosome number (2n = 24) in *Epipedobates*, although there was considerable variation in chromosome morphology, in the amount and distribution of heterochromatin and in the location of the NORs. Within the Hylodinae, the diploid number was the same (2n = 26) in *Hylodes* and *Crossodactylus* but varied among the three species of *Megaelosia* (2n = 28, 30 and 32). *Crossodactylus* had the most conserved karyotype in terms of chromosome morphology and C-banding pattern, whereas there were differences between the two species of *Hylodes*. The NOR location did not vary within each genus. On the other hand, all of these parameters varied among the three species of *Megaelosia*. Ultrastructural analysis showed that *Epipedobates femoralis* was peculiar in that it possessed biflagellate spermatozoa. The presence of mitochondria within the undulating membrane of the other species also differentiated them from *E. femoralis*. Despite the great similarity among the spermatozoa of the three genera of Hylodinae, *Crossodactylus* sp. n. was most similar to other leptodactylids and to most of the remaining bufonoids. In contrast, *M. massarti* and *H. phyllodes* shared a similar shape in their axial and juxtaxonemal fibers. These results elucidated some inter- and intrageneric aspects within each group (Hylodinae and Dendrobatidae) but did not provide much information on the relationships between the two

groups. Even with the use of banding techniques, the karyotypical analysis provided no unambiguous homeologies indicative of the possible relationships between the Hylodinae and Dendrobatidae. On the other hand, the karyotypic peculiarities of *E. femoralis* suggested a unique position within the genus. Within the Hylodinae, the extensive karyotypical variability in *Megaelosia*, contrasted with the conserved karyotypes of *Hylodes* and *Crossodactylus*, and agreed with previously established relationships within the subfamily based on morphological analysis. The peculiarity of the spermatozoa of *E. femoralis* supported the divergent position of this species within the genus, as already indicated by others. The possible apomorphies shared by the remaining three species of *Epipedobates* (absence of a juxtaxonemal fiber and the presence of a short, extremely expanded undulating membrane), supported the retention of *E. trivittatus* in this genus, in agreement with molecular data and contrary to the suggestion of its placement in the genus *Phobobates*. Base mainly on the structure of the flagellar apparatus and also on similarities in the organization of the acrosomal complex, we conclude that the spermatozoa of dendrobatids are of the "bufonoid type", and differ strongly from the pattern in ranoid species. Within the Hylodinae, *Megaelosia* shared flagellar characteristics with *Hylodes*. These findings together with morphological and biochemical data, reinforce the retention of *Megaelosia* in the subfamily Hylodinae.

RESUMO

As relações de parentesco da família Dendrobatidae ainda são pouco esclarecidas. Alguns autores consideram uma maior afinidade com anuros da linhagem Ranoidea ou ainda, dentro desta, especificamente com ranídeos africanos relacionados ao gênero *Petropedetes*. Alternativamente, supostas relações com leptodactilídeos da subfamília Hylodinae (Bufonoidea) também têm sido aventadas. Nesta tese, espécies de Hylodinae e Dendrobatidae foram estudadas através da análise cariotípica, incluindo métodos de bandamento cromossômico, e análise da ultra-estrutura dos espermatozóides. O objetivo do presente trabalho foi buscar dados adicionais que pudessem ser utilizados na avaliação das relações entre esses grupos. Os cariótipos de quatro espécies do gênero *Epipedobates* (Dendrobatidae) (*E. flavopictus*, *E. trivittatus*, *E. femoralis e E. hahneli*) e sete espécies representantes dos três gêneros de Hylodinae (*Hylodes phyllodes*, *H. asper*, *Crossodactylus* sp. n., *C.* cf. *caramaschi*, *Megaelosia massarti*, *M. boticariana* e *M. lutzae*), foram analisados por métodos de coloração convencional, bandamento C e localização das regiões organizadoras de nucléolos (NORs). A análise da ultra-estrutura dos espermatozóides se concentrou nas quatro espécies de *Epipedobates* e em *Hylodes phyllodes*, *Crossodactylus* sp. n. e *Megaelosia massarti*. Os dados citogenéticos mostraram constância no número diplóide de cromossomos na espécies de *Epipedobates* (2n = 24), embora considerável variação tenha sido encontrada na morfologia cromossômica, na quantidade e distribuição da heterocromatina constitutiva e na localização das NORs. Para os hilodíneos, o número diplóide se mostrou constante para *Hylodes* e *Crossodactylus* (2n = 26), mas bastante variável para *Megaelosia* (2n = 28, 30 e 32). *Crossodactylus* mostrou-se o mais conservado em relação ao padrão de banda C, localização das NORs e morfologia cromossômica, enquanto em *Hylodes*, embora a localização das NORs tenha se mantido constante para as duas espécies, variações na banda C e na morfologia cromossômica foram encontradas. Já nas espécies de *Megaelosia*, todos esses parâmetros mostraram-se variáveis. Na análise ultra-estrutural, *Epipedobates femoralis* se destacou dentro do gênero por apresentar espermatozóide biflagelado. Nas demais espécies, a presença singular de mitocôndrias no interior da membrana ondulante também as diferenciou de *E. femoralis*. Em Hylodinae apesar de uma grande semelhança nos espermatozóides, *Crossodactylus* sp. n. mostrou-se mais parecido com outros leptodactilídeos e com a maioria dos demais bufonóideos,

enquanto *M. massarti* e *H. phyllodes* compartilharam um formato singular das fibras axial e juxta-axonemal (aqui denominado "taco de baseball"). Os resultados ajudaram mais na resolução de alguns aspectos inter e intra-genéricos dentro de cada grupo, do que na verificação das relações de parentesco entre eles. Pelos estudos cariotípicos, mesmo com o emprego de métodos de bandamento, não foram obtidas homeologias seguras que pudessem indicar possíveis relacionamentos entre Hylodinae e Dendrobatidae. Por outro lado, algumas peculiaridades cariotípicas apresentadas por *E. femoralis* apontaram sua singularidade dentre as demais espécies. Em Hylodinae, a grande variabilidade cariotípica em *Megaelosia*, em oposição ao conservadorismo de *Hylodes* e *Crossodactylus*, confirmaram as relações estabelecidas por critérios morfológicos dentro da subfamília. A análise ultra-estrutural por sua vez suportou a proposta, já levantada na literatura, de que a posição taxonômica de *E. femoralis* fosse revista. Além disso, as possíveis apomorfias compartilhadas pelas três espécies restantes (ausência de fibra juxta-axonemal e membrana ondulante curta e extremamente dilatada), parecem indicar a retenção de *E. trivittatus* neste gênero, concordando com dados moleculares e contrariando a sugestão de sua alocação no gênero *Phobobates*. Considerando-se principalmente a estrutura do aparato flagelar e também semelhanças na organização do complexo acrossomal, podemos concluir que os espermatozóides dos dendrobatídeos são "do tipo bufonóideo", diferindo bastante do padrão encontrado para espécies de ranóideos estudados. Dentro dos hilodíneos, *Megaelosia* compartilha características flagelares com *Hylodes*, o que possivelmente pode se unir a dados morfológicos e bioquímicos para reforçar sua retenção na subfamília.

1. Introdução

1.1. Os anfíbios anuros: aspectos gerais e de relações filogenéticas

Cerca de 90% das espécies da Classe Amphibia pertencem à Ordem Anura, a qual conta com aproximadamente 4800 espécies descritas (Frost, 2002) e abriga os sapos, as rãs, as pererecas e formas afins. Estas espécies atualmente estão agrupadas em 29 Famílias, sendo que pelo menos 1.600 delas ocorrem na América do Sul. Os anuros estão distribuídos por todo o mundo, exceto nas extremas latitudes e na maioria das ilhas oceânicas (Frost, 2002). Ainda que apresentem esse grande número de espécies, encontradas numa grande diversidade de habitats, os anuros são bastante conservados morfologicamente, o que dificulta a utilização de caracteres morfológicos como ferramenta para estudos filogenéticos (Hillis,1991). De acordo com Inger (1967), apesar das adaptações para a vida fossorial, aquática ou terrestre, a variação morfológica nos Anura é discreta.

Segundo Blair (1973), a morfologia externa dos anuros é propensa a refletir pressões adaptativas e, por isso, é mais apta a revelar similaridades resultantes do processo de convergência do que em apontar afinidades filogenéticas. Morescalchi (1973) defende que não só os processos de convergência, mas também a ocorrência de paralelismos em várias das características anatômicas e biológicas durante a evolução dos Anura, representam problemas para a interpretação dessas características.

Para Hillis (1991) e Hillis *et al.* (1993), não há nenhuma proposta filogenética robusta que seja capaz de relacionar a maioria das famílias de anuros. Hillis (1991) considera que essa dificuldade está também, em grande parte, relacionada ao curto período de tempo de divergência entre a maioria das famílias desta Ordem, tornando a filogenia dos anuros um dos maiores "quebra-cabeças" dentro da evolução dos tetrápodes. Além do curto período de diversificação dos anuros, Hillis *et al.* (1993) apontam algumas outras razões que têm tornado a filogenia desse grupo tão difícil. Dentre estas razões os autores enfatizam a utilização, principalmente em estudos moleculares e de poucos exemplares como representantes de grandes grupos, o que tem gerado árvores filogenéticas com topologias complicadas, inconclusivas e inconsistentes. Ruvinsky & Maxson (1996) consideram que as relações dentro do grupo dos chamados "anuros modernos" (subordem Neobatrachia) permanecem obscuras e voltam a enfatizar a escassez de dados filogeneticamente

informativos quando da análise de características morfológicas. Estes autores ressaltam também que os métodos moleculares têm se mostrado úteis para a elucidação de relações em níveis de espécies e gêneros, mas, no entanto, parecem não ser efetivos na resolução de relações inter-familiares.

Em um estudo recente, Emerson *et al.* (2000) enfatizaram que análises com ambos os conjuntos de dados (morfológicos e moleculares) têm sido problemáticas, uma vez que os estudos morfológicos têm fornecido poucos caracteres, alguns dos quais de difícil interpretação, e que as análises moleculares têm abordado poucos táxons ou se baseado em poucos caracteres potencialmente informativos. Por outro lado, a análise combinada dos dados dessas duas fontes, bem como de fontes alternativas como dados bioquímicos, comportamentais, ultra-estruturais e citogenéticos, podem ajudar no esclarecimento ou no suporte de algumas hipóteses filogenéticas, levando a hipóteses mais robustas sobre a filogenia do grupo de interesse (veja Hillis, 1987). Nesse sentido, os dados moleculares de Hillis *et al.* (1993) por exemplo, aliados aos dados morfológicos de Duellman & Trueb (1986) se mostraram capazes de suportar hipóteses como a de merofiletismo dos Archeobatrachia.

Portanto, na tentativa de entender as relações de parentesco entre os Anura, ferramentas alternativas como a análise citogenética e da ultra-estrutura dos espermatozóides, somadas aos conhecimentos atuais, têm muito a contribuir. De acordo com Futuyma (1992), diferentes caracteres podem ter taxas evolutivas diferentes, o que justifica uma investigação dos anuros por meio destas ferramentas alternativas, as quais podem revelar caracteres mais divergentes e discriminativos que aqueles advindos principalmente do estudo da morfologia externa, que são a base da classificação filogenética dos Anura.

1.2. O estudo citogenético nos Anura

Problemas de ordem sistemática bem como de relações filogenéticas entre grupos de Anura têm sido avaliados por meio de estudos comparativos dos cariótipos de diferentes espécies. Tais estudos têm contribuído também para um melhor conhecimento e entendimento dos mecanismos cromossômicos operantes no decorrer do processo evolutivo

(Bogart & Wasserman, 1972; Bogart, 1970, 1973, 1991; Morescalchi, 1973; Cole, 1974; De Lucca & Jim, 1974; Batistic, 1984; Qumisiyeh & Baker, 1988, Busin *et al.*, 2001).

Até o final da década de 70 e início da década de 80, a citogenética dos anuros esteve alicerçada principalmente em comparações do número e morfologia cromossômica, e da posição das constrições secundárias em cariótipos submetidos à coloração convencional. Estes estudos mostraram a existência de grupos com grande homogeneidade no número e morfologia cromossômica como na Família Bufonidae (Bogart, 1973) e no grupo das *Hylas* Holoárticas (Anderson, 1991) e, por outro lado, revelaram também grupos com grande variação nessas características como os Leptodactylidae (Denaro, 1972) e, em menor grau, os Dendrobatidae (Bogart, 1991). Em grupos como *Physalaemus* (Leptdodactylidae) por exemplo, embora as espécies compartilhem um número diplóide de 2n=22 cromossomos, diferenças podem ser observadas na morfologia e tamanho de alguns pares, bem como no número e posição da constrição secundária (Silva *et al.*, 2000).

Através de análise cariotípica convencional observou-se também que espécies muito semelhantes morfologicamente e que poderiam ser consideradas, à primeira vista, como uma única espécie poderiam apresentar diferenças no número e/ou na morfologia cromossômica. A exemplo, isto foi observado em *Hyla brunnea* (2n=34) e *Hyla septentrionales* (2n=24) (Cole, 1974) e em *Megaelosia massarti* (2n=28) (Melo *et al.*, 1995) e *Megaelosia boticariana* (2n=30) (Giaretta & Aguiar-Jr., 1998).

Embasamentos adicionais para hipóteses originadas de estudos morfológicos também foram proporcionados pela análise cariotípica, como no caso das relações apontadas por Lynch (1971) entre os gêneros *Telmatobufo* e *Caudiverbera*, as quais foram substanciadas pela grande similaridade cariotípica (em número e morfologia cromossômica) encontrada entre eles nos estudos de Formas & Espinoza (1975).

Desde os estudos de Morescalchi (1973) tem-se considerado uma tendência evolutiva para redução no número de cromossomos nos anuros, uma vez que números elevados e presença de microcromossomos e cromossomos telocêntricos foram detectados associados às famílias consideradas basais dentro da Ordem. Beçak (1968), na tentativa de explicar variações numéricas encontradas nas Famílias Hylidae (2n=22, 24, 26 e 30) e Leptodactylidae (2n=18, 20, 22 e 26), já havia considerado que mecanismos de fusão cêntrica pareciam ter predominado durante a evolução dos Anura e levado à redução no número de cromossomos. No entanto, a autora também admitiu que em algumas espécies

esse fenômeno não era tão evidente e que rearranjos adicionais como inversões pericênticas ou outros tipos de translocações poderiam ter mascarado o processo de fusão.

Estudos posteriores mostraram que as diferenças numéricas encontradas em alguns grupos podem também ser devidas ao processo contrário, o de fissão cêntrica (Cole, 1974; Kuramoto & Allison, 1989; Miura, 1995; Busin *et al.*, 2001), levando a um aumento no número de cromossomos. Além disso, para *Eleutherodactylus* (Leptodactylidae), Bogart (1991) e Bogart & Hedges (1995) admitem a ocorrência de ambos os processos, combinados com eventos de translocações e inversões, para explicar a grande diversidade numérica (2n=18 – 36) observada no gênero (veja Kuramoto, 1990; King, 1990). As análises de Miura (1995) e Busin *et al.* (2001) contaram também com o auxílio do bandamento C além da análise dos cromossomos corados convencionalmente. Sem a utilização de métodos de coloração diferencial dos cromossomos como o bandamento C, cuja importância será discutida ainda nesta seção, a tentativa de eleger fusão e/ou fissão como mecanismos predominantes na evolução de certos grupos se torna uma tarefa árdua. Bogart (1991), por exemplo, não encontrou evidências na coloração convencional que indicassem o mecanismo operante no surgimento do cariótipo 2n=31 encontrado em *Eleutherodactylus glandulifer*.

Além dos rearranjos citados acima como possíveis agentes atuantes na diferenciação cariotípica nos anuros, o estudo citogenético também foi capaz de identificar o fenômeno de poliploidização como um importante mecanismo de especiação nesse grupo (Bogart & Wasserman, 1972). De acordo com esses autores, a especiação por poliploidização deve ser considerada não como um evento isolado, mas como um importante mecanismo evolutivo na Ordem. Cariótipos poliplóides já foram encontrados em cerca de 38 espécies em cinco Famílias (Leptodactylidae, Bufonidae, Ranidae, Hylidae e Pipidae) (Tymowska, 1991).

Apesar dos estudos baseados nos cariótipos convencionalmente corados com Giemsa terem trazido grande contribuição para a proposição de mecanismos evolutivos entre os anfíbios anuros, a citogenética comparativa avançou enormemente com o desenvolvimento e o emprego das técnicas de bandamento (principalmente bandamento C) e também de impregnação pela prata para detecção das NORs. Essas técnicas têm facilitado o reconhecimento de homeologias cromossômicas e auxiliado na compreensão dos mecanismos evolutivos envolvidos na diferenciação cariotípica da Ordem (Schmid, 1978 a,

b, 1980; King, 1980, 1991; Miura, 1995; Schmid *et al.*, 1990, 1995, Lourenço *et al.*, 1999; Silva *et al.*, 1999, dentre outros).

1.2.1. Bandamento C

O método de bandamento C é o principal método de bandamento utilizado para os Anura, uma vez que a utilização dos bandamentos G, Q e R não têm apresentado resultados satisfatórios como aqueles obtidos para outros grupos de vertebrados (Sumner, 1990). O bandamento C detecta regiões de heterocromatina constitutiva, cujo DNA apresenta sequências de bases altamente repetitivas. A técnica mais empregada é a de Sumner (1972), com pequenas modificações, a qual apresenta três etapas principais: tratamento ácido com HCl, tratamento básico com solução aquosa de hidróxido de bário [$Ba(OH)_2$] e, finalmente, incubação em uma solução salina de cloreto e citrato de sódio (2x SSC) aquecida a 60^0C. Conforme proposto por Holmquist (1979), cada uma das três etapas desempenha um papel específico no processo de bandamento. O tratamento ácido é responsável pela depurinação do DNA, enquanto o tratamento com hidróxido de bário causa a quebra dos sítios apurínicos bem como a denaturação irreversível do DNA. Durante o tratamento salino final ocorre a extração de segmentos de DNA por um processo de β-eliminação nos sítios depurinados. Por razões ainda discutíveis, esse DNA é mais facilmente extraído das regiões de banda C negativas, resultando em uma fraca coloração quando comparada com regiões de heterocromatina constitutiva (banda C positivas).

O bandamento C vem sendo empregado no estudo de um grande número de espécies e populações de anuros, na busca de possíveis polimorfismos e/ou variações (importantes indicadores da taxa de evolução cromossômica), no entendimento das inter-relações filéticas e na tentativa de caracterização de táxons (Schmid, 1978*a, b*, 1980; King, 1980; Kasahara *et al.*, 1996; Lourenço *et al.*, 1998; Silva *et al.*, 1999; Busin *et al.*, 2001).

De acordo com King (1991), nas espécies de Archeobatrachia analisadas até o momento, uma quantidade relativamente pequena de heterocromatina foi detectada. Em todas essas espécies, blocos banda C positivos foram sempre detectados coincidindo com a NOR ou adjacente a ela. Dentro dos Neobatrachia, embora em alguns casos a quantidade e a distribuição da heterocromatina apresentem regularidades capazes de caracterizar grupos de espécies (Kasahara, 1996), para a maioria dos anuros estudados esses parâmetros

mostram-se bastante variáveis em nível interespecífico (King, 1991) (exemplos em King, 1980; Green, 1986; Anderson, 1991; Formas & Cuevas, 2000).

Variações intra-específicas, embora pouco freqüentes, já foram relatadas para *Rana ridibunda* (Schmid, 1978b), *Pyxicephalus adspersus* (Schmid, 1980), *Rana chensinensis* (Green & Borkin, 1993); *Physalaemus petersi* (Lourenço *et al.*, 1999), *Physalaemus cuvieri* (Silva *et al.*, 1999) e *Alytes muletensis* (Odierna *et al.*, 2000). Ainda em nível intra-específico, polimorfismos em relação ao tamanho de certas regiões heterocromáticas também já foram descritos (Schmid, 1978*a*; 1980; King, 1991; Miura *et al.*, 1995). De acordo com John (1988), é provável que a quantidade e a distribuição da heterocromatina sejam fatores variáveis que têm contribuído de modo significativo para o mecanismo de isolamento cromossômico, uma vez que mudam a uma taxa extremamente rápida no curso da evolução.

O aumento na quantidade de heterocromatina é considerado como uma tendência evolutiva em anuros (Imai, 1991; King, 1991), embora este último autor não descarte a possibilidade de ocorrência de diminuição em certos grupos. King (1991) aponta possíveis mecanismos pelo quais esse aumento pode ocorrer. Um deles seria pela de adição de segmentos heterocromáticos a sítios cromossômicos específicos através da amplificação de segmentos de DNA repetitivo. Algumas evidências apontam que a adição de segmentos de heterocromatina em sítios específicos é capaz de proteger a eucromatina adjacente de modificações estruturais acarretadas pela recombinação. Um segundo mecanismo seria o de transformação da eucromatina, ou seja, blocos eucromáticos pré-existentes se transformariam em segmentos heterocromáticos provavelmente por um processo de amplificação de múltiplas seqüências repetitivas interespaçadas contidas na eucromatina. King (1991) deixa claro que processos de deleção de seqüências heterocromáticas não podem ser descartados na evolução dos anfíbios. A esse favor, Formas & Cuevas (2000) assumem a perda de heterocromatina, ao apontarem a ausência de banda centromérica no par no. 1 de *Telmatobufo bullocki*, como uma condição derivada dada sua presença em *Caudiverbera*, gênero monotípico tido com grupo irmão de *Telmatobufo*.

A variabilidade no padrão de banda C entre espécies relacionadas tem sido considerada como um pobre indicador de homeologias (Kaiser *et al.*, 1996). No entanto, alguns trabalhos têm obtido sucesso ao tratarem os padrões de banda C (quantidade e localização) como caracteres potenciais para análises filogenéticas as quais incluem

polarização das mudanças ocorridas e proposições de cladrogramas baseados nessas características (Green, 1986; Sessions & Kezer, 1987). Além disso, de acordo com Green & Borkin (1993), estudos detalhados de bandamento, como aquele realizado por Schmid *et al.* (1990), revelam que os cromossomos contêm uma riqueza de informações que podem evoluir a uma taxa maior do que aquelas características que são extraídas dos cariótipos submetidos à análise morfológica convencional.

1.2.2. Regiões Organizadoras do nucléolo (NORs)

Essas regiões são sítios cromossômicos de genes que transcrevem para os RNAs ribossômicos, que são sintetizados no nucléolo e posteriormente tornam-se parte integrante do ribossomo maduro no citoplasma. Esses sítios, portanto, são segmentos cromossômicos de cuja atividade se constituirá o nucléolo observado no núcleo das células interfásicas (Schwarzacher & Wachtler, 1983, 1993).

A primeira demonstração citogenética da localização desses segmentos cromossômicos portadores das NORs foi realizada por Matsui & Sasaki (1973), através de uma técnica que incluía extração de histonas e coloração com Giemsa, denominada banda N. A identificação das NORs, no entanto, ficou mais simples a partir da técnica desenvolvida por Goodpasture e Bloom (1975), na qual uma solução de prata amoniacal era empregada. Posteriormente, foram feitas simplificações por Howell & Black (1980), sendo este o método mais utilizado até hoje. Alternativamente, a localização da NOR também pode ser feita através de outras técnicas citoquímicas como coloração com mitramicina ou cromomicina, uma vez que esses fluorocromos são específicos para regiões ricas em GC, ou ainda por hibridação "*in situ*" com sondas de DNAr.

A especificidade pela prata se dá devido à reação desta com proteínas denominadas argirofílicas (ou proteínas Ag-NOR) que permanecem associadas ao DNAr mesmo durante a mitose (Roussel *et al.*, 1996) e pelo menos até o paquíteno da meiose. Algumas proteínas como a RNA polimerase I, o UBF (principal fator de transcrição do DNAr), a DNA topoisomerase I e a nucleolina (Howell, 1977; Schwarzacher *et al.*, 1978, Hernandez-Verdum, 1993) já foram caracterizadas como proteínas argirofílicas. Portanto, considera-se que somente as NORs que estiveram ativas durante ciclo celular precedente são evidenciadas pelo método. A marcação consiste de um precipitado metálico derivado da

redução do íon prata (Ag+) que possivelmente interage com grupamentos sulfidril (SH), dissulfeto (S-S), fosfato ou carboxila presentes nas proteínas argirofílicas associadas à NOR (De Capoa *et al.*, 1982; Buys & Osinga, 1984; Hubbell, 1985).

A presença de blocos de heterocromatina constitutiva em associação com NOR parece ser resultante da existência de seqüências repetitivas de DNA nas regiões espaçadoras entre os cístrons ribossomais 18S+28S, que são evidenciadas pelo método de banda C (King, 1991).

Geralmente as NORs se localizam em regiões de constrição secundária (Hsu *et al.*, 1975) (exceções em King, 1980; Schmid, 1982; Baldissera *et al.*, 1999; Formas & Cuevas, 2000). Heteromorfismos de tamanho da NOR entre cromossomos homólogos são comumente encontrados em anuros (Schmid, 1982) e têm sido atribuídos a fenômenos de amplificação dos cístrons ribossomais, os quais podem levar a duplicações ou mesmo triplicações destes cístrons (King, 1990). De acordo com Schmid *et al.* (1990), fenômenos de crossing-over desigual ou ainda trocas desiguais entre cromátides-irmãs, também podem contribuir para o aparecimento de NORs heteromórficas.

A localização e o número de NORs tendem a ser constantes para espécies ou grupos relacionados (Schmid, 1978*a*, *b*, Schmid *et al.*, 1990), embora essas características possam variar entre as famílias, gêneros e até mesmo dentro de espécies (Foote *et al.*, 1991; Kaiser *et al.*, 1996; Baldissera *et al.*, 1999; Lourenço *et al.*, 1999; Silva *et al.*, 1999). Lourenço et al (1998) encontraram sete padrões diferentes de distribuição da NOR entre 15 espécimes analisados de *Physalaemus petersi* pertencentes ao denominado grupo cariotípico I, fato que, segundo os autores demonstrava uma elevada taxa de evolução cromossômica naquele grupo

Ruiz *et al.* (1981) basearam-se em polimorfismos (diferenças no número e posição) na localização da NOR para discutir as possíveis relações evolutivas entre espécies poliplóides do gênero *Odontophrynus*. Anderson (1991) utilizando-se também do método Ag-NOR foi capaz de distingüir dois subgrupos que comportam a maioria das *Hyla* Holoárticas, um deles com a NOR no cromossomo 6 e o outro com a NOR nos cromossomos 10 ou 11, sendo o primeiro grupo suportado também por dados adicionais de morfologia cromossômica e padrão de banda C. Essas análises permitiram a proposição de que durante a evolução cromossômica das *Hyla* Holárticas pelo menos uma mudança na

localização dos cístrons ribossomais, além da modificação nos padrões distribuição da heterocromatina, deve ter ocorrido.

Baldissera *et al.* (1999), estudando 4 grupos de espécies do gênero *Bufo* de ocorrência na América do Sul, observaram diferenças na localização da NOR entre eles. Enquanto que nas espécies dos grupos *marinus* e *crucifer* a NOR foi detectada no par 7, no grupo *granulosus* ela foi encontrada no par 5 e no grupo *margaritifer* foi observada no par 10. Segundo esses autores, dentro do grupo *granulosus* a presença comum da NOR na região telomérica do braço curto do par 5 em *Bufo granulosus* e *Bufo pygmaeus* suportam uma possível linhagem evolutiva comum para as espécies deste grupo. Além disso, os resultados obtidos com a técnica de Ag-NOR parecem, segundo esses autores, corroborar o agrupamento morfológico das espécies sul-americanas do gênero *Bufo*, e a presença da NOR no par 10 em *B. margaritifer* parece suportar indiretamente a hipótese de relações entre os grupos da América do Sul e grupos Africanos.

Portanto, pelos trabalhos acima citados fica clara a importância que a detecção da NOR nas diferentes espécies de anuros tem tido na busca do entendimento da evolução cariotípica deste grupo e no reconhecimento de possíveis homeologias entre populações e espécies e de rearranjos cromossômicos atuantes durante a evolução cariotípica da Ordem.

1.3. A ultra-estrutura dos espermatozóides em Anura

A análise ultra-estrutural dos espermatozóides tem se mostrado uma valiosa ferramenta de geração de novos caracteres que têm permitido uma reavaliação das relações filogenéticas em diversos grupos animais tais como insetos, peixes, moluscos e répteis (Baccetti, 1979; Dallai & Mazzini, 1983; Jamieson, 1987; Mattei, 1991; Jamieson & Leung, 1991; Teixeira *et al.*, 1999).

Para os anuros, segundo levantamento feito por Garda (2002), 72 espécies já tiveram seus espermatozóides estudados em nível ultra-estrutural, estando o maior número de representantes analisados nas famílias Bufonidae, Hylidae, Leptodactylidae e Ranidae, embora representantes de famílias de Archeobatrachia também tenham sido incluídas.

O panorama geral que se pode delinear mediante estes estudos mostra que a morfologia dos espermatozóides dos anuros é bastante diversificada. Isso inclui espécies onde a cauda é formada exclusivamente pelo axonema, a exemplo das espécies já

analisadas do gênero *Rana* (Pugin-Rios, 1980; Kwon & Lee, 1995) e das famílias Pipidae e Microhylidae (Bernardini et al, 1986; Jamieson, 1999) e, por outro lado, espécies onde fibras protéicas acessórias ao axonema (fibras juxta-axonemal e axial), além de uma membrana ondulante, podem ser vistas. Nesta última condição inclui-se a grande maioria das espécies da família Leptodactylidae, Bufonidae e Hylidae (Pugin-Rios, 1980; Kwon & Lee, 1995; Meyer *et al.*, 1997).

Quanto às estruturas da cabeça, geralmente se observa uma vesícula acrossomal que pode ser cônica e recobrir a parte anterior do núcleo (condição tida como plesiomórfica) como encontrado na maioria das famílias dos bufonóideos (Pugin-Rios, 1980; Báo *et al.*, 1991; Lee & Jamieson, 1993), ou ainda ser uma estrutura sacular que ocupa apenas o topo do núcleo, uma condição apomórfica observada em espécies de *Rana* e *Xenopus* (Pugin-Rios, 1980; Bernardini *et al.*, 1996). Abaixo do acrossomo encontra-se o cone subacrosomal, descrito pela primeira vez em *Ascaphus truei* (Jamieson *et al.*, 1993), podendo ser maciço ou fibrilar. Esta estrutura tem sido sugerida como homóloga ao denominado "perforatório cônico" presente na maioria das espécies de bufonóideos já estudadas (Jamieson, 1999). Em *A. truei*, além do cone subacrossomal, um perforatório na forma de um bastão maciço com uma porção endonuclear também é observado (Jamieson *et al.*, 1993). Por outro lado, em *Pleurodema* (Leptodactylidae) e nas espécies já analisadas de Ranidae e Racophoridae o cone subacrossomal não é encontrado (Pugi-Rios, 1980; Kwon & Lee, 1995), deixando o acrossomo apoiado diretamente no envoltório nuclear.

Considerando aspectos da peça intermediária, o ângulo formado entre os centríolos proximal e distal é tido por Kwon & Lee (1995) como indicador da posição sistemática da espécie em estudo, sendo o aumento na angulação entre eles considerado uma tendência evolutiva. Em *Telmatobufo australis*, espécie com espermatozóide biflagelado (Pugin-Rios & Garrido, 1981), ambos os centríolos dão origem a axonemas independentes e portanto se dispõem paralelamente. Tal arranjo também foi observado em *Leiopelma hochstetteri* (Scheltinga *et al.*, 2001) embora esta espécie possua espermatozóide monoflagelado.

O acúmulo dessas informações tem propiciado o delineamento de possíveis tendências evolutivas do espermatozóide neste grupo e, embora com exceção do trabalho de Scheltinga *et al.* (2001) nenhum trabalho tenha colocado os dados ultra-estruturais numa matriz de caracteres para análise filogenética, grande parte dos trabalhos tem fornecido discussões relevantes a respeito de possíveis implicações desses dados para a filogenia dos

Anura (Lee & Jamieson, 1993; Jamieson *et al.*, 1993; Kwon & Lee, 1995; Scheltinga *et al.*, 2001). Jamieson (1999), esclarece que uma vez deduzidos caractéres plesiomórficos para Lissamphibia (Urodela, Gymnophiona e Anura), dada sua presença nos espermatozóides de possíveis Tetrapoda ancestrais e amniotas basais, algumas tendências evolutivas e sinapomorfias podem ser consideradas para os anuros ainda que sem enfoque cladístico. Como exemplo, Lee & Jamieson (1993) apontaram a simplificação do espermatozóide dos anuros, com a perda das estruturas acessórias à cauda (bastão axial e membrana ondulante), como uma tendência evolutiva possivelmente resultante da aquisição secundária da fertilização externa.

Além disso, possíveis sinapomorfias também foram propostas para determinados grupos. Lee & Jamieson (1993), ao re-examinarem a filogenia dos neobatráquios da linhagem Bufonoidea sob a luz dos dados ultra-estruturais propuseram o monofiletismo deste grupo tendo como base a presença do perforatório cônico subacrosomal, que diferia da condição plesiomórfica observada em *Ascaphus* (Jamieson *et al.*, 1993), onde um perforatório endonuclear em forma de bastão era encontrado. Esta proposta contrariava a natureza parafilética proposta para esse grupo nos estudos de Duellman & Trueb (1986). Outra sinapomorfia foi capaz de unir hilídeos, bufonídeos e os leptodactilídeos do Novo Mundo num grupo denominado Eubufonoidea. Trata-se da presença de um colar de mitocôndrias bem desenvolvido observado ao redor da porção inicial do flagelo, separado deste por um canal citoplasmático (Lee & Jamieson, 1993). Essa característica, não observada nos Myobatrachidae, garantiu a separação desta família (tida como "leptodactilídeos Australo-asiáticos") dos demais bufonóideos. De fato, o agrupamento de Myobatrachidae em Bufonoidea tinha pouco respaldo em caractéres morfológicos somáticos e essa separação já havia sido proposta, embora de forma não decisiva, por Duellman & Trueb (1986).

Kwon & Lee (1995), sumarizaram os dados disponíveis na literatura acerca destas tendências e dos estados de caráter (apomorfias e plesiomorfias) e suas implicações na filogenia dos Anura. Recentemente, as tendências evolutivas para alguns caractéres foram revistas após a descrição do espermatozóide de *Leiopelma hochstetteri*, uma espécie tida como basal para todos os anuros (veja Scheltinga *et al.*, 2001).

Além das proposições em níveis taxonômicos superiores, algumas contribuições, embora em menor grau, também têm sido oferecidas pela ultra-estrutura dos

espermatozóides para elucidação de questões dentro de famílias e gêneros. Garrido *et al.* (1989), valendo-se da análise ultra-estrutural em espécies de *Batrachyla* (Leptodactylidae), mostraram que esses dados corroboravam análises etológicas e cariotípicas que indicavam grande proximidade entre *B. antartandica* e *B. taeniata*, sendo ambas mais distantes de *B. leptopus*. Meyer *et al.* (1997) encontraram evidências espermatológicas suficientes para suportar a alocação de *Litoria alboguttata* dentro do gênero *Cyclorana*, como previamente indicada por estudos moleculares, morfológicos e cariotípicos. Ainda nesses níveis, os dados ultra-estruturais têm se mostrado divergentes quanto a outras metodologias em alguns casos. A exemplo, de acordo com Kwon & Lee (1995), os dados obtidos para espécies do gênero *Discoglossus* apontam-no como o mais basal dentro da família Discoglossidae, uma interpretação que não é respaldada por dados cariotípicos e anatômicos.

Em suma, os dados ultra-estruturais têm se aliado aos de morfologia externa (que são o alicerce da classificação atual dos Anura), moleculares e também citogenéticos (veja Lee & Jamieson, 1992; Meyer *et al.*, 1997), na busca do entendimento das relações de parentesco entre os diferentes grupos. Por outro lado, faz-se necessária a análise de um número maior de espécies na busca de novos caractéres que possibilitem uma futura análise filogenética cladística para os Anura.

1.4. Os grupos em estudo:

1.4.1. A subfamília Hylodinae (Leptodactylidae) (*sensu* Savage, 1973)

Esta subfamília é constituída de rãs de hábitos diurnos, principalmente de riachos serranos do sudeste e sul do Brasil e norte da Argentina (Lutz, 1931; Lynch, 1971; Heyer *et al.*, 1975). Atualmente são reconhecidos três gêneros: *Hylodes* (17 espécies), *Crossodactylus* (10 espécies divididas em três grupos) e *Megaelosia* (5 espécies) (Frost, 2002, Giaretta *et al.*, 1993; Bastos & Pombal-Jr., 1995). *Crossodactylus* e *Hylodes* são pequenos (30mm de comprimento) em relação a *Megaelosia*, que pode apresentar até 5 vezes o tamanho dos outros sendo um gênero endêmico da Mata Atlântica, e também o menos conhecido devido à sua difícil captura.

Dados cariotípicos já disponíveis de *Hylodes* e *Crossodactylus* mostram que a maioria das espécies apresenta 2n=26 cromossomos, exceção feita a *Hylodes nasus* (2n=24) (Denaro, 1972). Em ambos os gêneros observa-se uma grande uniformidade cariotípica, que é mais evidente nos cariótipos de *Crossodactylus* do que em *Hylodes*, no qual existem pequenas variações na morfologia de alguns cromossomos, as quais foram atribuídas a mecanismos de inversões pericêntricas por De Lucca & Jim (1974) (outros estudos com ambos os gêneros em Beçak, 1968; Bogart, 1970; Brum-Zorrilla & Saez, 1968, Bogart, 1991). O gênero *Megaelosia*, no entanto, é o que mais varia dentro da subfamília, possuindo espécies com 2n=28 (Melo *et al.*, 1995) e 2n=30 (Giaretta & Aguiar-Jr., 1998).

1.4.2. A família Dendrobatidae

Esta família compreende atualmente 207 espécies agrupadas em 9 gêneros - *Aromobates*, *Allobates*, *Colostethus*, *Cryptophyllobates*, *Dendrobates*, *Epipedobates*, *Mannophryne*, *Nephelobates*, *Phyllobates* – (Frost, 2002), distribuídos pelo sul da América Central, em toda a região Amazônica e também Centro e Sudeste do Brasil. O gênero *Colostethus* é o maior deles, com 113 espécies e apresenta características reprodutivas consideradas primitivas dentro da família (Frost, 2002).

A grande maioria das espécies tem atividade diurna, à exceção de *Aromobates nocturnus* (Myers *et al*, 1991), e muitas delas apresentam um complexo comportamento reprodutivo de cuidado à prole e combate. Os dendrobatídeos são conhecidos pelo aposematismo e toxicidade de muitas espécies. Seus venenos normalmente são alcalóides que se encontram na pele e são usados por algumas tribos de índios da América do Sul para a caça.

Dados cariotípicos da família Dendrobatidae já disponíveis na literatura indicam uma tendência de redução cromossômica de 2n=24 até 2n=18. Bogart (1991) sugere que o cariótipo 2n=24 seja o ancestral da família uma vez que é encontrado nos gêneros *Epipedobates* e *Colostethus* tidos como menos especializados do ponto de vista de comportamento reprodutivo. *Dendrobates* é o gênero mais especializado (em temos de comportamento reprodutivo) e apresenta variação no número cromossômico (2n = 20 ou 18).

Myers & Ford (1986) consideram os Dendrobatidae como uma unidade monofilética mas, no entanto, a identidade de seus grupos irmãos ainda é bastante controversa e sua posição sistemática dentro dos anuros não é consensual (veja também Ford, 1993).

1.5. Problemática de interesse e justificativa

Ainda existem controvérsias quanto ao ancestral a partir do qual os dendrobatídeos teriam se originado. Alguns autores consideram a família como sendo derivada, ou pelo menos próxima aos Leptodactylidae da América do Sul relacionados a *Hylodes* ou *Crossodactylus* (Noble, 1931; Lynch; 1971; Ardila-Robayo, 1979; Bogart, 1991) ou ainda com os Ranidae africanos relacionados a *Petropedetes* (Griffiths, 1959, 1963; *apud* Bogart, 1991, Ford, 1993).

Segundo Ford (1993), as hipóteses divergentes com relação a essa problemática de possíveis relacionamentos entre os dendrobatídeos e ranídeos (superfamília Ranoidea) ou leptodactilídeos (superfamília Bufonoidea), surgiram da análise de um número limitado de caracteres essencialmente morfológicos e de interpretações diferentes de certos caracteres e, por isso, a afiliação dos dendrobatídeos a qualquer um desses grupos parece depender do conjunto de caracteres analisados.

Além da análise de caracteres morfológicos, outras ferramentas já foram usadas na tentativa de elucidação desse problema. Do ponto de vista citogenético, Morescalchi (1973) analisando cariótipos por coloração convencional sugeriu que os cariótipos dos dendrobatídeos eram do "tipo Leptodactylidae". Bogart (1991) estudando cariótipos de espécies de *Hylodes, Colostethus, Epipedobates* e *Minyiobates*, bem como de espécies de *Petropedetes*, assumiu que os petropedetíneos tinham cariótipos muito mais semelhantes a outros ranídeos que aos Dendrobatidae. Por outro lado, Bogart sugeriu que os cariótipos de *Hylodes* e de outros leptodactilídeos hilodíneos (embora o autor não tenha analisado espécies de *Cossodactylus* e *Megaelosia*) eram similares aos observados nos dendrobatídeos, e que a presença de 2n = 24 cromossomos em *H. nasus*, número modal para Dendrobatidae, reforçava essas similaridades. Ainda segundo este autor, a presença deste número levava a crer que uma possível redução no número de cromossomos de 2n = 26 (modal para Leptodactylidae) para 2n = 24 teria ocorrido num leptodactilídeo ancestral

antes da evolução dos Dendrobatidae. A presença do cromossomo número 1 bem maior que os demais do complemento nas espécies analisadas de Dendrobatidae e Hylodinae também foi usada por Bogart (1991) na proposição da similaridade cariotípica.

Hay *et al.* (1995), utilizando comparações de seqüências de genes ribossomais mitocondriais para inferir as relações filogenéticas entre as famílias da Classe Amphibia, concluíram que os dendrobatídeos estariam mais próximos aos bufonóideos. Ainda segundo estes autores, essa hipótese estaria em conformidade com dados biogeográficos dos dois grupos, uma vez que os ranóideos são amplamente distribuídos pelo Velho Mundo, enquanto os dendrobatídeos aparentemente se originaram no Novo Mundo.

Ford (1993), ao reavaliar os caracteres utilizados para a proposição de ambas as hipóteses, atentou para a necessidade de obtenção de novos caracteres para uma investigação mais substanciada.

Tendo em vista a já mencionada contribuição que a citogenética (principalmente através das técnicas de bandamento) e a ultra-estrutura dos espermatozóides têm trazido para elucidação de questões sistemáticas dentro dos anuros, os objetivos desta tese são os que se seguem abaixo.

1.6. Objetivos

1.6.1. Contribuir com a caracterização citogenética da subfamília Hylodinae analisando espécies dos gêneros *Hylodes*, *Crossodactylus* e *Megaelosia*, e da família Dendrobatidae pela análise de espécies do gênero *Epipedobates* (*E. flavopictus*, *E. trivittatus* e *E. femoralis* e *E. hahneli*), não só pelo emprego de coloração convencional mas também pela utilização de bandamento C e localização da NOR, inéditos para esses grupos.

1.6.2. Comparar esses dados dentro de cada grupo e com aqueles disponíveis na literatura.

1.6.3. Comparar os dados obtidos entre os dois grupos, na tentativa de reconhecimento de possíveis homeologias cromossômicas capazes de auxiliar na avaliação das supostas relações filogenéticas.

1.6.4. Caracterizar a ultra-estrutura dos espermatozóides de alguns dendrobatídeos (gênero *Epipedobates)* e de Hylodinae (gêneros *Hylodes*, *Crossodactylus* e *Megaelosia*), contribuindo para aumentar o número de espécies de anuros analisadas por esta ferramenta e buscando evidências de relacionamento entre Hylodinae e Dendrobatidae

1.6.5. Analisar o conjunto de dados (citogenéticos e de ultra-estrutura) discutindo sua contribuição no estabelecimento de possíveis relações de parentesco entre esses grupos.

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2. Análise Citogenética

Artigo I

Herpetologica, 58(3), 2002, 293–303

CYTOGENETIC ANALYSIS OF FOUR POISON FROGS OF THE *EPIPEDOBATES* GENUS (ANURA: DENDROBATIDAE)

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ABSTRACT: We determined the overall karyotypic structure, positions of NORs, as well as the distribution of constitutive heterochromatin for *Epipedobates flavopictus, E. trivittatus, E. femoralis,* and *E. hahneli.* Despite a conserved chromosome number (2N = 24), morphological differences were seen in the group of small chromosomes. *Epipedobates femoralis* presented a distinctive karyotype compared to the other species analyzed. All species examined had NORs on different chromosomes. The C-banding pattern showed a considerable variation among the species. *Epipedobates flavopictus* is remarkably different from the others in possessing only centromeric C-bands. The distribution of heterochromatin varied among species and seems to evolve as a species-specific trait. We suggest that NOR location variability indicates that some rearrangement mechanisms have taken place during the evolutionary history of this group, because Dendrobatidae is considered a monophyletic taxon. We do not discard that some general pattern in either C-band or NOR location, or both, may emerge when more species of this genus are analyzed.

Key words: C-banding; Cytogenetics; Dendrobatidae; *Epipedobates*; NOR

POISON frogs are common leaf-litter inhabitants of neotropical rainforests. Six genera and 157 species belong to this family, which is characterized by presumably aposematically colored frogs with diurnal activity (Ford, 1993). The genus *Epipedobates* (sensu Myers, 1987) comprises an assemblage of 29 named species (D. Frost, http://research.amnh.org) distributed in lowland and Andean forest, from northern South America to lower Central America. It is recognized by a suite of characters that are considered mostly plesiomorphic within Dendrobatidae (Myers, 1987). The species currently belonging to this genus were formerly classified in other genera, such as *Dendrobates, Allobates,* and *Phyllobates* (D. Frost, http://research.amnh.org), which indicates the difficulty of classifying this group of species. According to Myers (1987), the classification of dendrobatids has never been satisfactory, mainly because they share such a similar morphology that taxonomists had access to few characters.

Karyotypic analysis has been used by systematists in formulating more rigorous hypotheses concerning phylogenies of some groups, and has also been used by cytogeneticists to deduce the chromosomal rearrangements incorporated in the course of evolutionary history (Batistic, 1984; Beçak, 1968; Bogart, 1970, 1973, 1991; Bogart and Wasserman, 1972; Cole, 1974; De Lucca and Jim, 1974; Green, 1986; Morescalchi, 1973, Qumisiyeh and Baker, 1988). Further, comparative cytogenetics has advanced extensively with the development of techniques for differential staining of mitotic chromosomes, such as C-banding and silver-staining of nucleolus organizer regions (NORs) (King, 1980, 1991; Lourenço et al., 1998, 1999; Miura, 1995; Schmid, 1978*a,b*, 1980; Schmid and Almeida, 1988; Schmid et al., 1990*a,b*, 1995).

Regarding dendrobatids, a few studies have taken advantage of karyotype analysis as an additional tool in the analyses of phylogenetic relationships (Bogart, 1991; Morescalchi, 1973; Rasotto et al., 1987), but no study has used differential staining methods on chromosomes in this group. A more extensive cytogenetic analysis using a greater number of species and genera could be useful in the understanding of the evolutionary history of dendrobatids.

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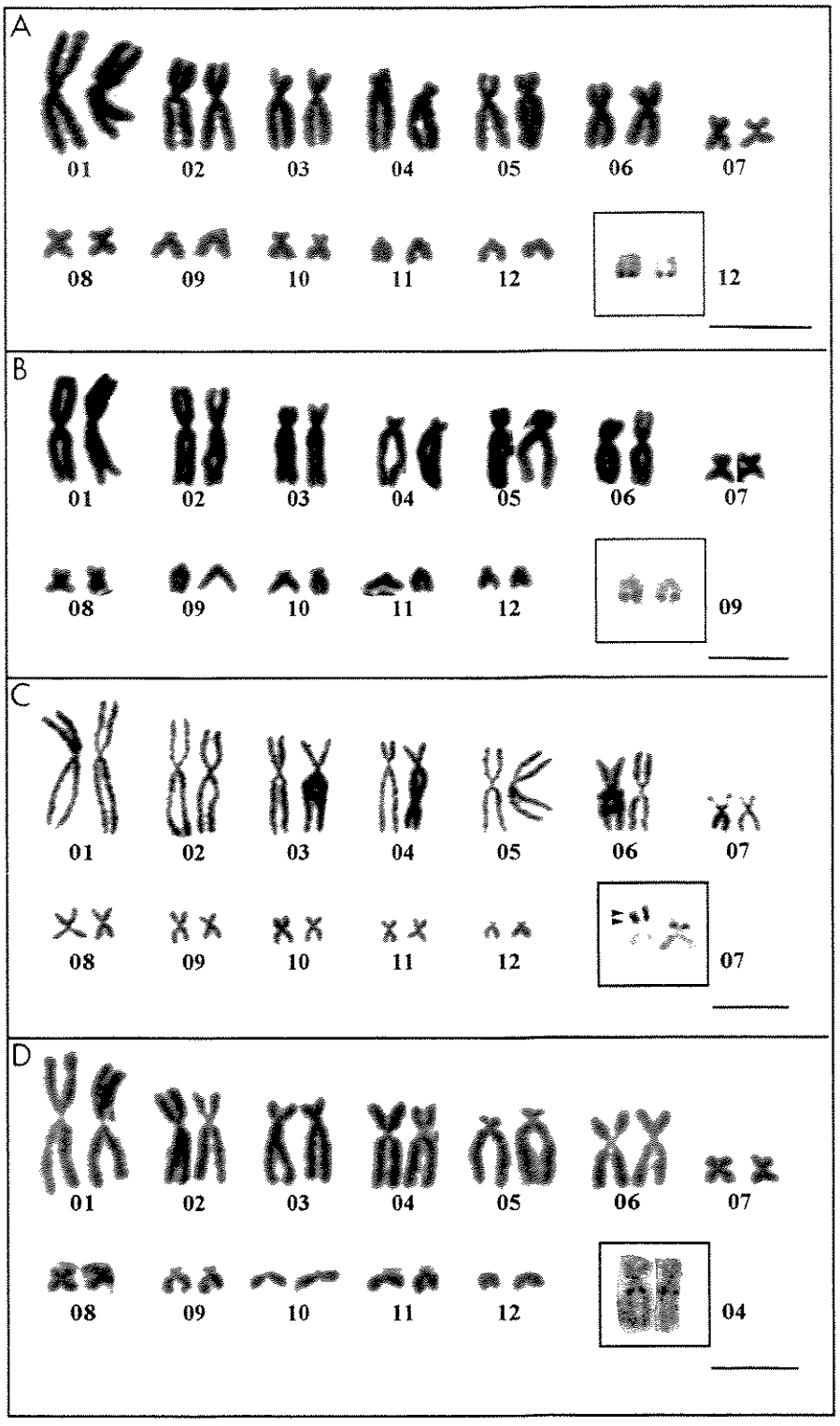
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In the present study, we characterize the karyotypes of *Epipedobates flavopictus*, *E. trivittatus*, *E. femoralis*, and *E. hahneli* with the aim of contributing new data for understanding patterns of chromosomal variation, and the potential of these data to help resolve intra- and intergeneric relationships in the Dendrobatidae.

Materials and Methods

We carried out karyotype analysis on six specimens of *E. flavopictus* (four males, two females), eight *E. trivittatus* (five males, three females), seven *E. femoralis* (four males, three females) and 10 *E. hahneli* (eight males, two females). The specimens were collected in the rainy seasons of 1997 and 1999, with authorization from the Brazilian Institute of Environment (IBAMA) (02005.001367/99-58-AM).

We collected *E. trivittatus* and *E. hahneli* in lowland Amazonia, approximately 40 km south of Manaus, Amazonas, Brazil (3° 37′ 10.4″ S, 59° 86′ 78.4″ W) in the municipality of Castanho, at Km 12 on the road to Autazes. Specimens of *E. femoralis* were collected in a 10,000-ha tropical rainforest reserve, Reserva Florestal Adolfo Ducke (Reserva Ducke), located 25 km northeast of Manaus, Amazonas (03° 08′ S, 60° 04′ W), and *E. flavopictus* were from Serra do Cipó, Santana do Riacho municipality, Minas Gerais State.

All animals received an intraperitoneal injection of 2% colchicine solution 4–6 h before they were sacrificed. Mitotic chromosomes were obtained directly from the intestine and testes after this in vivo colchicine treatment. The techniques used for the preparation of cell suspensions have been described previously (Schmid, 1978*a*; Schmid et al., 1979). Conventional staining with a 10% Giemsa solution was used for analyses of chromosome morphology, and silver nitrate labelling for nucleolar organizer regions (Ag-NOR) followed Howell and Black (1980).

C-banding patterns were obtained by the technique of Sumner (1972). In this procedure, slides were incubated for 30 min at room temperature in 0.2N HCl, then for a variable time at 50 C in a saturated $Ba(OH)_2$ solution, and briefly washed in distilled water. Immediately thereafter, the preparations were incubated for 1 h in 2SSC at 65 C, washed in distilled water, and finally stained with Giemsa (10%, pH 6.8).

Results

All four species had 2N = 24 chromosomes and their karyotypes consisted of six pairs of large chromosomes and six pairs of small chromosomes (Fig. 1; Table 1). Clear karyotypic distinction among the species could be seen from the considerable differences in chromosome arm ratios (Table 1), and most of these were observed among the smaller chromosome pairs (Fig. 1). The number of telocentric chromosome pairs was three in the *E. flavopictus* (chromosomes 9, 11, 12; Fig. 1A) and *E. hahneli* (10, 11, 12; Fig. 1D), two in *E. trivittattus* (9, 11; Fig. 1C), and none in *E. femoralis* (Table 1). Metacentric and submetacentric morphologies prevailed in the larger chromosomes of all four species, although one subtelocentric pair was also found in each of the karyotypes of *E. flavopictus* and *E. trivittatus* (pair no. 4 in both) and *E. hahneli* (pair no. 5) (Table 1).

The karyotype of *E. femoralis* was remarkably different from the others. It had meta- and submetacentric chromosomes in most pairs (eight pairs of meta- and three pairs of submetacentrics), and only one subtelocentric pair (pair no. 12, Fig. 1C; Table 1). Only this species had a conspicuous secondary constriction, on pair no. 7 (heteromorphic among the two homologues) (Fig. 1C).

In spite of these differences, some homeologies can be identified between the four karyotypes. Pairs no. 1–3 and 6–8

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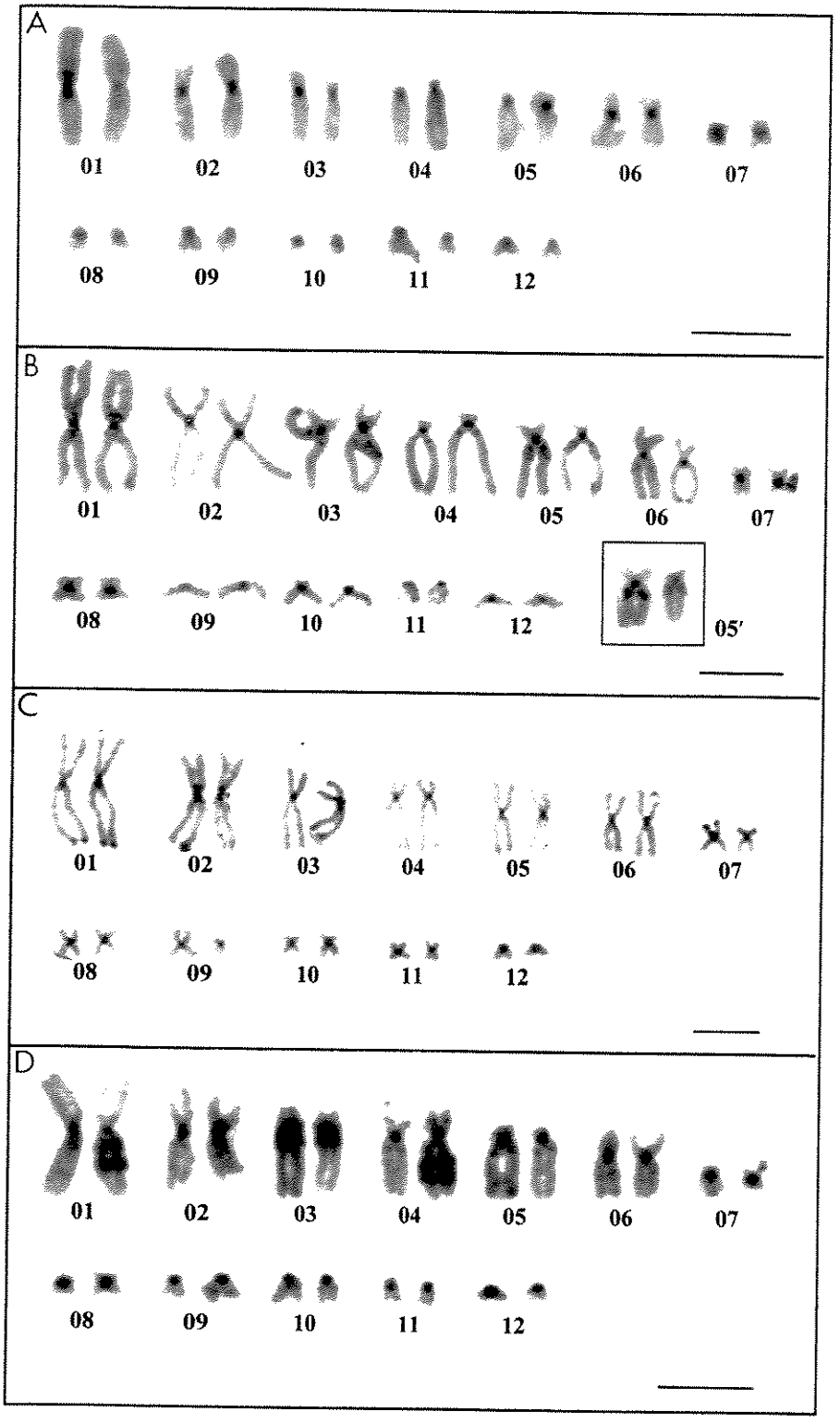
FIG. 1.—Mitotic chromosomes of (A) *E. flavopictus*, (B) *E. trivittatus*, (C) *E. femoralis*, and (D) *E. hahneli* with Giemsa staining. The isolated square inside each graph shows the NOR-bearing chromosome pair in each karyotype. The arrowheads in C indicate the duplicated NOR. Scale bar = 10 μm.


A
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B
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04
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06
07
08
09
10
11
12
05′
C
01
02
03
04
05
06
07
08
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11
12
D
01
02
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11
12

TABLE 1.—Morphometrical data of the chromosomes of the four species of *Epipedobates* (classification according to Green and Sessions, 1991).

	Chromosome											
	1	2	3	4	5	6	7	8	9	10	11	12
						Epipedobates flavopictus						
RL	20.0	13.5	12.1	11.1	10.5	9.7	5.5	4.3	3.5	3.5	3.1	3.1
AR	1.07	1.43	1.91	4.33	2.05	1.54	1.0	1.08	9.0	1.5	8.0	8.0
CP	m	m	sm	st	sm	m	m	m	t	m	t	t
						Epipedobates trivittatus						
RL	15.9	14.8	12.2	10.6	11.5	10.0	4.8	4.4	4.4	4.3	3.78	3.7
AR	1.21	1.50	2.54	4.66	2.70	1.60	1.06	1.33	8.0	2.50	6.6	3.20
CP	m	m	sm	st	sm	m	m	m	t	sm	t	st
						Epipedobates femoralis						
RL	17.3	15.1	12.5	11.5	11.0	9.7	5.3	5.1	3.8	3.7	2.8	2.3
AR	1.42	1.20	1.72	2.75	1.15	1.11	1.76	1.0	1.14	1.07	1.20	3.50
CP	m	m	sm	sm	m	m	sm	m	m	m	m	st
						Epipedobates habneli						
RL	20.0	13.7	12.6	11.8	10.2	10.2	5.5	4.3	3.5	3.1	2.7	2.7
AR	1.21	1.33	2.55	2.0	5.50	1.16	1.33	1.20	3.5	7.0	6.3	6.6
CP	m	m	sm	sm	st	m	m	m	st	t	t	t

RL = relative length, AR = arm ratio, CP = centromere position, m = metacentric, st = subtelocentric, sm = submetacentric, and t = telocentric.

seem to be nearly identical in overall morphology, arm ratios, and centromere positions (Fig. 1; Table 1).

$AgNO_3^-$ staining of the chromosomes revealed that the NORs are located on different chromosome pairs in the four species. The NORs are present at the telomeric region of pair no. 12 in *E. flavopictus*, on the interstitial region of pair no. 9 in *E. trivittatus* (being slightly heteromorphic and adjacent to a heterochromatic band), on pair no. 7 in *E. femoralis* (being heteromorphic and coincident with the secondary constriction), and on the long arm of pair no. 4 in *E. hahneli* (squares in Fig. 1A–D).

The centromeric regions of all chromosomes carry constitutive heterochromatin that is darkly stained (Fig. 2A–D). Considerable interspecific variation in C-banding pattern was observed among the karyotypes examined because of the differences in number, location, and size of interstitial, pericentromeric, and telomeric heterochromatic regions. The remarkable exception was the karyotype of *E. flavopictus*, which possesses only centromeric heterochromatin (Figs. 2A, 3A). Some of the non-centromeric bands present gray staining, that in some cases is not easily observed (Fig. 2A–D, 3A–D).

In *E. trivittatus*, the C-band detected on the long arm of the chromosome pair no. 5 is sub-divided in small blocks [Fig. 2B (square), 3B]. Moreover, telomeric bands are found in the long arm of pairs no. 6, 10, and 12, and the two telocentric chromosomes showed pericentromeric C-bands (Fig. 2B, 3B). Except for the secondary constriction region, the short arm of chromosome pair no. 7 in *E. femoralis* is totally heterochromatic. A distinctive C-band can be seen in the pericentromeric region of the metacentric pair no. 10, and another peculiar band is located in the telomeric region of chromosome pair no. 1 (Fig. 2C, 3C).

The heterochromatic segment on the long arm of chromosome no. 4 of *E. hahneli* was coincident with the ribosomal

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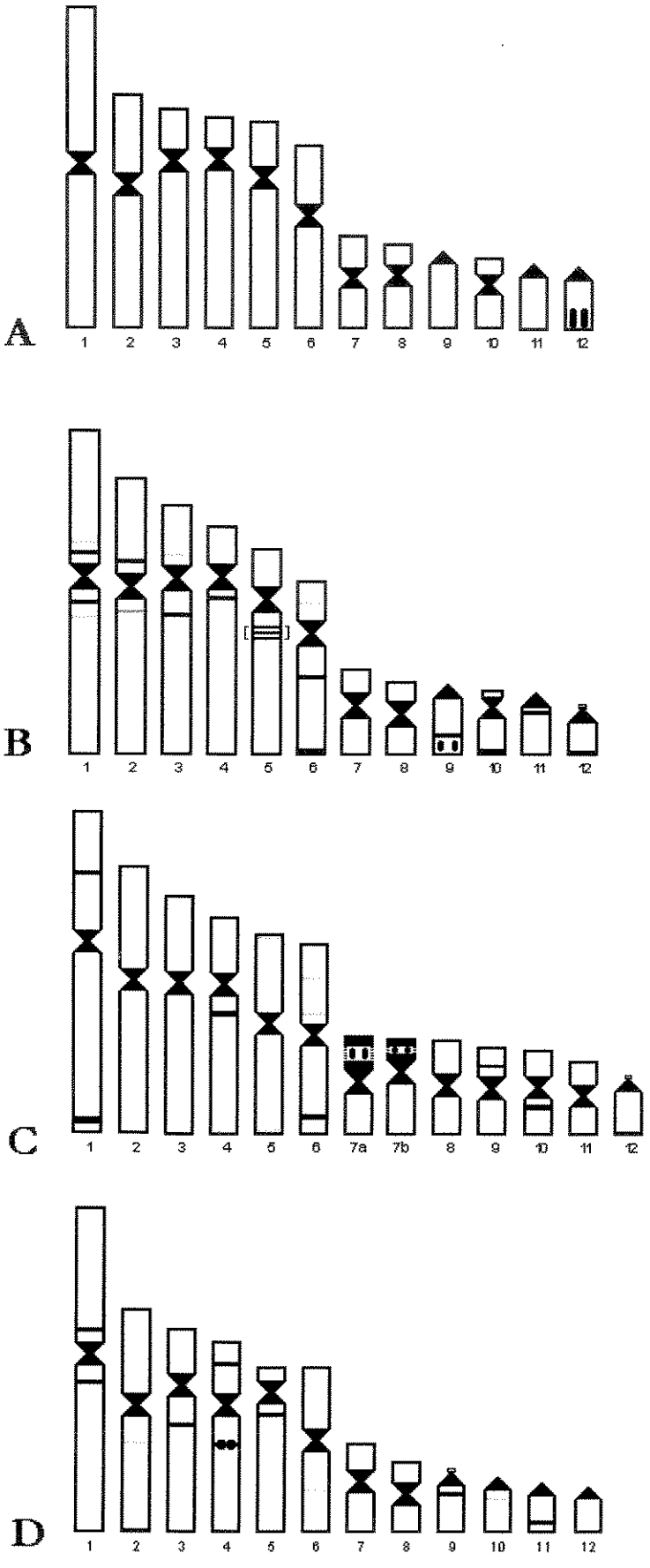
FIG. 2.—C-banded chromosomes: (A) *E. flavopictus*, (B) *E. trivittatus*, (C) *E. femoralis*, and (D) *E. hahneli*. The square in (B) indicates the chromosome that had a differential phenotype of the proximal C-bands. Scale bar = 10 μm.


A
1 2 3 4 5 6 7 8 9 10 11 12
B
1 2 3 4 5 6 7 8 9 10 11 12
C
1 2 3 4 5 6 7a 7b 8 9 10 11 12
D
1 2 3 4 5 6 7 8 9 10 11 12

gene loci. Large centromeric blocks are distinctive in this species. Moreover, in pair no. 4, two large blocks of heterochromatin are apparent on the pericentromeric region of the long and the short arm. Two of the three telomeric pairs (no. 10 and 11) show C-bands in either pericentromeric (pair no. 10) or subtelomeric positions (pair no. 11) (Fig. 2D, 3D).

DISCUSSION

The diploid chromosome number (2N = 24) of the four species analyzed here is in agreement with those presented by Bogart (1991) for *E. trivittatus*, *E. femoralis*, and *E. pictus*. The karyotypes of *E. trivittatus* and *E. femoralis* described by Bogart (1991) are quite similar to those described here, although the secondary constrictions detected in those karyotypes were not observed in our analysis. Bogart (1991) examined only female specimens of *E. femoralis* and also found the heteromorphism in pair no. 7. The heteromorphic pair was regarded as a chromosomal polymorphism or a sex-related characteristic. We analyzed male and female karyotypes and verified that this heteromorphism, found in all of the specimens, is due to the different length of the secondary constriction (in the short arm) between the two homologous and, therefore, is probably not sex-related.

Although the four species have the same chromosome number, they can be easily separated from each other by some differences in chromosome morphology. Whereas we found no telocentric chromosomes in *E. femoralis*, we observed two in *E. trivittatus* and three in *E. flavopictus* and *E. hahneli*. Also, the large group of chromosomes of *E. femoralis* has only meta- and submetacentric pairs, while the other species also have one pair of subtelocentric chromosomes (pair no. 4 in *E. femoralis* and *E. trivttatus*, and pair no. 5 in *E. hahneli*). Such differences in chromosome morphology can also be observed in the karyotypes of *Colosthethus* and *Dendrobates* that have been described; and in the latter, chromosome number variation (2N = 18 to 20) was detected also (Bogart, 1991; Rasotto et al., 1987).

Telocentric chromosomes have been found in all dendrobatid genera examined, including those species with lower chromosome number, regarded as an apomorphic state (Bogart, 1991; Rasotto et al., 1987). This led Bogart (1991) to conclude that the analysis of the number of chromosome arms would be of little value for understanding karyotype evolution in the family Dendobatidae and that chromosomes have undergone extensive restructuring via translocations and inversions in this group. In the species of *Epipedobates*, such restructuring seems to have occurred without any change in the chromosome number. The karyotypic differences found here can also suggest that the four species have probably acquired the same chromosome number by the process of convergence, as indicated by Bogart and Hedges (1995) for *Eleutherodactylus gossei* and *E. pantoni*. The results obtained for *Epipedobates femoralis* seem to be additional data that point out the peculiarity of this species within this genus. Behavioral, dietary, and molecular data have also provided evidence that proposes the classification of this species in a separate clade, more closely related to the genus *Colostethus* (Caldwell, 1996; Clough and Summers, 2000; Toft, 1995; Vences et al., 2000; Zimmermann and Zimmermann, 1988).

A general feature of the anuran karyotypes, as indicated by Schmid (1978*a*) and Schmid et al. (1990*b*), is the presence of only one pair of NORs, usually located on the same chromosome region in species

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FIG. 3.—Representative ideogram of the number of the chromosome, Ag-NOR location and C-banding pattern: (A) *E. flavopictus*, (B) *E. trivittatus*, (C) *E. femoralis*, and (D) *E. hahneli*. The dark areas indicate the strongly stained heterochromatin, and the gray sectors indicate the faintly stained one. The dark circles show the location of the NORs. The rectangle in the proximal region of chromosome 5 in (B) indicates the variable phenotype of these bands.

belonging to the same or closely related groups. In the species of *Epipedobates* analyzed in the present work, the NORs are located on different chromosome pairs. Such interspecifc variation was also found in some hylid (Anderson, 1991; Schmid, 1978*a*,*b*) and some bufonid species (Anderson, 1991; Baldissera et al., 1999). According to Schmid et al. (1990*b*), these examples of interspecific variability in NOR location indicate that chromosomal rearrangements occurred in the NOR-bearing chromosome segments during evolution. Translocations and inversions could be the mechanism implicated in the transfer of NORs between chromosomes as complete packages (Schmid, 1978*a*).

The NOR size heteromorphism between homologous chromosomes, like that found in *E. trivittatus*, also occurs in all other vertebrate classes and seems to be a general property of nucleolus organizers (Schmid et al., 1990*b*). In anurans, a high frequency of NOR heteromorphisms has been shown, and some of them are due to tandem duplication (as observed in chromosome no. 7 of *E. trivittatus*) or triplications in one of the two NORs (Schmid, 1982; Schmid, 1978*a*). Schmid (1982) and Schmid et al. (1990*b*) suggest two mechanisms to explain such development of heteromorphic NORs: unequal meiotic crossovers or sister chromatid exchanges. Gold (1979) also considered that mechanisms of disturbance in the DNA duplication could be responsible for spontaneous additions or deletions of NOR material. The slight heteromorphism on pair no. 9 in *E. trivittattus* probably reflects a simple amplification of ribosomal cistrons.

Heterochromatin associated with rDNA, like that observed in the NOR-bearing chromosome 4 of *E. hahneli*, was also found in a species of *Bufo* examined by Kasahara et al. (1996). King (1991) suggested that the spacer regions between the 28S+18S cistrons are probably composed of highly repetitive DNA, which could explain the staining of such segments by the C-banding technique.

Our study is the first report of C-banding analysis on any member of the family Dendrobatidae. The karyotypes of the species of *Epipedobates* presented variation in the stainability of the bands and in the amount and distribution of heterochromatin. Variation in the stainability of some C-band positive regions after alkaline denaturation has also been reported in other studies (King, 1980; Miura, 1995) and may indicate that the constitutive heterochromatin has a heterogeneous composition, as predicted by Arrighi and Hsu (1971) and Schmid (1978*b*). King (1991) suggests that the increase in the amount of heterochromatin, by addition at particular chromosomal sites or by the transformation of euchromatic regions to constitutive heterochromatin, seems to be an apomorphic characteristic in anuran karyotypes (see also review by Imai, 1991). If such processes also take place in the chromosome evolution of the genus *Epipedobates*, we can infer that the karyotype of *E. flavopictus* is more plesiomorphic, because only centromeric bands were found. Nevertheless, we cannot discard that such a striking difference in the amount of heterochromatin may be related with the occupation of a distinct habitat in relation to the Amazon species.

The high variability in amount and distribution of C-band observed in our analysis is in agreement with that described for other species of neobatrachian anurans. According to King (1991), most neobatrachian species analyzed by the C-banding technique show enormous interspecific diversity in both the distribution and amount of heterochromatin within their genomes; although, in some cases, they present regularities that can be used to characterize species groups (Kasahara et al., 1996). Such noticeable interspecific variation on the C-banding pattern found in these *Epipedobates* karyotypes precludes the recognition of synapomorphies among them, and thus makes a secure inference of their phylogenetic relationships difficult. Kaiser et al. (1996) and Bogart and Hedges (1995) analyzed some of the 30-chromosome species of *Hyla* and some species of *Eleuthrodactylus* respectively, and they also considered that heterochromatin variability is a poor indicator of homeology, providing little information useful to de-

termine the relationships among species. Kaiser et al. (1996) also suggested that, because the chromosome number (2N = 30) is retained in all karyotypes of the *Hyla* that they examined and the banding analyses did not enable a detailed identification of homeologous regions on chromosomes, the majority of the differences in morphotypes of the chromosomes can be attributed to inversions. This is also the probable mechanism that has occurred in the karyotypes of *Epipedobates* described here. Green (1986) also found some differences in chromosome morphology and C-band pattern in species of *Rana*. Using the known karyotypes of the species of the *R. pipens* group as an outgroup, he coded chromosomal character-state data matrix for a phylogenetic analysis, and the results reinforced earlier morphologically based taxonomies. Green (1986) concluded that, while chromosome bands provide more characters in individual karyotypes, determination of homeologies between chromosome pairs remains a problem. For the moment, phylogenetic analyses, like those of Green (1986), are not possible in Dendrobatidae due to the scarcity of karyotypic data.

In conclusion, the variability in the NOR and C-banding patterns observed here reinforce the hypothesis that dendrobatid chromosomes have undergone extensive restructuring via translocations and inversions, as proposed by Bogart (1991). We do not discard the possibility that these interspecific variations for the genus *Epipedobates* can be minimized when more species have been analyzed. According to Bogart (1973), when the karyotypes of a large number of related species are examined, patterns usually emerge. Also, we do not discard that such variations may be a consequence of the possible paraphyly of the genus *Epipedobates*, recently indicated by molecular analysis (Vences et al., 2000).

According to King (1990), karyotypic similarities and differences may be used to infer phylogeny if chromosome numbers, C-bands, sites of secondary constrictions, or other "markers" can be traced to a common ancestral karyotype in a monophyletic lineage. Although the present work contributes new data on the karyotypes of the poison frogs, there is still insufficient information for phylogenetic reconstructions. When more species have been analyzed, including those belonging to the currently accepted basal Dendrobatidae genera (*Colostethus* and *Aromobates*), we will be open to the possibility of coding individual chromosome pairs in a phylogenetic character matrix, following Green (1986) and Borwick (1995).

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APPENDIX I

Specimens Examined

Specimens were deposited in the Herpetology Collection "Gloria Moreira" of the Instituto Nacional de Pesquisas da Amazônia (INPA) and in the Museu de História Natural "Prof. Adão José Cardoso" (ZUEC) at the State University of Campinas, as follows: *E. flavopictus* (ZUEC 11428–11431, 11434 and 11436), *E. trivittatus* (INPA 77.83, 77.85–77.89, 77.91 and 77.94) and (ZUEC 11739 and 11740), *E. femoralis* (ZUEC 11455–11458, 11748, 11752 and INPA 77.81), and *E. hahneli* (INPA 77.69, 77.71, 77.72, 77.75–77.80 and ZUEC 11769).

Artigo II

(no prelo – Copeia)

Obs: Este artigo foi realizado em colaboração com Cristina Rosa, tendo sido apresentado também como parte de sua tese de Mestrado.

Karyotypic variation in the genus *Megaelosia* (Anura, Hylodinae) with the first description of a B chromosome in a leptodactylid frog

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Key words.— *Megaelosia;* Hylodinae; B chromosomes; cytogenetics; C-band; Ag-NOR.

Running haed.— Cytogenetics of *Megaelosia*

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Three species of the genus *Megaelosia* (*M. massarti*, *M. boticariana* and *M. lutzae*) were studied karyotypically using conventional Giemsa staining, C-banding and NOR techniques. *Megaelosia lutzae* had 2n=32 chromosomes, a new diploid number for this genus and for the subfamily Hylodinae. Two morphotypes of B chromosomes were found in two specimens of *M. massarti*. C-banding patterns and NOR location varied among the species. Nevertheless, some chromosome pairs, as well as the NOR-bearing secondary constriction and the large pericentromeric C-block on the short arm of chromosome 10, were consistent among the three species. *Megaelosia* differs markedly from other Hylodinae and Dendrobatidae in terms of karyotype.

The subfamily Hylodinae (Leptodactylidae) includes diurnal anurans distributed in southern and southeastern Brazil and northern Argentina (Frost, 2000). The 32 species currently recognized in this subfamily are grouped into three genera: *Hylodes* (17 spp.), *Crossodactylus* (10 spp.) and *Megaelosia* (5 spp.). *Megaelosia* differs markedly from the other hylodines in morphology. However, the presence of paired lateral vocal sacs, considered a derived condition, in *M. lutzae* and *M. massarti*, which are similar to *Hylodes*, supports the retention of *Megaelosia* in the Hylodinae (Lynch, 1971). *Hylodes* and *Crossodacylus* have a conservative chromosome number (2n=26), although a 24-chromosome karyotype was described for *Hylodes nasus* (Denaro, 1972; De Lucca and Jim, 1974; Bogart, 1991). *Megaelosia* has two karyotypes, with 2n=28 chromosomes in *M. massarti* (Melo et al., 1995) and 2n=30 in *M. boticariana* (Giaretta and Aguiar-Jr., 1998). The larger and variable chromosome number, the large body size (about four times larger than other hylodines), the dietary habits such as batrachophagy, and the absence of announcement call (Giaretta et al., 1993) are special characteristics of *Megaelosia* species not found in the other two hylodine genera.

The phylogenetic relationship between hylodines and dendrobatids remains controversial. Lynch (1971) proposed the origin of dendrobatids from the Hylodinae based on the morphological characteristics of *Hylodes* and *Crossodactylus,* and this was reinforced by Bogart (1991) based on karyological data for *Hylodes* species.

In this report, we provide a more complete cytogenetic analysis of three *Megaelosia* species, *M. massarti*, *M. boticariana*, and *M. lutzae*, aiming to contribute with chromosomal data for a future phylogenetic analysis within the Hylodinae and also to assess the Hylodinae-Dendrobatidae affiliation. We also describe a new chromosome number (2n=32) in *M. lutzae,* and a B chromosome with two morphotypes in *M. massarti.*

MATERIALS AND METHODS

Animals.—Four specimens of *M. massarti* (3 adult females and 1 sub-adult) from Paranapiacaba, Santo André, eight tadpoles of *M. lutzae* from Pindamonhangaba and four tadpoles of *M. boticariana* from Atibaia were studied. The three municipalities are located in São Paulo state. The adult and sub-adult specimens were deposited in the "Professor

Adão José Cardoso" Natural History Museum (ZUEC) of the Universidade Estadual de Campinas, Brazil, under accession numbers ZUEC 11395-11397 and 11553.

Chromosome preparations and techniques. — All the metamorphosed frogs received an intraperitoneal injection of 1% colchicine solution at least 4 h before killing, as described by Schmid et al. (1979). The tadpoles were kept alive in a 1% colchicine solution for 4-5 h. After treatment with colchicine, the animals were killed by narcosis with ether and slides were prepared from cells suspension of intestine epithelium and testis. For morphological studies, the slides were stained conventionally with Giemsa solution. Mean descriptive values for the karyotypes were calculated from information obtained from at least five well-spread mitotic metaphases for each specimen. The nomenclature of Green and Sessions (1991) was used to describe the chromosomal morphology. C-banding patterns were obtained by the technique of Sumner (1972), with slight modifications. Silver nitrate labeling for nucleolar organizer regions (Ag-NOR) was done as described by Howell and Black (1980).

RESULTS

Karyotype.— *Megaelosia boticariana* had 2n=30 chromosomes (Fig. 1A). Pairs 10-15 were metacentrics, while pairs 1, 3-5, 7-9 were submetacentrics and pairs 2 and 6 were subtelocentrics (Figs. 1A, 2A and Table I). *Megaelosia lutzae* showed 2n = 32 chromosomes consisting of seven pairs of metacentrics (pairs 8-10, 12-14, 16), five pairs of submetacentrics (2-6), one subtelocentric pair (pair 1) and three pairs of telocentrics (7, 11, 15) (Figs 1B, 2B and Table I). A secondary constriction was observed on the short arm of pair in 1 *M. boticariana* and on pair 3 in *M. massarti*, while in *M. lutzae* the constriction was located in the pericentromeric region of telocentric pair 15. The secondary constriction showed size heteromorphism in the three species (Figs. 1A-B, 2A-B, 3A and 4A).

In two specimens of *M. massarti,* there was one supernumerary (B-) chromosome in all of the cells examined. However, the B-chromosome had a different morphology in each specimen analyzed, being a small metacentric in ZUEC 11396 (165 metaphases analyzed) (Figs. 3B, 4B and Table I) and a large submetacentric in ZUEC 11397 (160 metaphases analyzed) (Figs. 3C, 4C and Table I). In both karyotypes with B-chromosomes (2n=28+1),

the most chromosomes of the A complement had the same morphology as in specimens without B-chromosomes (Figs. 3A, 4A and table I), except for pairs 1 and 2 of the karyotype carrying the large B-chromosome, in which they were classified as submetacentric and metacentric, respectively. A secondary constriction was also detected on the long arm of pair 3 (Figs. 3B, C, and 4B, C).

C- banding. — In the three species, almost all the centromeric and telomeric regions of the chromosomes showed darkly stained bands (Figs. 5A–C, 6A-B, 2 and 4). Differences in the number, location and size of the heterochromatin regions revealed interspecific variation in the C-banding pattern among the species. In *M. massarti,* some of the non-centromeric bands showed gray staining, which was not easily observed (Fig. 5A). The short arm of pair 10 showed a large block of pericentromeric heterochromatin in the three species. Also, the secondary constrictions (pairs 3, 1 and 15, respectively) in the three karyotypes had associated heterochromatin (Figs. 5and 6). The small B-chromosome was totally heterocromatic (Fig. 6A, and 4B) and the large B chromosome had a C-band only in the telomeric region of both arms (Fig. 6B and 4C).

Nucleolus organizer region — The NORs were located on different chromosome pairs in the three species (Figs. 7, 2A-B and 4A). In *M. massarti*, both the 28 and 29 chromosome karyotypes had the nucleolar organizer region in pair 3. This NOR was heteromorphic and coincident with the large heterochromatic block and the secondary constriction. In *M. boticariana,* the NOR was located on the short arm of pair 1, and was strikingly heteromorphic between the homologues. *Megaelosia lutzae* had the NOR in the pericentromeric region of pair 15, and was heteromorphic and coincident with an heterochromatic block.

DISCUSSION

Our description of the karyotypes of *M. massarti* and *M. boticariana* are in general agreement with Melo et al. (1995) and Giaretta and Aguiar-Jr. (1998). They differ significantly from the 32-chromosome karyotype of *M. lutzae*, which constitutes a new diploid number for *Megaelosia* and the subfamily Hylodinae. The three *Megaelosia* species analyzed also differed from each other in the morphology of most of the chromosome pairs. Only pairs 8-10, 12 and 14 showed some homologies, despite the slight differences in arm size in chromosomes 8, 9 and 14. A conspicuous NOR-bearing secondary constriction with a darkly stained associated heterochromatic segment is identical in the three karyotypes.

The C-banding pattern varied considerably among the three species. Only the large heterochromatic block found in the short arm of pair 10 and the banding pattern of the NOR-carrying chromosome were consistent. The short arm of pair 13 in *M. massarti* and *M. boticariana* was totally heterochromatic. The differential C-banding response of other pairs was rather complex and difficult to interpret, and precluded any unambiguous recognition of homologies. It was not possible to compare and use the amount of heterochromatin as an indicator of the evolutionary trend among these karyotypes (see King, 1980, 1991).

Interspecific variability in the amount and distribution of heterochromatin is a general feature of most anurans, even in species with a conservative karyotype (King, 1991; Bogart and Hedges, 1995; Spasic-Boskovic et al., 1997; Formas and Cuevas, 2000). All three species had a pronounced amount of heterochromatin concentrated in the telomeric region of most chromosome pairs. The variable intensity of staining in some heterochromatic regions indicated that the heterochromatin had a heterogeneous composition in these species (see Hsu and Arrighi, 1971).

As stated above, the NOR-carrying secondary constriction appeared to be homologous among the three species studied. Such homology reinforces the hypothesis that these karyotypes have undergone extensive rearrangements, which have altered the chromosome number and NOR location since in the most closely related anuran species the NOR position is almost always stable (Schmid, 1982; King, 1990; Amaral et al., 2000).

The heteromorphism among homologous NORs observed here appear to involve the amplification of the ribosomal cistrons in one of a pair of homologues, as also suggested

for most NOR heteromorphisms in the Anura (King, 1990). Schmid et al. (1990) proposed that unequal meiotic crossovers or sister chromatid exchanges could also give rise to heteromorphic NORs.

Within the Anura, supernumerary chromosomes have been described in the Leiopelmatidae, Pelobatidae, Hylidae and Ranidae (reviewed by Green, 1991). B chromosomes, such as occur in *M. massarti*, are not known in any other Leptodactylidae frog. The small B chromosome shows the common morphological properties which classically characterize supernumerary chromosomes, i.e., it is small and totally heterochromatic (see Green, 1990; Camacho et al., 2000). However, the large B found in ZUEC 11397 showed only small telomeric C-blocks on both arms. The karyotype with this large extra chromosome showed a slight change in the size of chromosome pairs 1 and 2 (shifting the position in the karyogram). Restructuring of the A set in karyotypes carrying supernumeraries is very uncommon and may indicate that additional rearrangements have occurred. In Anura, the presence of distinct morphotypes for B chromosomes in a population has been reported for *Leiopelma hochstetteri*, which has the greatest variation in supernumerary chromosomes among vertebrates (Green et al., 1987; Green, 1988, 1991). The variation in the remaining anuran species is restricted to the number of these extra elements (Green, 1991).

Different mechanisms have been proposed to explain the origin, maintenance and frequency of B chromosomes, the most accepted being that they may have derived from A chromosomes as a by-product of the standard karyotype (Camacho et al., 2000). An independent origin for these chromosomes has also been suggested (Jones and Rees, 1982). According to Green (1990, 1991), there is no evidence to indicate that these chromosomes are evolutionary vestiges of ancestral karyotypes, and their low frequency of occurrence across species suggests a random process of generation. As reported by Oliveira et al (1997), the structure, function and behavior of supernumerary chromosomes are quite peculiar in different groups, making it difficult to generalize about their importance for the species. Any hypothesis about the origin and differentiation of supernumeraries in *M. massarti* is, at best speculative. Nevertheless, these chromosomes contribute to the karyotypic variability in *Megaelosia*.

In conclusion, *Megaelosia* differs considerably from *Hylodes* and *Crossodactylus*, which have a uniform karyotype within each genus (Bogart, 1970; Denaro, 1972; De Lucca

and Jim, 1974). Lynch (1971) argued that *Megaelosia* species have certain primitive characteristics and that the genus belongs to the subfamily Hylodinae only because of the derived condition of the vocal apparatus shared with *Hylodes*. The similar external morphology of *Megaelosia* and *Hylodes* species may simply be the result of convergence. Our data agree with the distinctiveness of *Megaelosia* within the Hylodinae but are unable to provide any evidence toward assessing Hylodinae-Dendrobatidae affiliations due to both, the high intrageneric karyotypic variation and the absence of homologies with those dendrobatid karyotypes already analyzed under conventional staining methods and C-banding techniques (see Aguiar-Jr. et al., 2002).

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Table 1 – Morphometric analysis of the chromosomes of three *Megaelosia* species, and two specimens with supernumerary chromosomes. CH= chromosome, IC=centromeric index, RL=relative length, CP= centromeric position, M=metacentric, SM=submetacentric, ST=subtelocentric and T=telocentric. Classification according to Green and Sessions (1991).

Megaelosia massarti

CH	1	2	3	4	5	6	6'	7	8	9	10	11	12	13	14
IC	0.80	0.26	0.27	0.37	0.15	0.31	0.35	0.32	0.42	0.44	0.43	0.44	0.43	0.34	0.30
RL	12.38	11.97	10.32	10.15	7.49	7.48	7.47	6.91	5.8	5.39	4.98	4.76	4.41	4.00	3.22
CP	M	SM	SM	M	ST	SM	SM	SM	M	M	M	M	M	SM	SM

Megaelosia boticariana

CH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
IC	0.33	0.24	0.34	0.27	0.25	0.23	0.29	0.33	0.36	0.42	0.43	0.40	0.42	0.46	0.45
RL	13.52	12.39	10.44	9.31	8.64	7.71	6.29	4.87	4.81	4.46	4.18	3.74	3.31	3.31	3.02
CP	SM	ST	SM	SM	SM	ST	SM	SM	SM	M	M	M	M	M	M

Megaelosia lutzae

CH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15'	16
IC	0.24	0.37	0.37	0.25	0.27	0.32	0	0.39	0.43	0.43	0	0.44	0.45	0.46	0	0	0.46
RL	11.53	9.95	9.12	8.17	7.85	6.69	6.40	5.43	5.05	4.82	4.51	4.33	3.96	3.7	3.6	4.82	3.39
CP	ST	SM	SM	SM	SM	SM	T	M	M	M	T	M	M	M	T	T	M

***Megaelosia massarti* (small B chromosome)**

CH	1	2	3	4	5	6	6'	7	8	9	10	11	12	13	14	B
IC	0.39	0.30	0.29	0.38	0.19	0.33	0.34	0.28	0.41	0.40	0.40	0.39	0.32	0.32	0.29	0.43
RL	12.0	11.10	10.20	9.83	7.55	7.48	7.33	6.80	5.32	5.10	4.88	4.56	4.11	3.90	3.18	4.86
CP	M	SM	SM	M	ST	SM	SM	SM	M	M	M	M	M	SM	SM	M

***Megaelosia massarti* (large B chromosome)**

CH	1	2	3	4	5	6	6'	7	8	9	10	11	12	13	14	B
IC	0.25	0.39	0.34	0.38	0.17	0.31	0.35	0.34	0.39	0.44	0.44	0.45	0.47	0.45	0.30	0.37
RL	11.8	10.0	9.53	7.75	7.46	6.49	6.40	5.66	5.05	4.54	4.34	3.93	3.92	3.54	3.45	12.4
CP	SM	M	SM	M	ST	SM	SM	SM	M	M	M	M	M	SM	SM	SM

LEGENDS

Figure 1. Giemsa stained karyotypes of *M. boticariana* (A) and *M. lutzae* (B). Note the conspicuous secondary constriction (arrowheads) in the two karyotypes. Bar = 5μm.

Figure 2. Ideograms of the *M. boticariana* (A) and *M. lutzae* (B) karyotypes. Dark sectors indicate C-bands. Dotted areas represent the secondary constrictions. The gray areas indicate NOR sites.

Figure 3. Karyotypes of three females of *M. massarti* with 28 (A) and 29 chromosomes (B and C). The inset shows the small supernumerary in specimen ZUEC 11396 (B) and the large supernumerary in specimen ZUEC 11397 (C). In C, note the difference in morphology of chromosomes 1 and 2 compared to the standard 28-chromosome karyotype . The arrow-heads indicate the conspicuous secondary constrictions. Bar = 5μm.

Figure 4. Ideograms of the 28 (A) and 29-chromosome karyotypes of *M. massarti* with small (B) and large (C) supernumerary chromosomes. The dotted areas show the secondary constrictions and the gray areas represent the NOR. Dark sectors denote the C-band regions.

Figure 5 - C-banded chromosomes of females *M. massarti* (A), and tadpoles of *M. boticariana* (B) and *M. lutzae* (C). Note that most of centromeric region are C-banded. Note also the presence in the three karyotypes of a large C-block coincident with the secondary constriction and a dark pericentromeric and centromeric band on the short arm of pair 10. Bar = 5μm.

Figure 6. Banding pattern of 29-chromosome karyotypes of two females of *M. massarti*. The inset shows the fully heterochromatic small B chromosome (A) and the large B chromosome with weakly stained telomeric C-bands (B). Bar = 5μm.

Figure 7. NOR-bearing chromosome pairs in females of *M. massarti* from a standard karyotype (A), karyotype with small (B) and large (C) supernumerary chromosomes; and tadpoles of *M. boticariana* (D) and *M. lutzae* (E). Bar 5=μm.

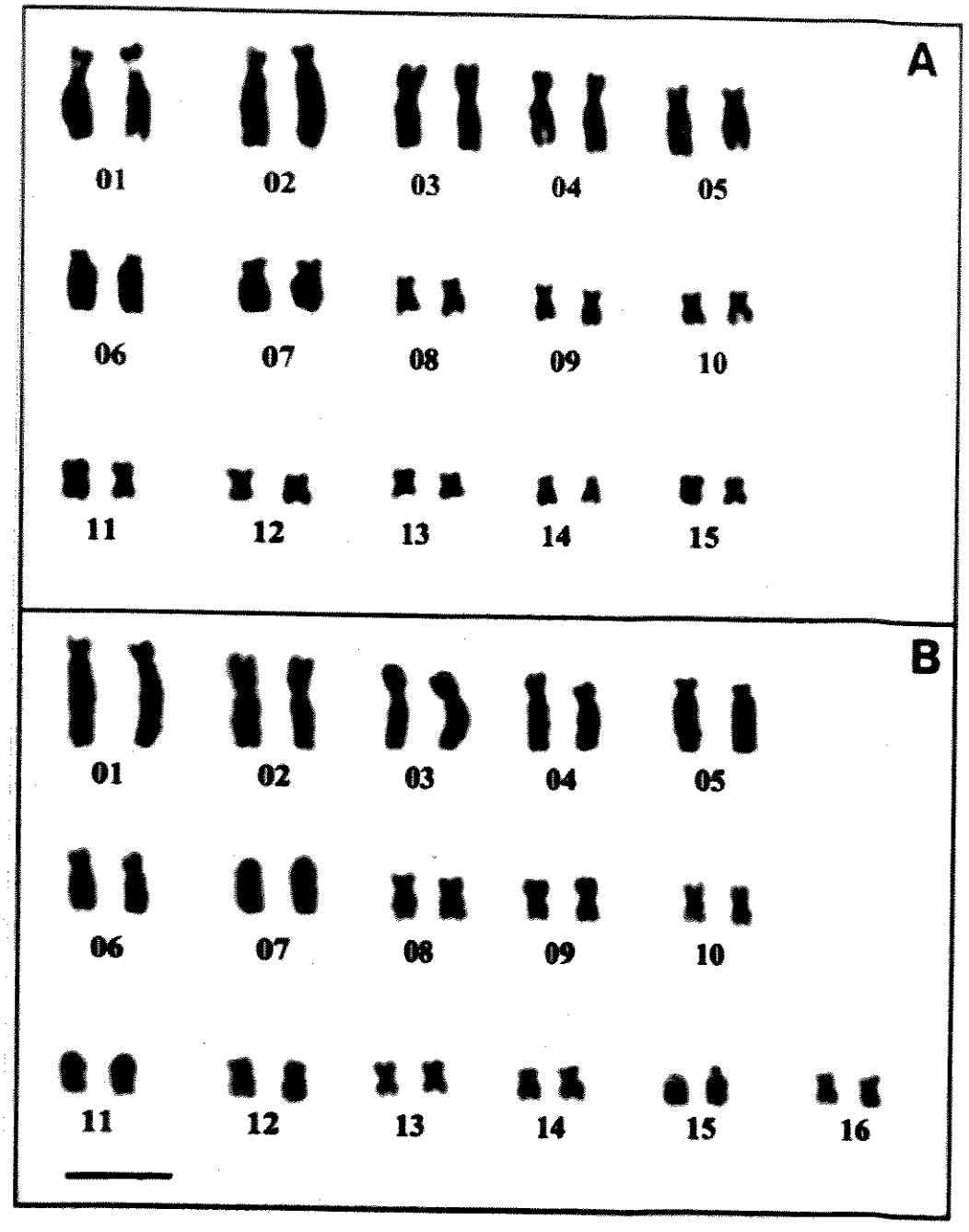
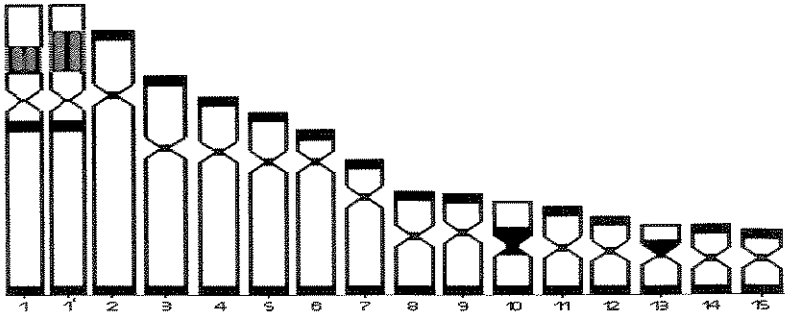
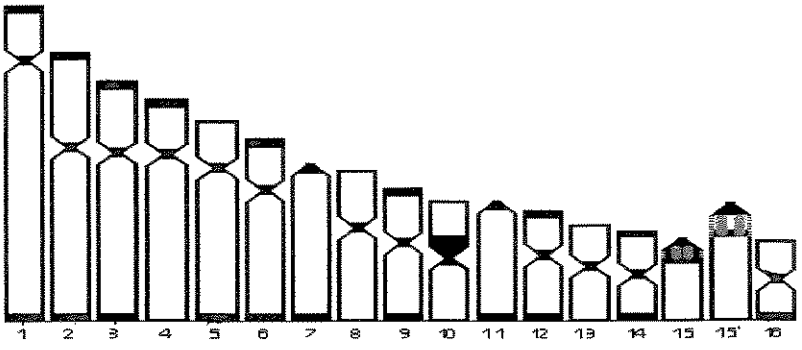


Figure 1



A



B

Fig. 2

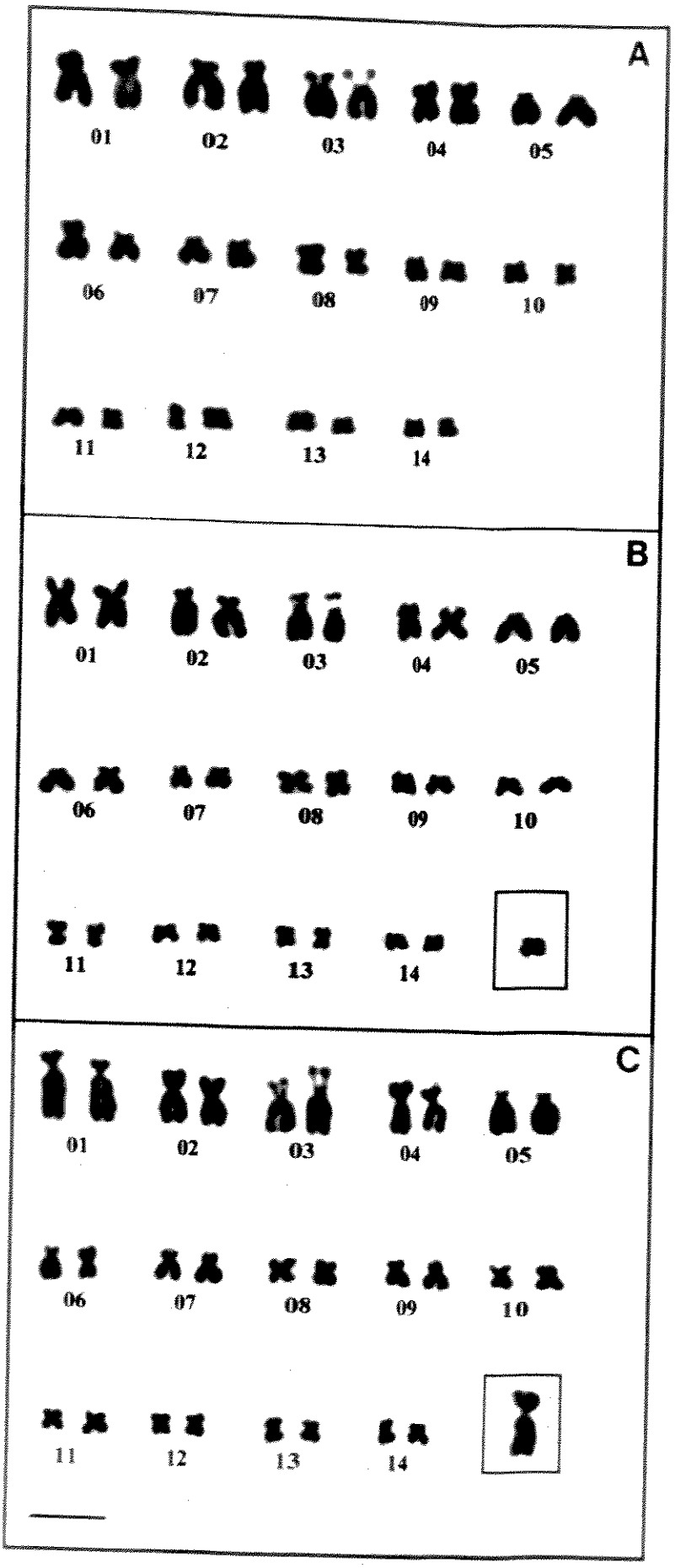

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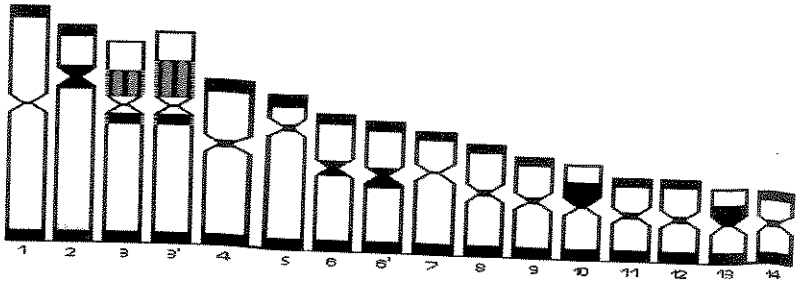
Figure 3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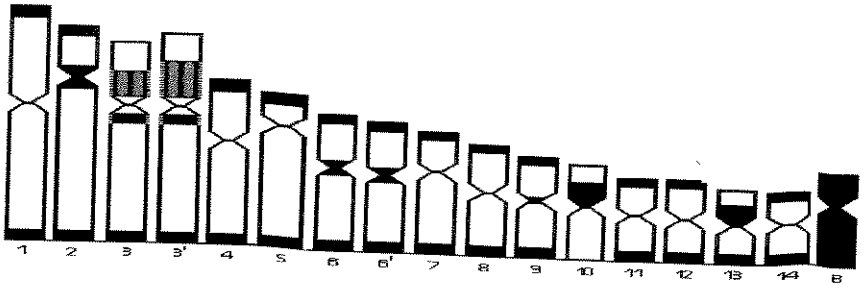
A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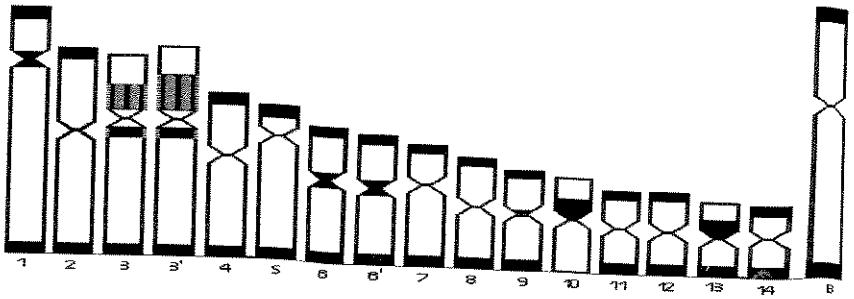
B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C

Fig. 4

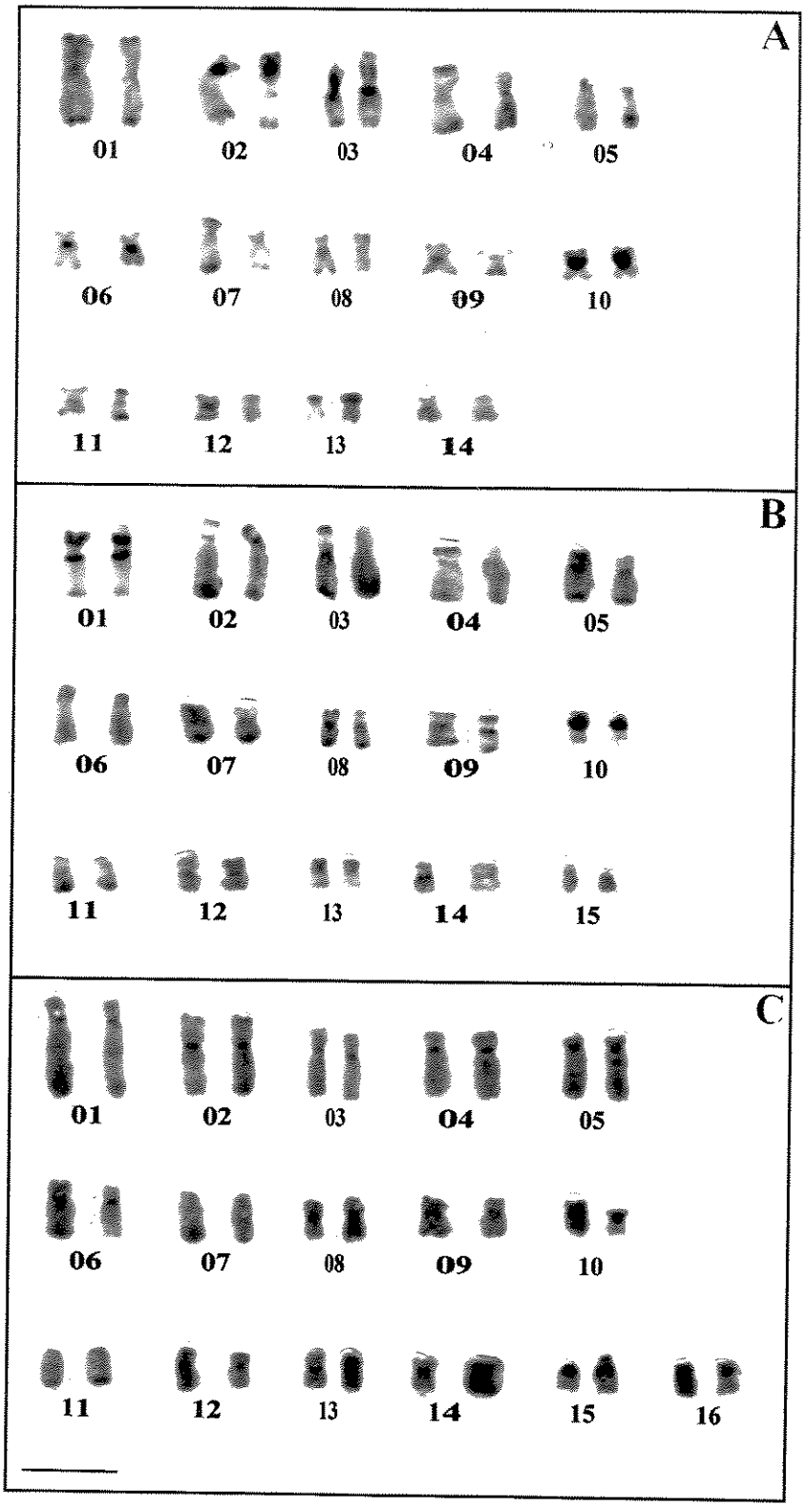
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Figure 5

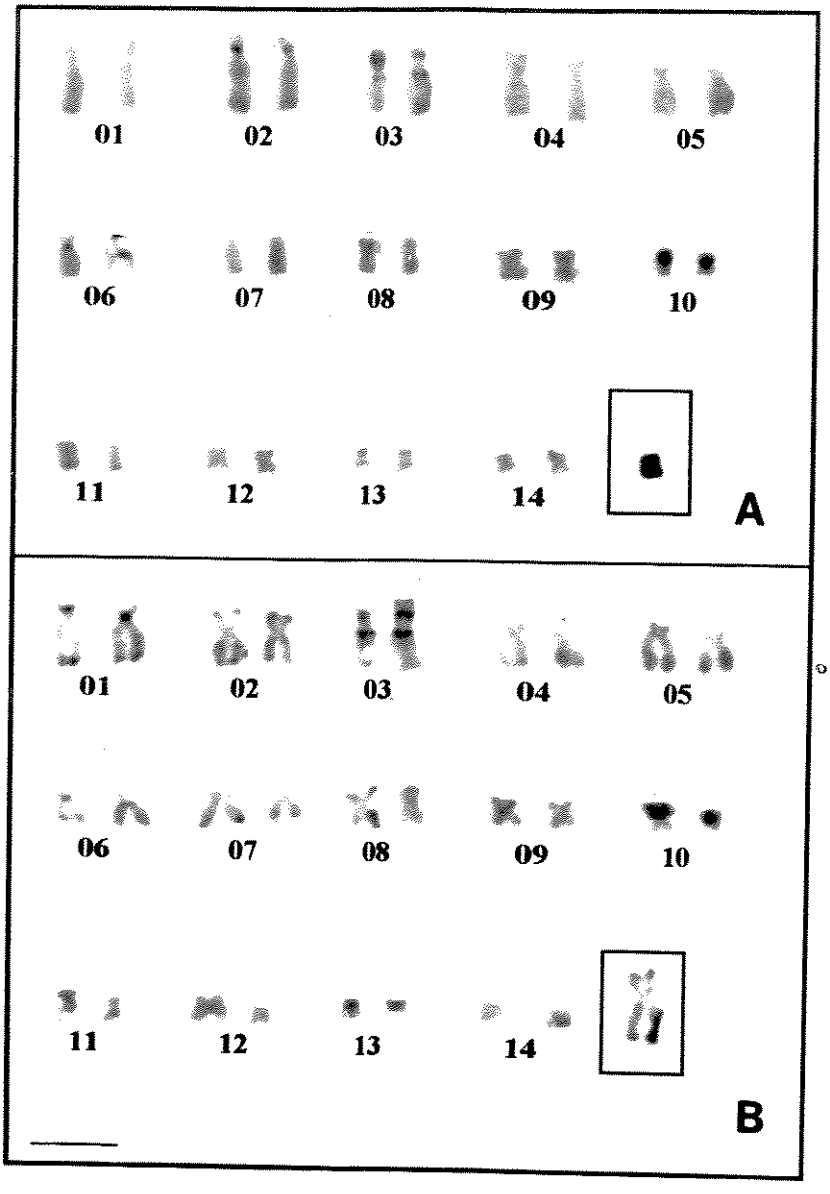
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Figure 6

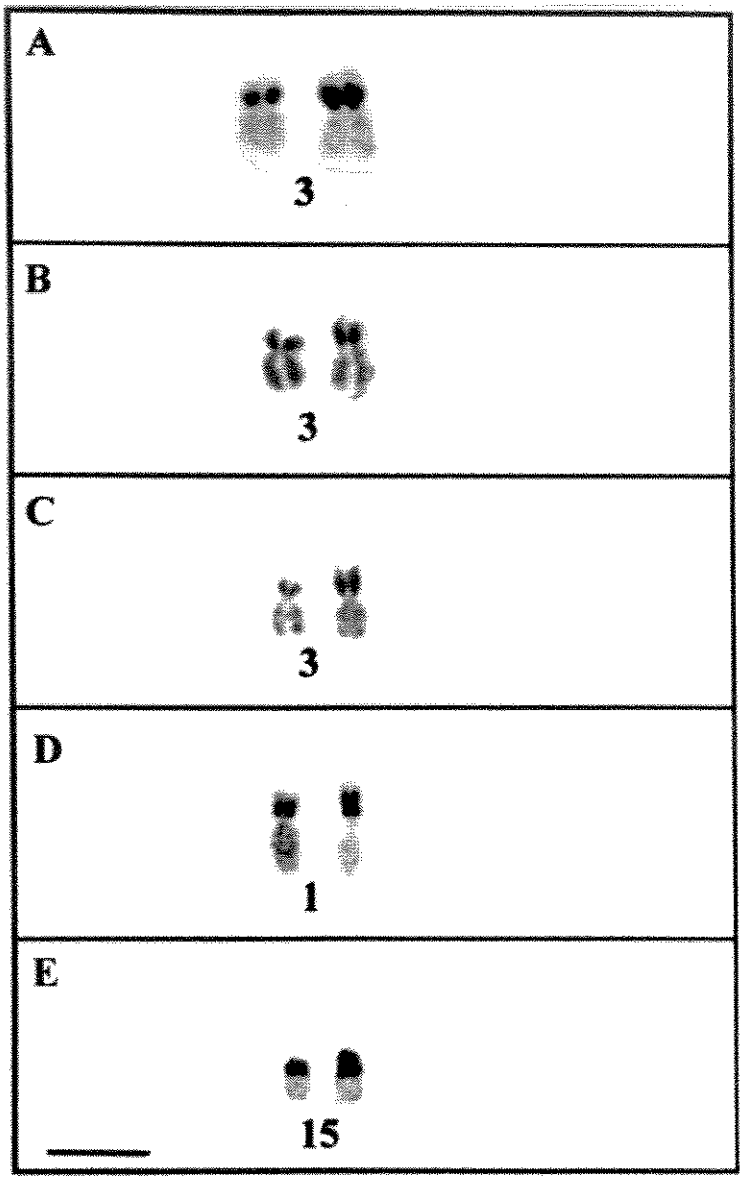

Figure 7

Artigo III

Cytogenetics of *Hylodes* and *Crossodactylus* (Anura, Leptodactylidae) with comments on Hylodinae/Dendrobatidae relationships

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Abstract: The karyotype, NOR location and C-banding pattern of two species of *Hylodes* (*H. phyllodes* and *H. asper*) and two of *Crossodactylus* (*Crossodactylus* sp. n. and *Crossodactylus* cf. *caramaschi*) were studied. All species had a diploid number of 2n=26, with differences in the chromosomal morphology of the *Hylodes* species while the two *Crossodactylus* species were cytogenetically indistinguishable. The NOR was located on pair 1 in both species of *Hylodes*, and on pair 8 in the *Crossodactylus* species. In the latter, the NOR was heteromorphic between the homologues. The NOR was coincident with a secondary constriction in the four species. Except to *H. phyllodes*, such secondary constrictions were clearly seen strongly stained after C-banding treatment. The C-banding pattern varied between the two species of *Hylodes*, but was identical in the *Crossodactylus* species. The results from conventionally stained karyotypes confirmed the uniformity within the genus *Crossodactylus*, and the relatively conserved karyotypes within *Hylodes*, in agreement with other literature reports. We conclude that the cytogenetic data do not provide further evidence which could be useful to corroborate the supoposed relationships between the hylodines and dendrobatids since there are no unambiguous homeologies between the karyotypes of these groups.

INTRODUCTION

Frogs of the subfamily Hylodinae are restricted to eastern Brazil and northern Argentina. *Hylodes* and *Crossodactylus* are the most speciose genera, with 19 and 10 described species, respectively, whereas *Megaelosia* comprises only five species (FROST 2002). The grouping of these genera within a subfamily is supported by a series of morphological characteristics which distinguish them from the remaining leptodactylids (LYNCH 1971). According to HEYER (1975), the Hylodinae frogs form a monophyletic group in which *Megaelosia* retains some peculiarities not shared with the other two genera (see also GIARETTA et al. 1993; MELO et al. 1995; GIARETTA and AGUIAR-JR. 1998; ROSA et al. *in press*, paper II, this thesis).

Morphological studies done by NOBLE (1931) and LYNCH (1971) indicated relationships between the Hylodinae and Dendrobatidae, with the former being considered the most basal group. In contrast, GRIFFTHS (1959, 1963) and FORD (1993) indicated the African ranid frogs as the group most closely related to the dendrobatids. These competing hypotheses were reviewed by FORD (1993) who emphasized the need for reanalyze, redefine, and/or confirm traditionally problematic characters.

In Anura, there is a general trend towards supplementing morphological data with alternative data sets, including karyological data, sperm ultrastructural features, allozyme patterns and mitochondrial gene sequences, all of which have provided new insights to the taxonomy and phylogeny of this group (MIYAMOTO 1983; ODIERNA et al. 2000; KWON and LEE 1995; LOURENÇO et al. 2000; VENCES et al. 2000; AGUIAR-JR. et al. 2002, paper I, this thesis; AGUIAR-JR. et al. 2003, paper V, this thesis).

Cytogenetic analyses of hylodine frogs based on conventional staining methods have indicated a close relationship with the Dendrobatidae (MORESCALCHI 1973; BOGART 1991). These analyses have also revealed that *Crossodactylus* is the most karyotypically conserved genus in terms of chromosome number (2n = 26) and morphology, whereas *Megaelosia* is the most diversified genus, with a diploid number ranging from 28 to 32 chromosomes (BEÇAK 1968; BOGART 1970; DE LUCCA and

JIM 1974; GIARETTA and AGUIAR-JR. 1998; MELO et al. 1995; ROSA et al. *in press*, paper II, this thesis).

In this report, we present further karyotypical data on *Crossodactylus* and *Hylodes* species, which provide new insights on the intergeneric relationships within the Hylodinae and on the affinities between this subfamily and dendrobatid frogs.

MATERIAL AND METHODS

The cytogenetic analysis involved 20 specimens of *Hylodes phyllodes* (18 adult males and two sub-adults) and 26 specimens of *Hylodes asper* (six adult males, four females and sixteen tadpoles). Specimens of both *Hylodes* species were caught in Paranapiacaba, São Paulo State, Brazil. Twelve specimens of *Crossodactylus* sp. n. (eight adult males, one female and three tadpoles) from the Parque Florestal do Itapetinga, in the municipality of Atibaia, and four adult males of *Crossodactylus* cf. *caramaschi*, from the municipality of Botucatu, both located in São Paulo State, Brazil, were also analyzed.

Voucher specimens of all species studied were deposited in the Museu de História Natural "Professor Adão José Cardoso" (ZUEC), Universidade Estadual de Campinas, Brazil, under the following catalog numbers: *H. phyllodes* ZUEC 11419-11421 and 11554, *Crossodactylus* sp. n. ZUEC 11437 and 11438, *H. asper* ZUEC 11439-1441, 11447 and 11454-11556 and *Crossodactylus* cf. *caramaschi* ZUEC 11422-11425.

Mitotic chromosomes were obtained from intestinal and testicular cell suspensions, as described by SCHMID (1978) and SCHMID et al. (1979). The nucleolar organizer regions (NORs) were detected by silver nitrate staining (HOWELL and BLACK 1980). The C-banding technique was that of SUMNER (1972), with modifications in the alkaline treatment.

RESULTS

All four species had 2n=26 chromosomes. Except for the presence of two pairs of telocentric chromosomes (pairs 7 and 8) in *H. phyllodes*, the karyotype of this species closely resembled that of *H. asper*. In both karyotypes, meta- and submetacentric

chromosomes prevailed. Pairs 1, 2, 4, and 10-13 were metacentric and pairs 5 and 6 were submetacentric in the both species. Pair 9 was metacentric in *H. phyllodes* and submetacentric in *H. asper*, and pair 3 was submetacentric in *H. phyllodes* and subtelocentric in *H. asper* (Figs. 1A, B; 3A, B and Table 1). The latter had an arm ratio close to the value for the transition from submeta- to subtelocentric chromosomes, as classified by GREEN and SESSIONS (1991), indicating that this difference between the two karyotypes may be a measurement artifact (Table 1). In both species, pair 1 had a secondary constriction in the pericentromeric region of the short arm (Figs. 1A, B and 3).

The two species of *Crossodactylus* had identical karyotypes in which metacentric chromosomes prevailed (pairs 1, 2 and 8-13); two submetacentric pairs (4 and 6) and two subtelocentric chromosomes (pairs 3 and 5) were also found (Figs. 2A, B and 3C, D and Table 1). A conspicuous secondary constriction was observed on the long arms of pair 8, and was heteromorphic between the two homologs (Fig. 3C, D).

The NORs were located on the secondary constrictions of all four species, i.e. pair 1 in *Hylodes* species and pair 8 in *Crossodactylus* species. In the latter, the secondary constriction and the NOR were heteromorphic (inset in Figs. 1A, B and 2A, B).

After C-banding treatment, only centromeric bands were present in the *H. phyllodes* karyotype, whereas in *H. asper* the telomeric regions of pairs 1, 2, 3 and 4 also appeared positively stained. In addition, a darkly stained pericentromeric band was detected in the nucleolar constriction of pair 1 in the latter karyotype (Fig. 1C, D and Fig. 3). Both species of *Crossodactylus* showed an identical banding pattern in which the largest C-positive blocks were located mainly in the telomeric regions, except for the NOR-bearing pair which carried a large block coincident with the centromeric and NOR regions. Except for such C-blocks in the NOR region, no other interstitial band was detected. Chromosome pair 9 was peculiar since its short arm was entirely heterochromatic. Small C-blocks were found at the centromere of all pairs (Fig. 2C, D and Fig. 3).

DISCUSSION

Conventionally stained karyotypes

The diploid number of 2n=26 chromosomes found in the four species analyzed here agreed with that described for other species of both genera (BEÇAK 1968; BRUM-

ZORRILA and SAEZ 1968; DENARO 1972; DE LUCCA and JIM 1974; BOGART 1970, 1991). BOGART (1991) described a single exception, *Hylodes nasus*, with 2n=24 chromosomes. BRUM-ZORRILA and SAEZ (1968) described a karyotype for *Crossodactylus gaudichaudii* with 22 chromosomes. However, this description was restricted to the chromosome number and differed from the later data obtained by BOGART (1970) who reported a karyotype with 2n=26 for *C. gaudichaudii* in an analysis in which plates and morphometrical data were shown.

Five species of the genus *Hylodes* have already been karyotyped using conventional chromosome staining: *H. asper* (DENARO 1972), *H. glabrus* (DENARO 1972; BOGART 1991), *H. ornatus* and *H. pulcher* (DE LUCCA and JIM 1974), and *H. nasus* (BOGART 1991). For *H. lateristrigatus* (BRUM-ZORRILA and SAEZ 1968), only the diploid number was described. The karyotype of *H. asper* in the present study was very similar to that described by DENARO (1972), although there were some differences between them in their chromosome classification. Thus, whereas metacentric chromosomes prevailed in the *H. asper* karyotype described here, submetacentrics predominated in the description by DENARO. This discrepancy probably does not reflect a real difference within the species but rather is only a consequence of differences in the nomenclature used. The same classification for pairs 1, 2, 4-6 and 10-13 in *H. phyllodes* and *H. asper* possibly indicates homeologies between these two karyotypes.

The data on chromosome morphology indicate that the genus *Hylodes* is relatively well conserved, with most pairs classified as meta- and submetacentrics. The morphology of pair 1 is a particular feature of the genus, since this chromosome is metacentric and bears a secondary constriction adjacent to the centromere on the short arm in all species examined. A secondary constriction in the short arm of only one homolog of pair 6 in *H. glabrus* was also reported by DENARO (1972), but was not observed by BOGART (1991). Although pairs 2 to 6 showed slight differences in centromere position, they appeared homeologous among the *Hylodes* species. The greatest variations were concentrated in pairs 7 to 10. In this group, telocentric pairs such as those found in *H. phyllodes* (pairs 7 and 8) were also observed in *H. glabrus*, although they were classified as pairs 8 and 10 by DENARO (1972) or 8 and 9 in the specimen studied by BOGART

(1991). Although classified as subtelocentrics, pairs 13 in *H. ornatus* and pair 12 in *H. pulcher* also appear to be telocentric in the plates presented in DE LUCCA and JIM (1974).

If the reduction in chromosome number is a trend within *Hylodes*, as it seems to be in other leptodactylid groups (BEÇAK 1968; MORESCALCHI 1973; HEYER and DIMENT 1974), then the presence of telocentric pairs in *H. phyllodes* and *H. glabrus* suggests that a 24-chromosome karyotype such as that found in *H. nasus* is not exclusively the result of a single fusion-mediated event since a telocentric pair is also observed in the latter species. This conclusion reinforces the assumption by DE LUCCA and JIM (1974) that mechanisms of pericentric inversion must have predominated during the karyotypic evolution of this genus. In addition, comparison of the available karyotypes of *Hylodes* and *Crossodactylus* species shows that they can easily be related to one another by postulating a few rearrangements such as pericentric inversions, since most of pairs are identical in the karyotypes already described of both genera.

Only three species of the genus *Crossodactylus* have had their karyotypes described: *C. grandis* (BEÇAK 1968), *C. gaudichaudii* (BRUM-ZORRILA and SAEZ 1968; BOGART 1970) and *C. dispar* (DE LUCCA and JIM 1974). The data in these reports, together with our descriptions here reveal great karyotypic uniformity within this genus. Although minor differences in chromosome classification are present, the general chromosome morphology reported previously for other species is identical to that described here for *Crossodactylus* sp. n. and *Crossodactylus* cf. *caramaschi*. Most of the chromosomes are classified as meta- or submetacentric. The subtelocentric pairs are 3 and 5 in *C. gaudichaudii*, as in *Crossodactylus* sp. n. and *C.* cf. *caramaschi*, 3 and 4 in *C. dispar* and 4 and 5 in *C. grandis*, but are considered as acrocentric chromosomes in the latter species because of the different nomenclature adopted by BEÇAK (1968). The different positions of these subtelocentric pairs are probably a consequence of the similar size among pairs 3, 4 and 5 in these species. A secondary constriction was also observed in *C. gaudichaudii* and *C. grandis*. In the former species, the constriction occurred on the short arm of pair 8, as in the two species described here, whereas in the latter it occurred on pair 7, and was heteromorphic between the homologues in both cases. Together, these findings indicate that although *Crossodactylus* sp. n. and *C.* cf. *caramaschi* differ in body size and

pattern of vocalization (A. A. Giaretta, unpublished data), they cannot be distinguished from each other through conventionally stained karyotypes.

Ag-NOR labeling and C-banding pattern

The NOR location and C-banding pattern have not yet been described for any species of *Hylodes* or *Crossodactylus*. Within the Hylodinae, only three species of the genus *Megaelosia* have been analyzed using such methods (Melo et al., 1995; ROSA et al. *in press*).

Whereas the C-banding pattern was identical in the two species of *Crossodactylus*, it varied between *Hylodes phyllodes* and *H. asper*, which belong to different species groups within the genus (FROST, 2002). The variation illustrated by these two *Hylodes* species is the most common situation found in Anura, in which there is enormous interspecific variation in the distribution and amount of heterochromatin (GREEN 1986; KING 1991; LOURENÇO et al. 2000; AGUIAR-JR. et al. 2002*b*). Although even intraspecific variations in the C-banding pattern have also been described in some species (see ODIERNA et al. 2000, SILVA et al. 2000), in others such variations are not particularly marked, as in some species of the *Physalaemus beligonigerus* group (AMARAL et al. 2000), or may even be absent, as in some species of the genus *Bufo* (KASAHARA et al. 1996) and in the two *Crossodactylus* species studied here.

The amount of telomeric heterochromatin observed in *Crossodactylus* sp. n. and *C.* cf. *caramaschi* was comparable to that found in species of *Megaelosia* (ROSA et al. *in press*, paper II, this thesis). However, any considerations regarding this similarity would be purely speculative since no unambiguous homeology can be established between the *Crossodactylus* and *Megaelosia* karyotypes.

The NOR location is a conserved characteristic within *Hylodes* and *Crossodactylus*, in agreement with SCHMID et al. (1990) whose asserted that in closely related species the NORs are almost always located in the same chromosome region, although many exceptions have been found, e.g. in dendrobatid species of the genus *Epipedobates* (AGUIAR-JR et al. 2002, paper I, this thesis). Although no other species of *Hylodes* and *Crossodactylus* have been analyzed using Ag-NOR labeling, it seems reasonable to suppose that the NORs are also located in the same chromosome region in the remaining

species, since the NOR-carrying secondary constriction has counterparts in all of the already karyotypically studied species of these genera. In the case of *Megaelosia* (ROSA et al. *in press*, paper II, this thesis), although the NOR is located on different chromosome pairs, the chromosome region is also obviously homeologous among the three karyotypes described and may have arisen via a series of structural rearrangements which changed its position among the karyotypes.

Size heteromorphism among homologous NOR, such as that seen in the two *Crossodactylus* species analyzed here, has been recognized in several anuran species (SCHMID 1982; LOURENÇO et al. 2000; AGUIAR-JR. et al. 2002, paper I, this thesis) and seems, in the case here reported, to have resulted from the amplification of only some ribosomal sequences rather than of the entire NOR (duplication). As with the conventional staining of the chromosomes, the C-banding pattern and NOR location cannot differentiate between *Crossodactylus* sp. n. and *C.* cf. *caramaschi.*

The karyotypical data provided here and those reported in the literature for the Hylodinae, agree with that argued by BOGART (1970) who asserted that some genera of leptodactylid frogs have apparently undergone rapid karyotypic evolution while others have maintained fairly stable karyotypes.

Concluding remarks

Although MORESCALCHI (1973), RASOTO et al. (1987) and BOGART (1991) shared the opinion that karyotypical traits supported a relationship between the leptodactylids, or specifically of hylodines, and Dendrobatidae, the data they presented did not unambiguously support their hypothesis.

MORESCALCHI (1973) argued that dendrobatid karyotypes were of the "Leptodactylidae type" and differed from those of the Ranidae. This assumption was based on DUELLMAN (1967), who presented karyotypical data for two species of dendrobatids, *Dendrobates pumilio* and *Colostethus inguinalis*. However, only one metaphase plate was provided for *D. pumilio* without any of measurements or chromosomal classification, while for *C. inguinalis* only the diploid number was described. FORD (1993), in reviewing the competing hypotheses on the origin of the Dendrobatidae, argued that the assumption by MORESCALCHI was far from conclusive since it was based on data from advanced and

not basal dendrobatids, although the genus *Colostethus* was already considered to form a basal lineage in the monophyletic assemblage of toxic dendrobatids (MYERS et al. 1991). RASOTO et al. (1987) concluded that the variation in diploid number and the presence of telocentric chromosomes, which they found in *Dendrobates*, further supported the leptodactylid relationships. However, these authors did not present any convincing discussion of their assumption and, based on MORESCALCHI (1973), they assumed that in all Lissamphibia the only chromosome mutations were fusions or translocations, with a tendency to eliminate telocentric chromosomes. At the same time, they admitted that in a number of families, including South American leptodactylids, centric fission appeared to have occurred. Thus, if both the evolutionary trends were possible, the variation in the number and presence of telocentrics was of little value in supporting a relationship between the Dendrobatidae and the Leptodactylidae.

Based on an analysis of species of four dendrobatid genera and two species of *Hylodes*, BOGART (1991) concluded that the karyotypes of hylodine leptodactylids were very similar to those of dendrobatids, which lent to support a closer evolutionary relationship between the two groups. The two main characteristics in the *Hylodes* karyotypes noted by BOGART which lead to his conclusions were the presence of 24 chromosomes in *H. nasus*, the most common number found in the Dendrobatidae, and the large size of pair 1, also observed in the 20-chromosome species of *Dendrobates*.

The common occurrence of 24 chromosomes in the Dendrobatidae is due mainly to its presence in the two most studied genera, *Epipedobates* and *Colostethus*. In *Colostethus*, considered as the basal dendrobatid genus, a new chromosome number (2n=22) was recently described for three species (VEIGA-MENONCELLO et al. 2001). The genus *Colostethus* comprises more than 100 of the approximately 180 species of the Dendrobatidae. Considering the small number of species which have been karyotyped, it is difficult to affirm that 24 chromosomes is the modal or plesiomorphic state for the entire family. In addition, based on chromosomal relative length in the known dendrobatid karyotypes (BOGART 1991; AGUIAR-JR et al. 2002, paper I, this thesis), the size of pair 1 varies considerably and thus does not provide a useful characteristic for establishing relationships. As argued by KING (1990), a shared karyomorph between two groups

(generally families and subfamilies) does not necessarily imply any affinities between such groups. For these reasons, both characteristics do not support the assumed relationships.

Even with additional markers such as C-banding and Ag-NOR labeling, the karyotypical data seem to contribute more to the resolution of questions within the Hylodinae and Dendrobatidae than between them, since no reliable homeologies can be identified among the described karyotypes. As an example, ROSA et al. (*in press*, paper II, this thesis) observed remarkable karyotypical variability within *Megaelosia* and concluded that the karyological features of this genus do not provide useful insights for assessing the Hylodinae/Dendrobatidae relationship, although the data were useful for indicating a divergent position of *Megaelosia* within Hylodinae. Within Dendrobatidae, AGUIAR-JR. et al. (2002, paper I, this thesis) found interspecific differences in the number of telocentric chromosomes, C-banding pattern and NOR location in the karyotypes of four *Epipedobates*. This study was able to demonstrate the peculiarity of the *E. femoralis* karyotype, which appeared to support the allocation of *E. femoralis* to another taxon, as suggested by previous studies using different kinds of tools (TOFT 1995; CALDWELL 1996; VENCES et al. 2000).

The preceding discussion agrees with the conclusion by KING (1990) that the criteria for determining ancestral karyotypes suffer from a number of basic constraints when attempts are made to compare higher taxonomic levels and that above the generic level some taxa may be unrecognizable in terms of chromosome homeologies. The extensive restructuring of dendrobatid karyotypes via translocations and inversions proposed by BOGART (1991) may contribute to this lack of unambiguous homeologies. KING (1990) argued that differences and similarities may be used to infer phylogeny only if chromosome number, C-bands, sites of secondary constrictions or other markers can be traced to a common ancestral karyotype in a monophyletic lineage.

With additional cytogenetic data for dendrobatids and more species studied within the Hylodinae, it is possible that patterns in C-banding and NOR position will emerge. Such traits can become phylogenetically informative and then can be useful in analysis such as those done by MIYAMOTO (1983) and GREEN (1986). Molecular analysis is another tool that may be used to clarify these possible relationships, especially since it has been applied successfully to other anuran groups (see VENCES et al. 2000).

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Table I. Morphometrical data of the karyotypes of *Hylodes phyllodes*, *H. asper*, *Crossodactylus* sp. n. and *Crossodactylus* cf. *caramaschi* (Chromosome nomenclature follows GREEN and SESSIONS 1991)

	Chromossomes												
	1	2	3	4	5	6	7	8	9	10	11	12	13
						Hylodes phyllodes							
RL	17.2	13.2	11.1	10.5	9.3	7.8	5.3	4.9	4.7	4.3	4.3	3.7	3.5
AR	1.12	1.66	2.85	1.33	1.81	1.71	12.0	11.0	1.40	1.0	1.30	1.25	1.12
CP	m	m	sm	m	sm	sm	t	t	m	m	m	m	m
						Hylodes asper							
RL	19.4	17.7	11.2	11.0	9.2	6.4	5.3	4.9	4.5	4.0	3.6	3.4	3.0
AR	1.19	1.67	3.93	1.54	2.71	2.05	1.42	1.51	1.77	1.42	1.33	1.20	1.45
CP	m	m	st	m	sm	sm	m	m	sm	m	m	m	m
						Crossodactylus sp.n.							
RL	17.3	12.3	10.8	10.8	9.6	6.9	5.8	5.2	5.2	4.6	4.4	3.6	3.2
AR	1.25	1.66	3.66	1.33	3.16	2.0	1.30	1.25	1.25	1.0	1.37	1.10	1.37
CP	m	m	st	m	st	sm	m	m	m	m	m	m	m
						Crossodactylus cf. *caramaschi*							
RL	17.2	13.1	10.1	10.0	9.6	6.6	5.4	5.4	4.9	4.9	4.4	4.2	3.9
AR	1.18	1.65	3.50	1.05	3.87	1.70	1.44	1.20	1.0	1.50	1.25	1.42	1.0
CP	m	m	st	m	st	sm	m	m	m	m	m	m	m

RL= Relative Lenght, AR= Arm Ratio, CP= Centromeric Position, m= metacentric, sm=submetacentric, st=subtelocentrice and t= telocentric.

FIGURE LEGENDS

Figure 1. Karyotype of *Hylodes phylodes* (A) and *H. asper* (B) after conventional staining with Giemsa. Ag-NOR labeling localized the NOR site on the short arm of chromosome 1 in both species (squares on A and B). After C-banding, only centromeric bands were observed in *H. phyllodes* (C) whereas telomeric blocks and an NOR-coincident block were seen in *H. asper* (D). Bars = 5 μm.

Figure 2. Conventionally stained karyotypes of *Crossodactylus* sp. n. (A) and *Crossodactylus* cf. *caramaschi* (B). Heteromorphic NORs were located on the long arm of chromosome 8 in both species (squares on A and B). C-banded chromosomes showed large telomeric blocks in most chromosomes of both species (C and D). Bars = 5 μm.

Figure 3. Ideograms based on the morphometrical data of Table 1. *Hylodes phyllodes* (A), *H. asper* (B), *Crossodactylus* sp. n. (C), and *Crossodactylus* cf. *caramaschi* (D). Dark areas: C-banding positive regions. Grey circles: NOR sites. Open areas in A and B and dotted areas in C and D represent the secondary constrictions.

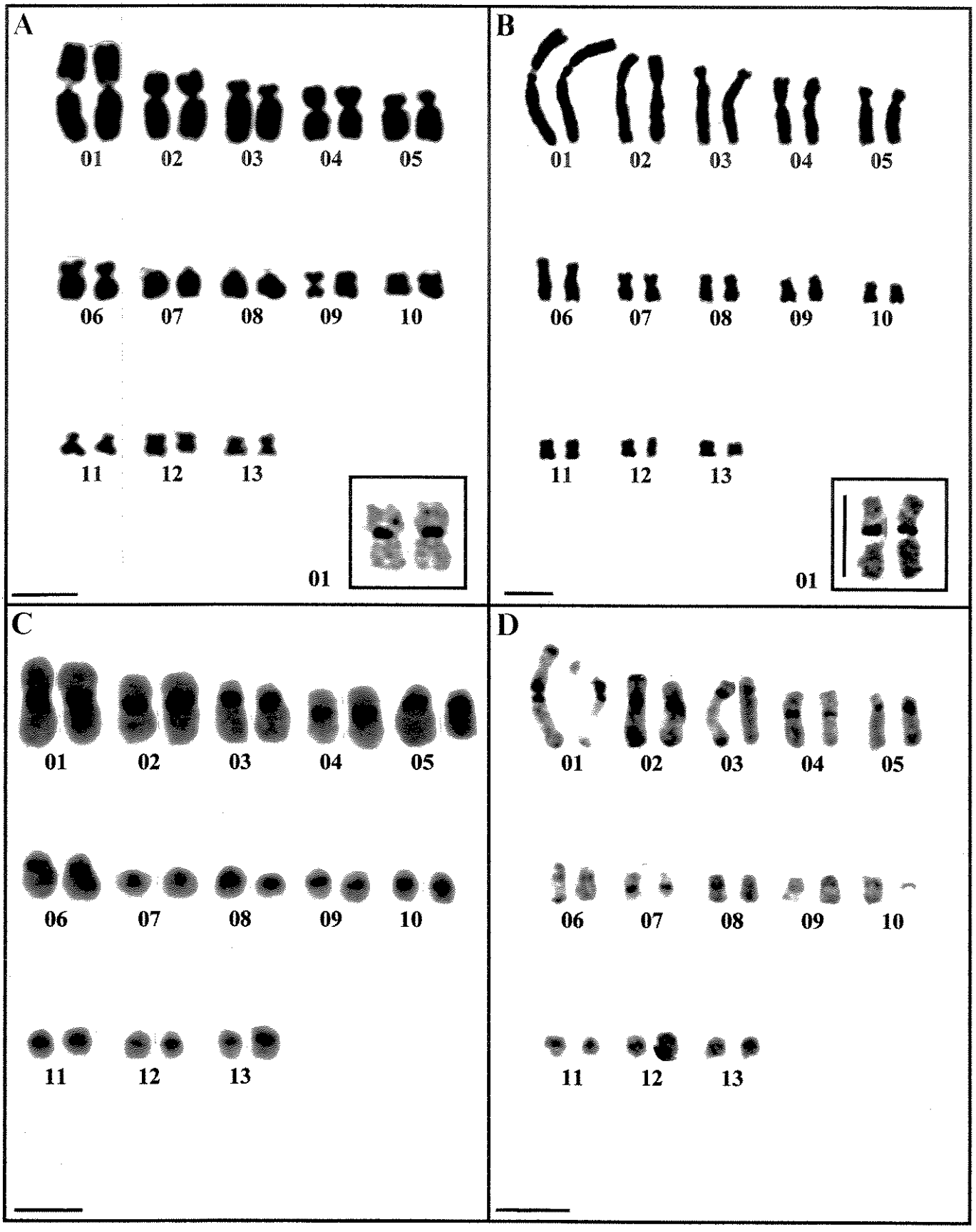
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Figure 1

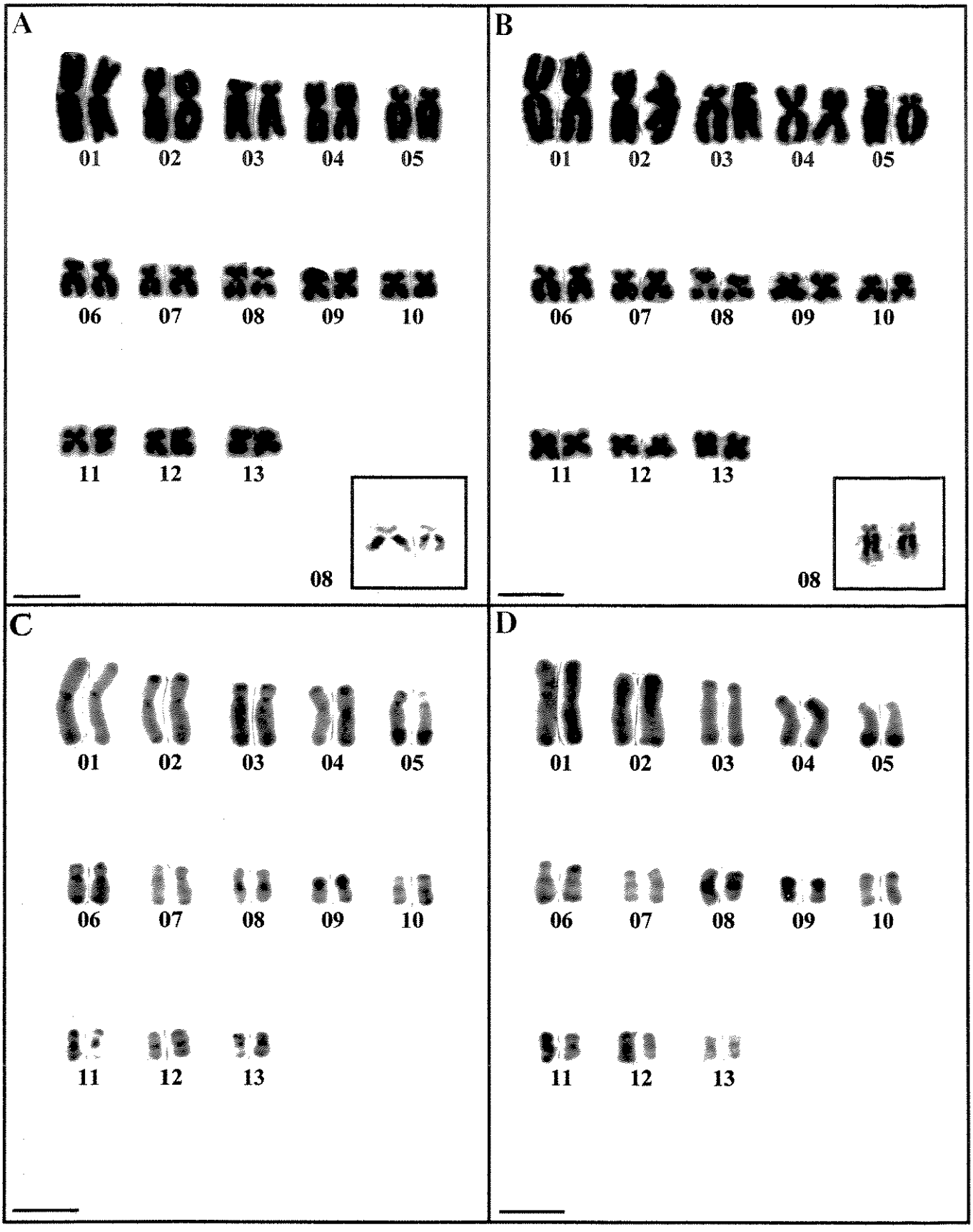
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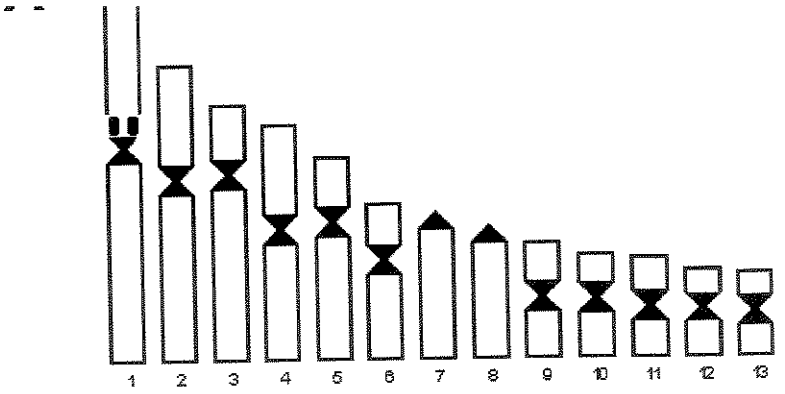
Figure 2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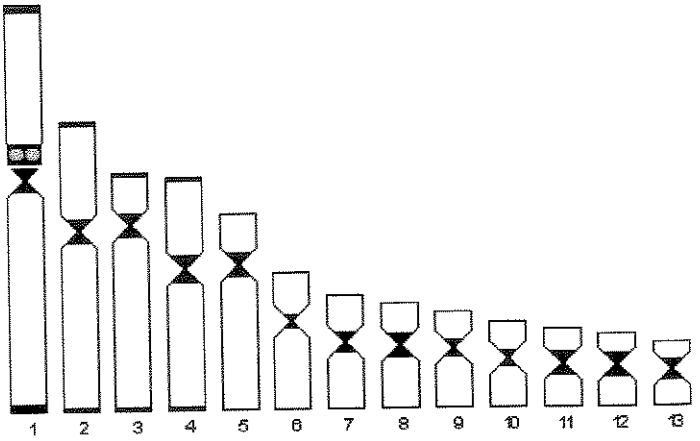
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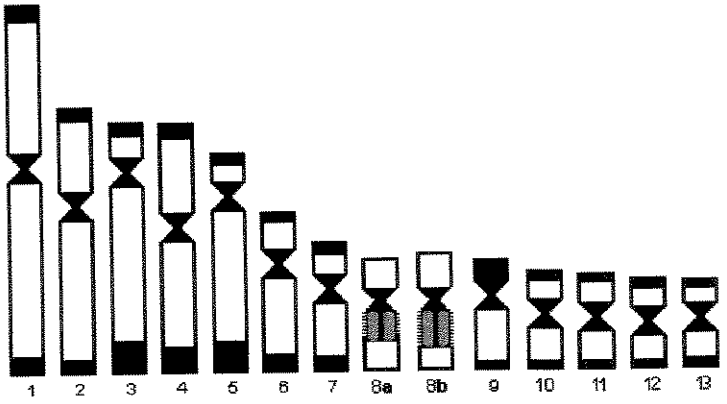
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D

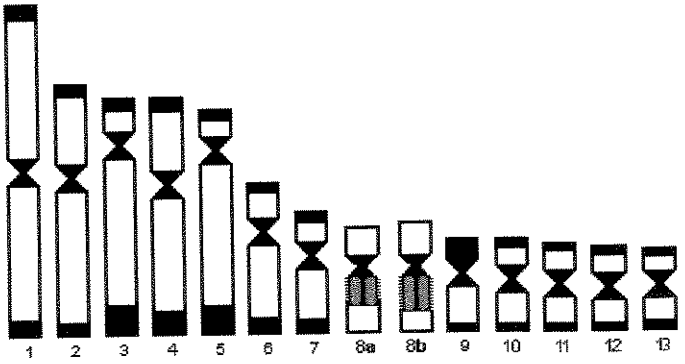
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Figure 3

3. Análise da ultra-estrutura dos espermatozóides

Artigo IV

Obs: Este artigo foi realizado em colaboração com Adrian Garda, tendo sido apresentado também como parte de sua tese de Mestrado.

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Tissue&Cell

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

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Abstract. We describe, for the first time, the spermatozoon ultrastructure of a dendrobatid frog, *Epipedobates flavopictus*. Mature spermatozoa of *E. flavopictus* are filiform, with a moderately curved head and a proportionally short tail. The acrosomal vesicle is a conical structure that covers the nucleus for a considerable distance. A homogeneous subacrosomal cone lies between the acrosome vesicle and the nucleus. The nucleus contains a nuclear space at its anterior end, and electron-lucent spaces and inclusions. No perforatorium is present. In the midpiece, the proximal centriole is housed inside a deep nuclear fossa. Mitochondria are scattered around the posterior end of the nucleus and inside the undulating membrane in the anterior portion of the tail. In transverse section the tail is formed by an U-shaped axial fiber connected to the axoneme through an axial sheath, which supports the undulating membrane. The juxta-axonemal fiber is absent. The spermatozoon of *E. flavopictus* has several characteristics not observed before in any anurans, such as a curved axial fiber, absence of a juxta-axonemal fiber, and presence of mitochondria in the typical undulating membrane. Our results endorse the view that, in anurans, the conical perforatorium and subacrosomal cone are homologous and that Dendrobatidae should be grouped within Bufonoidea rather than Ranoidea.© 2002 Published by Elsevier Science Ltd.

Keywords: sperm, ultrastructure, Anura, Dendrobatidae, *Epipedobates*

Introduction

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

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

The ultrastructure of spermatozoon has been used as an alternative data set to investigate the phylogeny of many taxa such as fishes (Jamieson, 1991; Tanaka et al., 1995), amphibians (Lee & Jamieson,1992, 1993; Jamieson et al., 1993; Scheltinga et al., 2001), reptiles (Jamieson, 1995; Teixeira et al., 1999a; Teixeira et al., 1999b), and invertebrates (Jamieson, 1987). An advantage of sperm ultrastructure data is that they provide more conservative characters for groups with highly derived body plans, such as Amphisbaenia, which cannot be scored for some traditional morphological traits (Teixeira et al., 1999b). Spermatozoon ultrastructure data have also been useful in clarifying relationships among Polyplacophora, where traditionally used characters are either too conserved or too variable (Buckland-Nicks, 1995). Spermatozoon morphology, therefore, seems to be useful for groups where, for some reason, external morphology cannot be scored, either because of evolutionary conservativeness (as in some traits of Polyplacophora) or specialization (as for Amphisbaenia).

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

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

Material and methods

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

Results

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

Acrosome complex

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

Nucleus

Below the nuclear envelope, a nuclear space that probably results from the condensation of chromatin process is seen

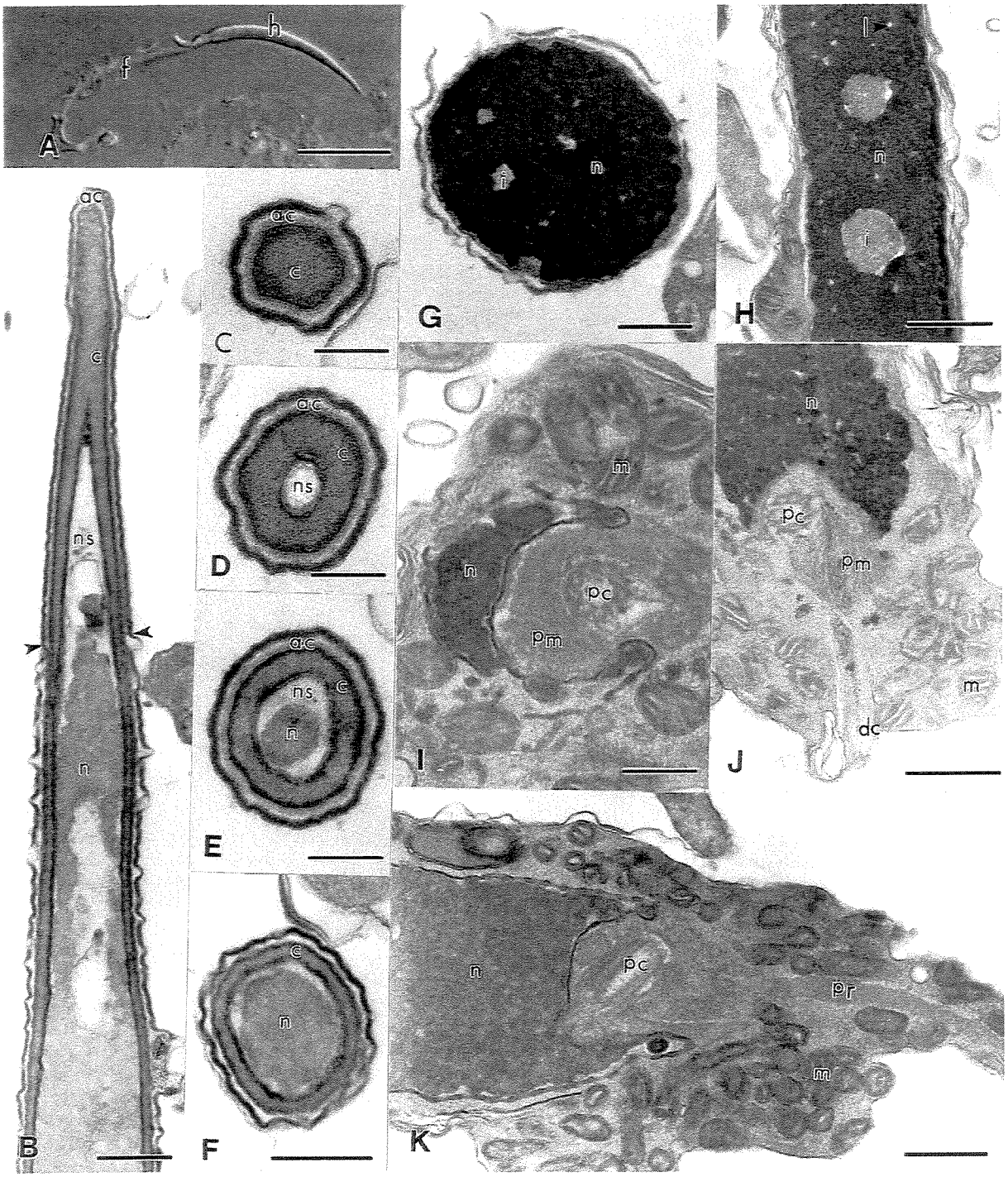

Fig. 1 Spermatozoa of *E. flavopictus*. (A) Light microscopy showing whole spermatozoon with head (h) and flagellum (f). ×1250. (B–K) Transmission electron micrographs of head and midpiece. (B) Longitudinal section of the head region showing the end of the acrosome vesicle (arrowheads). ×38 000. (C–F) Transverse sections of the head region showing the reduction of the acrosome and enlargement of the nucleus. ×116 000, ×100 000, ×94 000, ×54 000, respectively. (G & H) Transverse and longitudinal sections of the nucleus showing the lacunae (l) and inclusions (i). ×40 000, ×25 000, respectively. (I) Oblique section of the midpiece. Note the scattered distribution of mitochondria. ×50 000. (J & K) Longitudinal view of the midpiece at the level of the distal centriole and paraxonemal rod, respectively. ×28 000, both. Abbreviations: ac, acrosome vesicle; af, axial fiber; ax, axoneme; c, subacrosomal cone; dc, distal centriole; m, mitochondrion; n, nucleus; ns, nuclear space; pc, proximal centriole; pm, pericentriolar material; pr, paraxonemal rod; u, undulating membrane. Scale bars: (A) 20 μm; (B & I) 0.2 μm; (C) 0.05 μm; (D & E) 0.1 μm; (F & G) 0.3 μm; (H, J & K) 0.5 μm.

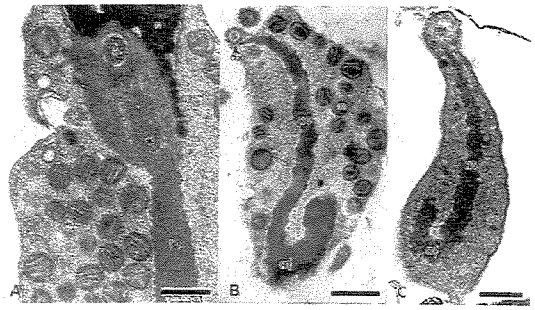

Fig. 2 Spermatozoa of *E. flavopictus*. Transmission electron micrographs of the midpiece and tail. (A) Longitudinal section through the midpiece showing the insertion of the paraxonemal rod in the nuclear fossa (*). ×42 000. (B) Transverse section of the tail showing the S-shaped paraxonemal rod, mitochondria inside the undulating membrane, U-shaped axial fiber in transverse section, and axial sheath. ×24 000. (C) Transverse section of the posterior portion of the tail; note the absence of mitochondria. ×60 000.

(Fig. 1B, D & E). The nucleus is circular in transverse section (Fig. 1G) and conical in longitudinal section (Fig. 1B). The anterior portion of the nucleus is enveloped by the acrosome complex and gradually tapers (nuclear shoulders absent) to a point within the subacrosomal cone (Fig. 1E & F). The chromatin is highly condensed and electron-dense. Despite the high degree of condensation, several small electron-lucent nuclear lacunae and inclusions are seen (Fig. 1G & H).

Midpiece

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

Tail

In transverse section, the tail of *E. flavopictus* sperm consists of the axoneme, undulating membrane and axial fiber (Fig. 2B). In transverse section the axial fiber is curved or U-shaped section and apparently of the same composition of an electron-dense structure that supports the undulating membrane, to which it is connected. Since other structures

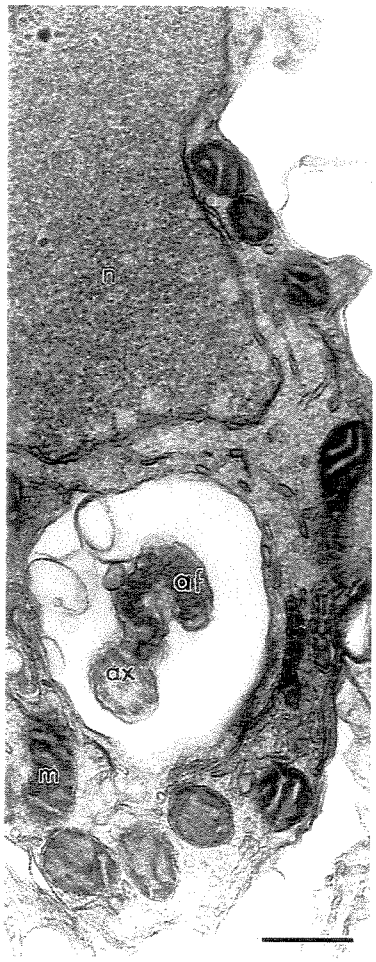

Fig. 3 Section of spermatid showing presence of mitochondrial collar in the early development of the sperm. ×40 300. Abbreviations: af, axial fiber; as, axial sheath; ax, axoneme; m, mitochondrion; n, nucleus; pc, proximal centriole; pm, pericentriolar material; pr, paraxonemal rod; u, undulating membrane. Scale bars: (A & F) 0.3 μm; (B & E) 0.5 μm; (C) 0.2 μm.

are found inside the undulating membrane of *E. flavopictus*, such as cytoplasm and mitochondria, it is necessary to name this structure to which the axial fiber is connected, hereafter called àxial sheath'. It is formally defined as the connection between the axial fiber and the axoneme (or juxta-axonemal fiber, when it is present). In *E. flavopictus* the axial sheath is directly connected to the axoneme through the doublet 3, without a juxta-axonemal fiber (Fig. 2B & C).

Mitochondria are observed inside the undulating membrane in the anterior portion of the tail (Fig. 2B). At the end of the tail, the undulating membrane no longer contains mitochondria and the plasma membrane is completely adhered to the axial fiber and axial sheath. In Figure 3, the intermediate piece of a spermatid is seen. Contrary to the condition seen in the mature spermatozoon, the mitochondria are organized in a mitochondrial collar around the flagellum.

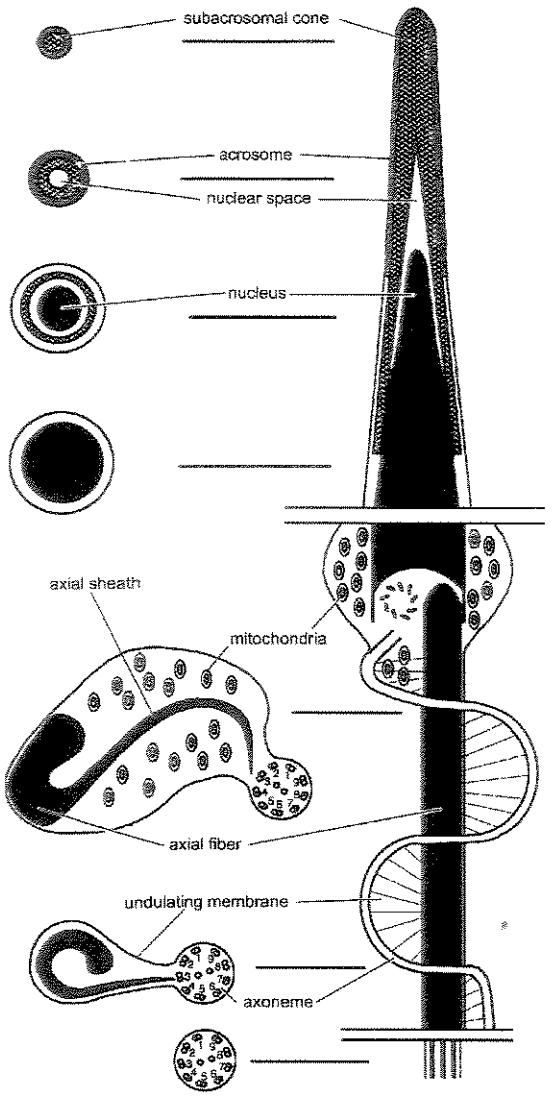

Fig. 4 Diagrammatic representation of the spermatozoon of the dendrobatid frog *E. flavopictus*.

Discussion

The basic structure of the spermatozoon of *E. flavopictus* (Fig. 4) is similar to that of most neobatrachians (Ford, 1993) described to date, such as *Bufo arenarum* (Burgos & Fawcett, 1956), *B. marinus* (Swan et al., 1980; Lee & Jamieson, 1993), *Melanophryniscus cambaraensis* (Báo et al., 2001), *Odontophrynus cultripes* (Báo et al., 1991), *Lepidobatrachus laevis* (Waggener & Carroll, 1998), and *Pachymedusa dacnicolor* (Rastogi et al., 1988). However, it possesses several traits not seen in these other species and which may possibly be shared with other dendrobatids.

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

We regard, like James (1970) and Jamieson (1999), that the conical perforatorium and the subacrosomal cone are homologous based on what follows. First, argument (1) above is a syllogism: bufonoids have a conical extranuclear perforatorium whereas *A. truei* has a rod-like, endonuclear perforatorium; hence, since the function of supporting the spermatozoon head is performed by the perforatorium, the subacrosomal cone of *A. truei* cannot be homologous with the conical perforatorium of bufonoids. Similarity of function, however, is not a requirement for homology (Lauder, 1994). Lee and Jamieson (1992, 1993) and Jamieson et al. (1993) were probably influenced by the earlier work of Burgos and Fawcett (1956), the first to suggest that the coarse strands of dense material, observed around the tapering end of the nucleus in the spermatozoon of *B. arenarum*, form a perforatorium. Had Burgos and Fawcett (1956) chosen a different name (without implying a function) for the same structure, say 'subacrosomal cone', argument (1) above would vanish.

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

Finally, if the view of Lee and Jamieson (1992, 1993) and Jamieson et al. (1993) is correct, six steps would be required during the evolution of anurans, when mapping the characters conical perforatorium and subacrosomal cone onto a cur-

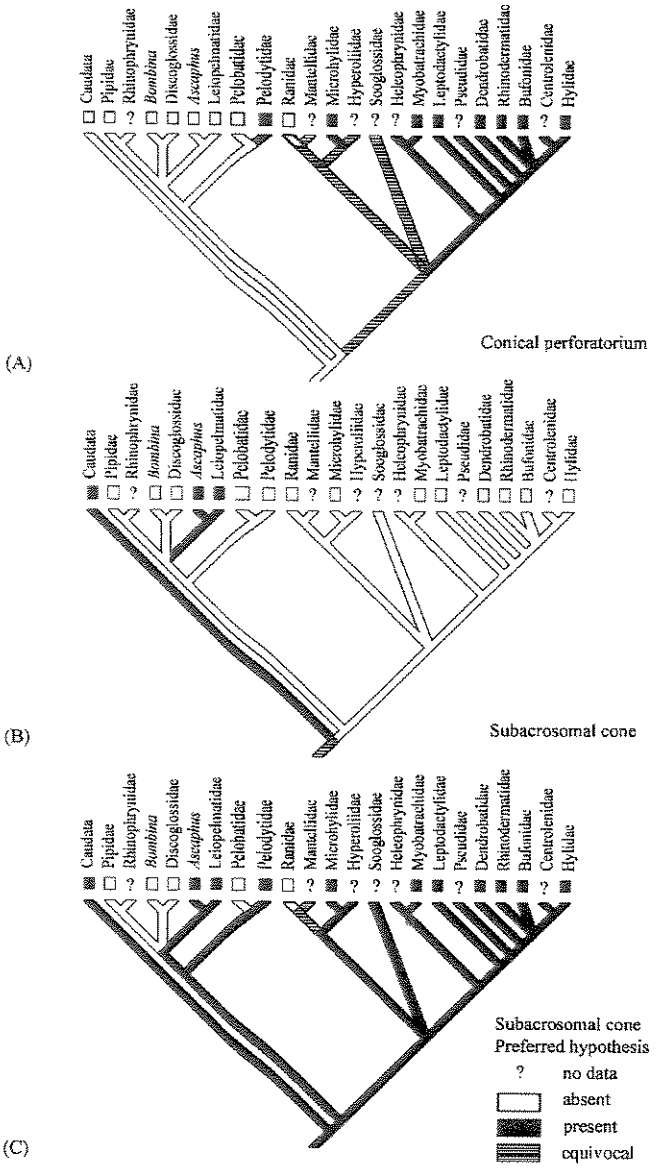

Fig. 5 Reconstruction of the evolution of sperm ultrastructure characters of *E. flavopictus*. Phylogeny of anurans from Hay et al. (1995). (A) Evolution of conical perforatorium, according to Jamieson et al. (1993) and Lee and Jamieson (1993), number of steps = 4. (B) Evolution of subacrosomal cone, according to Jamieson et al. (1993) and Lee and Jamieson (1993), number of steps = 2. (C) Preferred hypothesis for the evolution of the subacrosomal cone, when considering the conical perforatorium homologous to the subacrosomal cone in anurans, number of steps = 4. Data for families used in the analysis was obtain from the present work (Dendrobatidae) and from the following literature: Caudata (Selmi et al., 1997), Pipidae (Reed & Stanley, 1972; Bernardini et al., 1986), *Bombina* (Furieri, 1975; Pugin-Rios, 1980), Discoglossidae (Pugin-Rios, 1980; Lee & Kwon, 1996), *Ascaphus* (James, 1970; Jamieson et al., 1993), Leiopelmatidae (Scheltinga et al., 2001), Pelobatidae (James, 1970; Asa & Phillips, 1988), Pelodytidae (Pugin-Rios, 1980), Ranidae (Poirier & Spink, 1971; Pugin-Rios, 1980), Microhylidae (*Chiasmocleis albopunctata*, personal observation), Myobatrachidae (Lee & Jamieson, 1992), Leptodactylidae (Pugin-Rios, 1980; Báo et al., 1991; Waggener & Carroll, 1998; Amaral et al., 1999), Rhinodermatidae (Pugin-Rios, 1980), Bufonidae (Burgos & Fawcett, 1956; Lee & Jamieson, 1993; Báo et al., 2001), and Hylidae (Rastogi et al., 1988; Lee & Kwon, 1992; Lee & Jamieson, 1993).

rent phylogeny of the group (Hay et al., 1995) (Figs 4 & 5). According to this view, the conical perforatorium was absent in the common ancestor of anurans and salamanders and evolved once in the 'bufonoid' lineage (Fig. 4A). Furthermore, the subacrosomal cone was originally absent in anurans and evolved independently twice in the group (Fig. 4B). Conversely, if James (1970) was correct in regarding the conical perforatorium of bufonoids as homologous with the subacrosomal cone of *A. truei*, then only four evolutionary steps are required and the presence of the subacrosomal cone would be

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

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

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

The numerous and randomly distributed mitochondria distinguish *E. flavopictus* from several other Neobatrachia so far examined, which usually have a mitochondrial collar, as in Bufonidae (Burgos & Fawcett, 1956; Swan et al., 1980; Lee & Jamieson, 1993) and Leptodactylidae (Pugin-Rios, 1980; Báo et al., 1991). The presence of mitochondria creates a large separation between the axial sheath and the plasma membrane in the anterior tail region of *E. flavopictus*. In all other amphibians with an undulating membrane, the plasma membrane is closely adhered to the axial sheath. Interestingly, early spermatids of *E. flavopictus* have a mitochondrial collar detached from the midpiece (Fig. 2F), as in Bufonidae (Burgos & Fawcett, 1956; Swan et al., 1980; Lee & Jamieson, 1993).

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

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

The significance of anuran sperm ultrastructure needs to be evaluated under the scope of sound phylogenetic techniques. To do so, characters must be continuously evaluated and families yet to be studied (e.g. Sooglossidae, Centrolenidae, Microhylidae, Rhinophrynidae, Mantellidae, Hyperoliidae, and Brachycephalidae) must be investigated in order to built a consistent data set that enables parsimony analysis.

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Artigo V

Obs: Este artigo foi realizado em colaboração com Adrian Garda, tendo sido apresentado também como parte de sua tese de Mestrado.

Biflagellate Spermatozoon of the Poison-Dart Frogs *Epipedobates femoralis* and *Colostethus* sp. (Anura, Dendrobatidae)

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ABSTRACT This study describes the spermatozoa of the dendrobatids *Epipedobates femoralis* and *Colostethus* sp. using light and transmission electron microscopy. Both species possess a biflagellate spermatozoon, an unusual characteristic only previously reported in two anuran species belonging to the families Leptodactylidae and Racophoridae. The acrosomal complex of both species consists of a conical acrosomal vesicle and a subacrosomal cone, both of which cover the anterior portion of the nucleus, but to differing extents. In the midpiece, the centrioles are disposed parallel to each other and to the cell axis and give rise to two axonemes. Two paraxonemal rods were also seen entering the nuclear fossa. Both flagella are surrounded by a single mitochondrial collar. Each flagellum is formed by an axial fiber connected to the axoneme by an axial sheath; juxta-axonemal fibers are absent. Our data seem to support that *Epipedobates femoralis* should be placed in a separate clade possibly related to *Colosthetus* and that these two genera may not be monophyletic. J. Morphol. 255:114–121, 2003.

KEY WORDS: sperm ultrastructure; biflagellarity; Anura; Dendrobatidae; *Epipedobates; Colostethus*

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

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

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

Various datasets have been used to investigate dendrobatid systematic relationships, including cytogenetic and molecular markers (Morescalchi, 1973; Rasotto et al., 1987; Bogart, 1991; Hillis et al., 1993; Hay et al., 1995; Summers et al., 1999; Clough and Summers, 2000; Vences et al., 2000) and also

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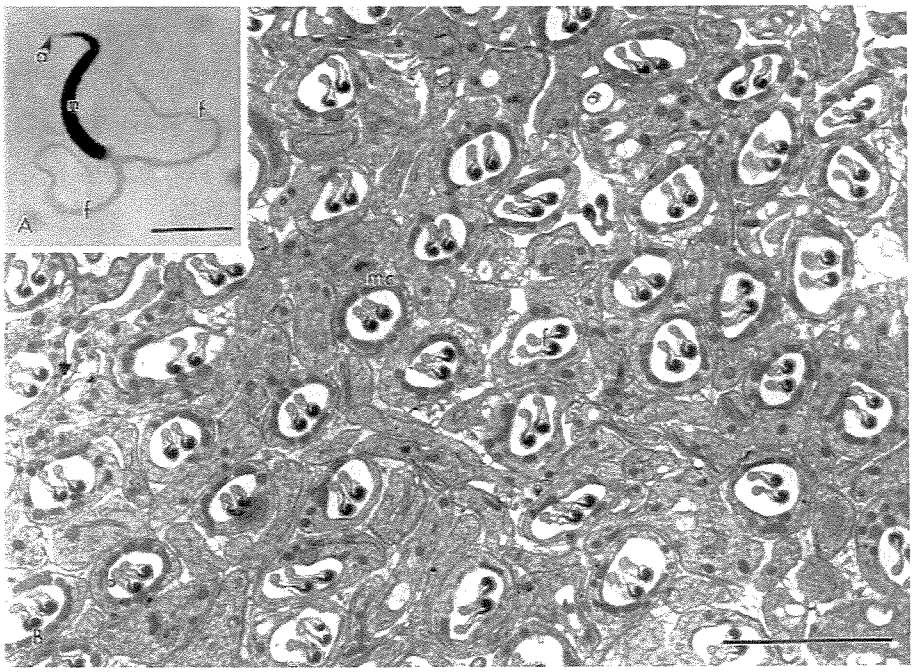

Fig. 1. **A:** The spermatozoa of *Epipedobates femoralis* showing the presence of two independent flagella, both with an undulating membrane. LM. **B:** A spermatocyst of *Colostethus* sp. where several pairs of flagella surrounded by mitochondrial collars can be seen. TEM. a, acrosome; f, flagellum;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

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

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

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

MATERIALS AND METHODS

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

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

RESULTS

Spermatozoa of *Epipedobates femoralis* and *Colostethus sp.* contain two flagella that are inserted into a short midpiece (Fig. 1A,B). The nucleus and

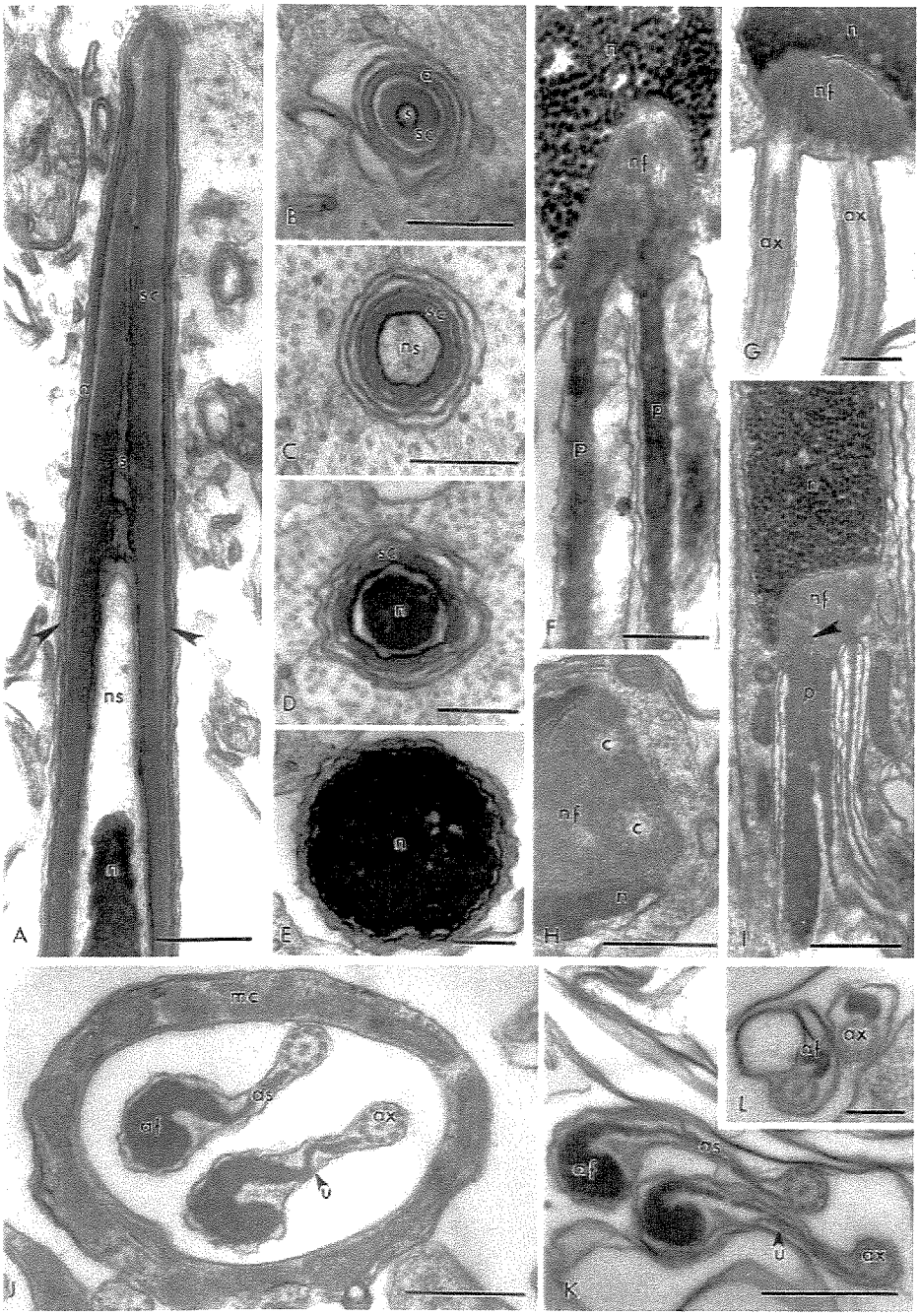

Fig. 2. Spermatozoa of *Colostethus* sp. **A:** Longitudinal section of the acrosome showing the end of the acrosomal vesicle (arrowheads). Also note the canal throughout the subacrosomal cone's length and the nuclear space. **B–E:** Transverse sections of the headpiece showing reduction of the acrosomal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

tail of *E. femoralis* are approximately 19.5 μm and 27.0 μm long, respectively. The paired flagella and mitochondrial collars are particularly evident in transverse sections through a spermatocyst (Fig. 1B).

Colostethus sp.

The acrosomal tip is blunt in longitudinal section and a conical acrosomal vesicle covers the anterior portion of the head and extends to the beginning of the nucleus, where a large nuclear space can be seen (Fig. 2A–C). Below the acrosomal vesicle, the subacrosomal cone ensheathes the nucleus (Fig. 2A,D). Above the nuclear membrane, the subacrosomal cone is traversed throughout its length by a canal, referred to here as the subacrosomal canal (Fig. 2A,B). In transverse section, at the anteriormost portion of the acrosome, the subacrosomal canal is surrounded by the subacrosomal cone and the de-

tached acrosomal vesicle (Fig. 2B). More posteriorly, the nuclear space is surrounded by the subacrosomal cone that adheres closely to the nuclear membrane (Fig. 2C). Subsequently, the nucleus is surrounded by the subacrosomal cone alone and, eventually, only by the plasma membrane (Fig. 2D,E). The transition from the tip of the acrosome to the end of the subacrosomal cone is smooth, with no nuclear shoulders.

In the midpiece, two paraxonemal rods enter the shallow nuclear fossa (Fig. 2F–I). The centrioles are disposed parallel to the longitudinal axis, with an axoneme showing the typical 9+2 pattern of microtubules emerging from each centriole (Fig. 2G,H). The axial sheath (sensu Garda et al., in press) and axial fiber coalesce near the point of insertion into the nuclear fossa to form the paraxonemal rod that projects slightly into the nuclear fossa (Fig. 2I). Several mitochondria are arranged around both flagella within the mitochondrial collar (Fig. 2J).

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

Epipedobates femoralis

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

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

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

DISCUSSION

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

The placement of the Dendrobatidae has shifted between the neobatrachian groups Ranoidea and Bufonoidea (Ford and Cannatella, 1993; Hillis et al., 1993; Ruvinsky and Maxson, 1996; Emerson et al., 2000). Besides the similarities in the acrosomal complex, which differ from that observed in ranoid groups (Kwon and Lee, 1995), the mitochondrial collar surrounding the axonemes in the species studied here is characteristic of most bufonoids, for which it is considered a synapomorphic characteristic (Lee and Jamieson, 1992). However, the presence of this characteristic does not necessarily imply affinities between bufonoids and dendrobatids because Scheltinga et al. (2001) reassessed such characteristics and argued that there is no arrangement of mitochondria that can be convincingly regarded as plesiomorphic for the Anura. Even within *Epipedobates*, an alternative pattern was found by Garda et al. (in press) in *E. flavopictus* sperm, in which the mitochondria are randomly distributed within the undulating membrane.

Biflagellate spermatozoa have been described in several animal groups. In fishes, for example, biflagellate sperm have evolved at least six times, without any link to phylogeny or internal fertilization (Mattei, 1988; Jamieson and Leung, 1991). The presence of two free flagella, widespread in Turbellaria, has been considered a plesiomorphic characteristic of platyhelminths with a tendency towards mono- and aflagellate conditions (Hendelberg, 1969; Justine, 1991). Franzén (1982) described the only case of biflagellarity in Annelida (the polychaete *Tomopteris helgolandica*). Among molluscs, biflagellate spermatozoa are known in two bivalves, *Corbiculla flamae* and *C. leana*, where they are considered a modification of the

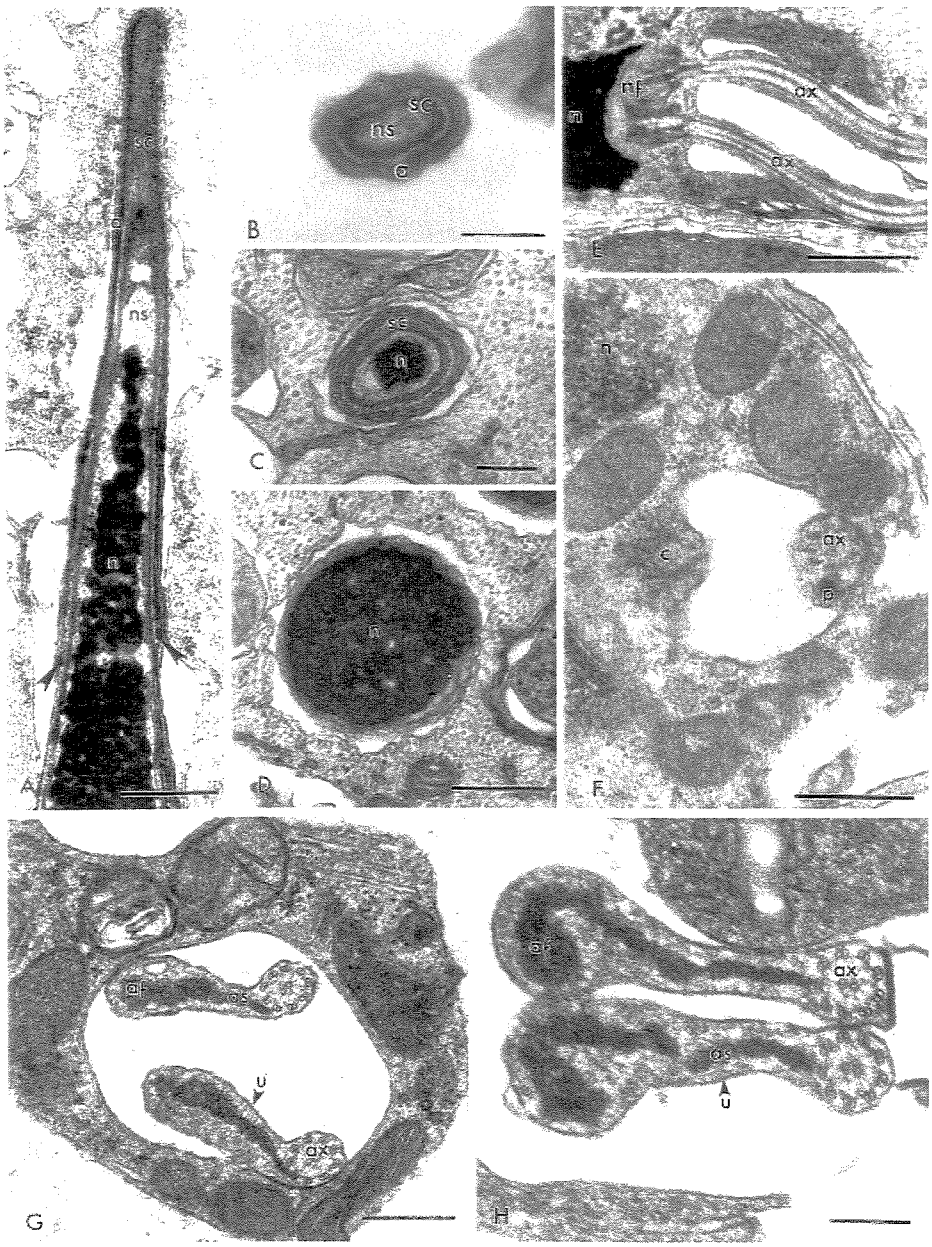

Fig. 3. Spermatozoa of *Epipedobates femoralis*. **A:** Longitudinal section of the acrosomal region. Note the absence of the subacrosomal canal. **B–D:** Progressive enlargement of the nucleus and reduction of the acrosomal vesicle and subacrosomal cone. **E:** Insertion of both axonemes into the nuclear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

primitive monoflagellar condition related to the specialized mode of reproduction found in these species (Komaru and Konishi, 1996). In insects, where sperm structure is highly diversified, a biflagellate condition is observed in some Hemiptera (Jamieson et al., 1999).

Within the Caudata of Lissamphibia, Austin and Baker (1964) suggested the presence of two flagella and two undulating membranes in *Pseudobranchus striatus axanthus*, a sirenid salamander. However, such analysis was restricted to light microscopy and the figures shown by these authors are not convincing as to the presence of such a peculiar tail. So much so that later Selmi et al. (1997), through an ultrastructural study of salamandrids sperm, emphasized that sperm ultrastructure of Sirenidae species was still unknown. In anurans, a number of studies have reported the existence of biflagellarity. In the Chilean leptodactylid *Telmatobufo australis*, the presence of two flagella was inferred to be a primitive characteristic related to the fertilization environment, although the exact mode of fertilization of this species was unknown (Pugin-Rios and Garrido, 1981). Mainoya (1981) indicated that anurans may have sperm tails with either one or two flagella and erroneously cited Nicander (1970), who

mentioned no case of biflagellarity in anurans. Lee and Jamieson (1993) argued for a general trend towards the simplification of sperm among the Anura (see also Jamieson et al., 1993) and suggested that the biflagellarity in *Rhacophorus* (Rhacophoridae), *Scaphiophus* (Pelobatidae), and *Telmatobufo* (Leptodactylidae) was apomorphic. This view was reinforced by Jamieson (1999) for *Chiromantis*, another racophorid genus. Although Mainoya (1981) asserted that sperm cells of *Chiromantis xerampelina* had only one tail flagellum with two axial filaments, Jamieson (1999), based on the report of Wilson et al. (1991), emphasized that a pair of free flagella comprise the tail piece in this species. However, *Rhacophorus* (Mizuhira et al., 1986) actually have two axonemes embedded in a matrix of hundreds of microtubules, forming a single tail rather than two free flagella. Likewise, the pelobatids *Scaphiophus holbrookii* (James, 1970) and *Megophrys montana* (Asa and Phillips, 1988) have a single tail with two axonemes. Waggener and Carrol (1998) also reported a biaxonemal condition in *Lepidobatrachus laevis*, but this conclusion was apparently based on a misinterpretation of micrographs: the presence of two axonemes united by membrane (fig. 5A–F in their report) resulted, in fact, from the same axoneme being hit twice by the same cut in the undulating tail; their figure 5G corroborates the common pattern found in neobatrachians, where the tail is formed by the axoneme, juxta-axonemal fiber, axial sheath, and axial fiber. We propose that true biflagellarity is a condition in which the two axonemes are independent and isolated by the plasma membrane. Thus, biflagellar sperm in anurans have been observed only in *Telmatobufo australis* (Pugin-Rios and Garrido, 1981), *Chiromantis xerampelina* (Wilson et al., 1991; Jamieson, 1999), and some dendrobatids (this study). The sperm of *Epipedobates femoralis* and *Colostethus* sp. represent the first cases in the Anura of two complete flagella, with an undulating membrane and axial rod, because the spermatozoa of *Telmatobufo australis* and *Chiromantis xerampelina* have two simple flagella.

It seems premature to ascribe any phylogenetic significance of the biflagellarity to the Anura as a whole. However, within Dendrobatidae the biflagellarity may support the placement of *Epipedobates femoralis* in a separate clade (*Allobates femoralis*, sensu Zimmermann and Zimmermann, 1988) possibly related to *Colostethus*, as previously indicated by molecular analysis (see Vences et al., 2000; Clough and Summers, 2000), which placed *A. femoralis* outside the clade containing the other toxic dendrobatids. Dietary data collected by Toft (1995) and Caldwell (1996) are also in agreement with such an idea. The genus *Colostethus* is characterized by the lack of skin toxins found in the remaining dendrobatids (Bogart, 1991; Toft, 1995) and is considered a basal lineage to the monophyletic assemblage of toxic genera (Myers et al., 1991). The biflagellarity of *E. femoralis*—also a nontoxic dendrobatid, as noted by Caldwell (1996)—contrasts with the condition found in other congeneric species, such as *E. flavopictus* (Garda et al., in press), *E. trivittatus* and *E. hahneli* (Aguiar–Jr. et al., unpubl. data). Hence, our results also seem to suggest that these two speciose genera (*Epipedobates* and *Colostethus*) may not be monophyletic in agreement with that proposed by Vences et al. (2000) through molecular data. Further ultrastructural studies of other species of *Epipedobates* should confirm whether close relatives of *E. femoralis* are to be retained in the genus, as noted by Myers et al. (1991). In addition, an analysis of *Aromobates nocturnus*—the presumed sister taxon of all other dendrobatids (Myers et al., 1991)—may shed light on the significance of biflagellarity in the Dendrobatidae. Also, we suggest that as many as possible genera need to be studied before the taxonomic significance of this character can be stated, considering the scarcity of sperm ultrastructure data of dendrobatids.

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Artigo VI

Sperm ultrastructure of the Brazilian Amazon poison frogs *Epipedobates trivittatus* and *Epipedobates hahneli* (Anura, Dendrobatidae)

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Abstract – The sperm ultrastructure of *Epipedobates trivittatus* and *E. hahneli* is described. The spermatological characteristics are identical in both species, except for the extension of the acrosomal vesicle. The structure of the acrosomal complex is shared with species of the Bufonoidea neobatrachian lineage, which differs from the condition observed to species included into the ranoid lineage. The phylogenetic significance of the mitochondrial collar, also seen in Eubufonoidea groups, is still unclear. Also the significance of the alternative mitochondrial arrangement seen here is unclear. The perpendicular arrangement of the centrioles is also shared with the Bufonoidea, and is considered a synapomorphic condition. An expanded undulating membrane and the absence of a juxtaxonemal fiber appear to be apomorphic characteristics of dendrobatid species, as also observed (homoplastically) for *Litoria* and *Cyclorana* species. However, the notable expansion of the undulating membrane appears to be an autapomorphy of Dendrobatidae frogs. The ultrastructural data presented here do not support the proposed placement of *E. trivittatus* in a separate genus.

Introduction

The genus *Epipedobates* was described by Myers (1987) and comprises 28 species. The members of this genus are distributed throughout Central America and northern South America, with a large number of representatives in the Brazilian Amazon (Frost, 2002). Within the genus, some monophyletic units have been hypothesized and organized into four nominal groups of species, namely *trivittatus*, with 3 species, *pictus*, with 9 species, *femoralis*, with 5 species and *petersi*, whith 2 species (Frost, 2002). According to Ford (1993), few unique apomorphies can be ascribed to the genus *Epipedobates*, originally described based on plesiomorphic characters (Myers, 1987), or to its species groups. In addition, molecular data have indicated a paraphyletic origin for this genus.

The grouping of genera within the Dendrobatidae have undergone a number of changes, including the erection of new genera and the shifting of species between genera (Myers, 1987; Zimmermann and Zimmermann, 1988; Myers et al., 1991; La Marca, 1992, 1994; Myers et al., 1991; Frost, 2002). Species currently belonging to *Epipedobates* have previously been placed in at least three other genera, *Dendrobates*, *Phyllobates*, and *Colostethus* (reviewed in Frost, 2002). Based on behavioral characteristics, Zimmermann and Zimmermann (1988) proposed two additional genera for the Dendrobatidae, *Allobates* and *Phobobates*, in which species of *Epipedobates* could be placed. However, the acceptance of these new taxa is not unanimous (see Myers et al., 1991; Frost, 2002).

A wide range of tools such as molecular markers, cytogentic data, ecological traits and sperm ultrastructural characteristics have been used to clarify the inter- and intrageneric relationships of the dendrobatids, as well as their relationships within the superfamily Neobatrachia (Bogart, 1991; Toft, 1995; Caldwell, 1996; Vences et al., 2000; Clough and Summers, 2000; Garda et al., 2002, paper IV, this thesis; Aguiar-Jr. et al., 2002, paper I, this thesis; Aguiar-Jr. et al., 2003, paper V, this thesis). Sperm ultrastructural analysis has been used in many anuran groups and is an alternative source of data for assessing taxonomic and phylogenetic inferences (Lee and Jamieson, 1993; Jamieson et al., 1993; Kwon and Lee, 1995; Meyer et al., 1997).

Although there have been few ultrastructural studies in dendrobatid frogs, such analyses can be very useful. Garda et al. (2002) described the sperm ultrastructure of *Epipedobates flavopictus* and identified characteristics which reinforced the placement of

the Dendrobatidae within the Bufonoidea lineage, in agreement with molecular data from nuclear and mitochondrial gene sequences (Hillis et al., 1993; Hay et al., 1995). In addition, Aguiar-Jr. et al. (2002b) analyzed the spermatozoa of *Epipedobates femoralis* and *Colostethus* sp. and noted considerable ultrastructural similarity between them. These spermatozoa also had a remarkable biflagellate structure, a condition previously reported in only two anuran species, *Telmatobufo australis*, a lepotdactylid frog (Pugin and Garrido, 1981) and *Chiromantis xerampelina*, a rhacophorid frog (Wilson et al., 1991 – *apud* Jamieson, 1999). These results reinforced the relationships between *E. femoralis* and species of *Colostethus* (Aguiar-Jr. et al., 2002b), as already suggested in the literature, and indicated the placement of *E. femoralis* in a separate clade within the Dendrobatidae (Toft, 1995; Vences et al., 2000; Clough and Summers, 2000).

In the present work, we describe the sperm ultrastructure of two additional species of *Epipedobates* (*E. trivittatus* and *E. hahneli*) as a contribution to our understanding of the inter- and intrageneric relationships of the genus. The data obtained may be useful for reevaluating the phylogenetic relationships within the family Dendrobatidae and its position within the Anura.

Material and methods

Three adult males of *Epipedobates trivittatus* and *E. hahneli* were collected in lowland Amazonia, approximately 40 km south of Manaus, Amazonas, Brazil (3° 37' 10.4" S, 59° 86' 78.4" W) in the municipality of Castanho, at km 12 on the road to Autazes. The specimens were collected under a permit from the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA) (02005.001367/99-58-AM). Voucher specimens were deposited in the "Célio Fernando Baptista Haddad (CFBH)" Amphibian Collection of the Departamento de Zoologia, Universidade Estadual Paulista (UNESP), São Paulo, Brazil, and in the Museu de História Natural "Prof. Adão José Cardoso" (ZUEC) of the Universidade Estadual de Campinas (UNICAMP), São Paulo, Brazil. The specimens received the following accession numbers: *E. hahneli* - ZUEC 11755 and 11769, and CFBH 5.232, and *E. trivittatus* - ZUEC 11747 and 11739, and CFBH 5.222.

The testes were removed by dissection, cut into small pieces and fixed overnight at 4°C in 0.1 M sodium cacodylate buffer, pH 7.2, containing 2% paraformaldehyde, 2%

glutaraldehyde, 3% sucrose and 5 mM $CaCl_2$. Postfixation was done for 1 h in the same buffer containing 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$. The tissues were subsequently rinsed in sodium cacodylate buffer and incubated for 2 h in 0.5% uranyl acetate. After rinsing in buffer, the material was dehydrated in an increasing acetone series and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a LEO 906 transmission electron microscope. To measure the total length of the head (acrosomal complex + nucleus) and flagellum, a suspension of spermatozoa was prepared in sodium cacodylate buffer, spotted on clean slides and observed and photographed using phase contrast microscopy.

Results

In light microscopy, the spermatozoa of *E. hahneli* consisted of a filliform head about 23.2 μm long and a single flagellum ~32μm long (Fig. 1A). The general morphology of the spermatozoa of *E. trivittatus* was very similar to that of *E. hahneli*, with a head 22.1 μm long and a single flagellum ~29 μm long (Fig. 2A). In both cases, an undulating membrane was clearly seen connecting the axoneme and the paraxonemal rod (Figs. 1A and 2A).

At the ultrastructural level, the spermatozoa of these species are almost identical. Thus, only one description is provided, with emphasis on the differences when necessary.

Acrosomal complex

In both species, the nucleus was cylindrical and elongated, with the anterior portion being covered by the acrosomal complex (acrosome + subacrosomal cone). The acrosome consisted of an electrondense conical vesicle (Figs. 1B and 2B). The acrosomal vesicle was less extensive in *E. hahneli*, and no longer extended to the anterior portion of the nuclear space (Fig. 1B). A subacrosomal cone was present under the acrosome, and contained diffuse electrondese material. This cone lay at the anterior portion of the nucleus and extended beyond the limits of the acrosomal vesicle (Figs. 1B and 2B). In transverse sections, the acrosomal complex was circular and the subacrosomal cone narrowed progressively towards the base of the nucleus to eventually disappear in the most basal

portion (Figs. 1C-F and 2C-F). Transverse sections of the anterior portion of the nucleus in *E. hahneli* showed that the acrosomal vesicle was already absent in this region (Fig. 1E).

Nucleus

The nucleus was conical in longitudinal sections and circular in transverse sections (Figs. 1B, E, F and 2B, E, F). The chromatin was not highly condensed in most of the spermatozoa. This aspect was clearer in the nucleus of *E. trivittatus* spermatozoa (Fig. 2E, F). In addition, a nuclear space was observed since the chromatin did not completely fill the nucleus (Figs. 1B and 2B).

Midpiece

In both species, the midpiece was short (Figs. 1G-J and 2G-I). In this region, the final portion of the nucleus had a pronounced concavity (the nuclear fossa) in which the proximal and distal centrioles were inserted and lay at an angle of 90° to each other (Figs. 1H, I and 2G-I). The distal centriole gave rise to the axoneme. The paraxonemal rod (*sensu* Jamieson et al., 1993) also entered the nuclear fossa adjacent to the proximal centriole (Figs. 1G, I and 2I). Numerous mitochondria were observed in a cytoplasmic expansion termed the mitochondial collar, which surrounded the end of the nucleus and the anterior portion of the flagellum (Figs. 1J, K and 2J).

Flagellum

In transverse sections, the flagellum consisted of an axoneme, an undulating membrane and an axial fiber. The latter was curved, had a high electrondensity and was connected to the axoneme through an "axial sheet" (*sensu* Garda et al., 2002) of the same electrondensity (Figs. 1J-N and 2J-M). The mitochondrial collar was separated by a cytoplasmic canal (Figs. 1J and 2J). Further down, numerous mitochondria were also observed within the undulating membrane (Figs. 1K, L, and 2K). In the most distal portions, the undulating membrane lacked mitochondria (Figs. 1M and 2L) and the axial sheet progressively shortened, leaving the axoneme and the axial fiber close together (Figs.

1N-O and 2M, N). At the end of the flagellum, only the axoneme was seen (Figs. 1P and 2O).

Discussion

The spermatozoa of *E. hahneli* and *E. trivittatus* were very similar to each other and to those of *E. flavopictus* (Garda et al., 2002, paper IV, this thesis), *E. femoralis* and *Colostethus* sp. (Aguiar-Jr. et al., 2003, paper V, this thesis), except for the presence of two flagella in the latter two species.

In agreement with previous work by Garda et al. (2002, paper IV, this thesis), the data obtained here on the general structure of the spermatozoa seems to suggest a possible grouping of the dendrobatids within the Bufonoidea neobatrachian lineage. This suggestion is based mainly on the tail arrangement which, in both cases, comprises an axoneme and an accessory axial fiber united by an axial sheath within an undulating membrane. This organization differs from that seen in ranoid species, in which the tail consists only of the axoneme. Lee and Jamieson (1993) indicated that the absence of accessory tail filaments was an evolutionary trend in anurans, although they emphasized that this trend (schematically illustrated in that work) could not be used as a phylogeny.

Beside the flagellar apparatus, the structure of the acrosomal complex in *E. trivittatus* and *E. hanhneli* was also very similar to that of the remaining dendrobatids and to that of most bufonoid species studied so far, but differed from the condition seen in species of the ranoid lineage (Mizuhira et al., 1986; Báo et al., 1991; Lee and Jamieson, 1993; Kwon and Lee, 1995; Meyer et al., 1997; Garda et al., 2002, paper IV, this thesis; Aguiar-Jr. et al., 2003, paper V, this thesis). However, the presence of a conical acrosomal vesicle is considered a plesiomorphic condition (Kwon and Lee, 1995; Jamieson, 1999). Beside this, since the subacrosomal cone is homologous with the putative conical perforatorium of bufonoids and with the subacrosomal cone of *Ascaphus* (see discussion in Garda et al., 2002, paper IV, this thesis), this characteristic (presence of a subacrosomal cone which lies under a conical acrosomal vesicle) also seems to be plesiomorphycally shared between dendrobatids and bufonoids. Therefore, despite of the spermatological similarities, synapomorphies must be established phylogenetically before the affinities between dendrobatids and species belonging to the bufonoid lineage can be confirmed. For

the moment, we consider it prudent to accept dendrobatids sperm as being of the "bufonoid-type" with no further phylogenetic implications.

Mitochondria arranged in a collar-like structure around the anterior portion of the flagellum was another trait shared by the bufonoids and dendrobatids, which might indicate affinities between these taxa. This mitochondrial collar is a well-developed structure in the testicular spermatozoa of dendrobatids (except for *E. flavopictus*, in which it is only observed in early spermatids) and bufonoids, and is considered a synapomorphy in the latter (Lee and Jamieson, 1993). However, Scheltinga et al. (2001) attributed a possible plesiomorphic status to this characteristic since it resembled a condition seen in many acanthopterygian fishes, but did not discard the possibility that such a condition represented a reversal to a pre-lisamphibian condition. In contrast, Garda (2002), based on a survey of 22 spermatological characters, suggested that the presence of a mitochondrial collar was an apomorphic condition when compared to the distribution of these organelles around the axial fiber observed in some species (as also proposed by Lee and Jamieson, 1992), but was plesiomorphic when compared with the mitochondria arranged adjacent to the axoneme. Lee and Jamieson (1993) observed a decrease in the number or even absence of mitochondria in the mature sperm of species in which seminal or ejaculated sperm were analyzed. These authors argued that this loss during ontogeny strongly suggested that the presence of many mitochondria was a primitive condition in the Anura.

In view of these uncertainties, the peculiar mitochondrial arrangement in the spermatozoa of *E. trivittatus* and *E. hahneli* (this work) and *E. flavopictus* (Garda et al., 2002, paper IV this thesis), in which these organelles are also seen scattered within the undulating membrane, becomes very difficult to interpret and no definite conclusions as to its significance can be made at the moment. Mitochondria within the undulating membrane have only been described in the unrelated species *Nectophrynoides occidentalis* (a bufonid) (Pugin-Rios, 1980) and *Ascaphus truei* (an archaeobatrachian species) (Jamieson et al., 1993). In *N. occidentalis*, the mitochondria are restricted to an expanded region of the undulating membrane adjacent to the axoneme and the dense sheet. In sperm of *A. truei*, the mitochondria are located in one or more longitudinal series of grooves in the paraxonemal rod, within a putative homolog of the undulating membrane. In the genus *Epipedobates*, *E. femoralis* is unique in that the mitocondria are restricted to the mitochondrial collar around the most anterior portion of the flagella (Aguiar-Jr. et al., 2003, paper V, this thesis).

Other intrageneric spermatological differences found within *Epipedobates*, such as the extent of the acrosomal vesicle, the presence of nuclear inclusions in *E. flavopictus* spermatozoa and the biflagellar nature of *E. femoralis* spermatozoa, are of unknown evolutionary significance (see Aguiar-Jr. et al., 2003, paper V, this thesis).

Concerning the midpiece, the perpendicular arrangement between the two centrioles in *E. trivittatus* and *E. hahneli* is also observed in bufonoid groups such as Bufonidae, Hylidae and Leptodactylidae, and differs from the oblique arrangement (140°) in Ranidae species. Kwon and Lee (1995) considered the oblique arrangement to be an apomorphic trait in the evolutionary trend towards an increase in the angle between the centrioles.

Considering the flagellar apparatus, the thick, short undulating membrane, and the absence of a juxtaxonemal fiber observed here appear to be apomorphic characteristics of dendrobatid spermatozoa (see also Garda, 2002, paper IV, this thesis; Aguiar-Jr. et al., 2003, paper V, this thesis). According to Scheltinga et al. (2001), a plesiomorphic flagellar pattern occurs in *Bufo*, *Litoria* and in most leptodactylid species (Pugin-Rios, 1980; Lee and Jamieson, 1992, 1993; Meyer et al., 1997) in which a thin, long undulating membrane, is found beside the juxtaxonemal and axial fibers. Meyer et al. (1997) considered the pattern in fossorial Australian hylids (*Litoria albogattata* and *Cyclorana* species, except for *C. cryptotis*) to be apomorphic. As in the Dendrobatidae, in these species the juxtaxonemal fiber is absent and the undulating membrane is short and enlarged (although not as expanded as in dendrobatids). These authors proposed that this pattern could have resulted from a secondary simplification from the complex condition found in non-fossorial hylids (*Litoria aurea* species group plus *C. cryptotis*), as well as in the remaining Eubufonoidea species and in urodeles. This suggestion agrees with the general trend towards sperm simplification reached by highly apomorphic taxa. The marked expansion of the undulating membrane appears to be an autapomorphic characteristic of Dendrobatidae frogs studied so far.

Based on behavioral traits, Zimmermann and Zimmermann (1988) proposed the placement of *E. trivittatus* in the genus *Phobobates*, a new genus which also accommodated two other species (*P. bassleri* and *P. silverstonei*) previously belonging to *Epipedobates*. The sperm ultrastructural data presented here do not support the removal of *E. trivittatus* from *Epipedobates*. Rather, the apomorphic condition of the flagellar apparatus shared with the remaining species of this genus indicates that this species should be retained in this

taxon, in agreement with the molecular data obtained by Clough and Summers (2000). In the latter study, *E. trivittatus* was grouped in a clade with other *Epipedobates* species and as the sister-taxon of *E. hahneli*. In addition, in this work by Vences et al. (2000) using mitochondrial rRNA genes, *Phobobates trivittatus* and *P. silverstonei* were grouped with *Epipedobates* species (*sensu stricto*). Based on their results, these authors suggested that *Phobobates* was not supported as a separate taxon and should be regarded as a synonym of *Epipedobates*, as also suggested by Myers et al. (1991), Toft (1995) and Caldwell (1996). Cytogenetically, the differences between the *E. trivittatus* karyotype and that of its congeneric species (Aguiar-Jr. et al., 2002, paper I, this thesis) are insufficient to warrant a reevaluation of its taxonomic status. The data here presented, together with those for *E. flavopictus* (Garda et al., 2002, paper IV, this thesis), reinforce *E. femoralis* (see Aguiar-Jr. et al., 2003; paper V, this thesis) as a distinct species within the genus because of the biflagellar condition of its spermatozoa.

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Figure Legends:

Figure 1. A. Spermatozoa of *E. hahneli* under light microscopy. **B-P.** Transmission electron microscopy. **B.** Longitudinal section of the head showing the acrosomal vesicle with a short extension (arrows), the subacrosomal cone, the nuclear space and the increasingly condensed chromatin. **C-F.** Transverse sections of the acrosomal complex showing the progressive narrowing of the subacrosomal cone and enlargement of the nucleus. **G.** Transverse section of the final portion of the nucleus at the nuclear fossa showing the proximal centriole and the adjacent paraxonemal rod. **I and J.** Sections of the posterior portion of the nucleus and midpiece. Note the perpendicular arrangement between the proximal and distal centrioles, and the insertion of the paraxonemal rod in the nuclear fossa. **J-P.** Transverse sections of the flagellar portion. **J.** Mitochondria occur in a collar-like structure around the anterior most portion of the flagellum, from which they are separated by a cytoplasmic canal. **K and L.** In a further down position, mitochondria are also seen scattered within the undulating membrane. **M.** Further down the undulating membrane lacks mitochondria. **N and O.** The undulating membrane and the axial sheath progressively shorten, bringing the axial fiber close to the axoneme. **P.** Only the axoneme is seen in the most distal region. **Scale Bars:** A = 10μm; B, F and H-J = 0.30μm; C, D and K = 0.20μm; E = 0.25μm, G = 0.45μm and L-O = 0.15μm.

Figure 2. A. Spermatozoa of *E. trivittatus* under light microscopy. **B-N.** Transmission electron microscopy. **B.** Longitudinal section of the acrosomal complex showing the conical acrosomal vesicle, which extends further than in *E. hahneli*, the subacrosomal cone and the nuclear space. Note that the chromatin is not highly compacted. **C-F.** Transverse sections of the acrosomal complex and nucleus. Note the progressive narrowing of the subacrosomal cone which disappears in the basal portion of the nucleus. **G-I.** Transverse sections of the nucleus and midpiece of spermatids in various stages of maturation. Note the perpendicular arrangement of the centrioles in the pronounced nuclear fossa. In **I**, the paraxonemal rod is seen penetrating the nuclear fossa. **J.** Mitochondrial collar around the anterior portion of the nucleus and separated from the flagellum by the cytoplasmic canal. **K.** Mitochondria scattered within the highly expanded undulating membrane. **L.** In a more

distal region, mitochondria are no longer seen in the undulating membrane, which remains expanded even in the absence of these organelles. **M and N.** Shortening of the undulating membrane and axial sheath and approximation between the axoneme and axial fiber. **O.** In the final portion of the flagellum, no accessory fibers are seen associated with the axoneme. **Scale Bars:** A = 10μm; B and F = 0.5μm; C-E, H, M and N = 0.25μm; G and I = 0.35μm; J-L = 0.45μm; O = 0.30μm and P = 0.20μm.

Abbreviations: av = acrosomal vesicle; af = axial fiber; ar = axial rod; as = axial sheath; ax = axoneme; cc = cytoplasmic canal; dc = distal centriole; m = mitochondria; mc = mitochondrial collar; n = nucleus; nf = nuclear fossa; ns = nuclear space; pc = proximal centriole; pr = paraxonemal rod; sc = subacrosomal cone; um = undulating membrane.

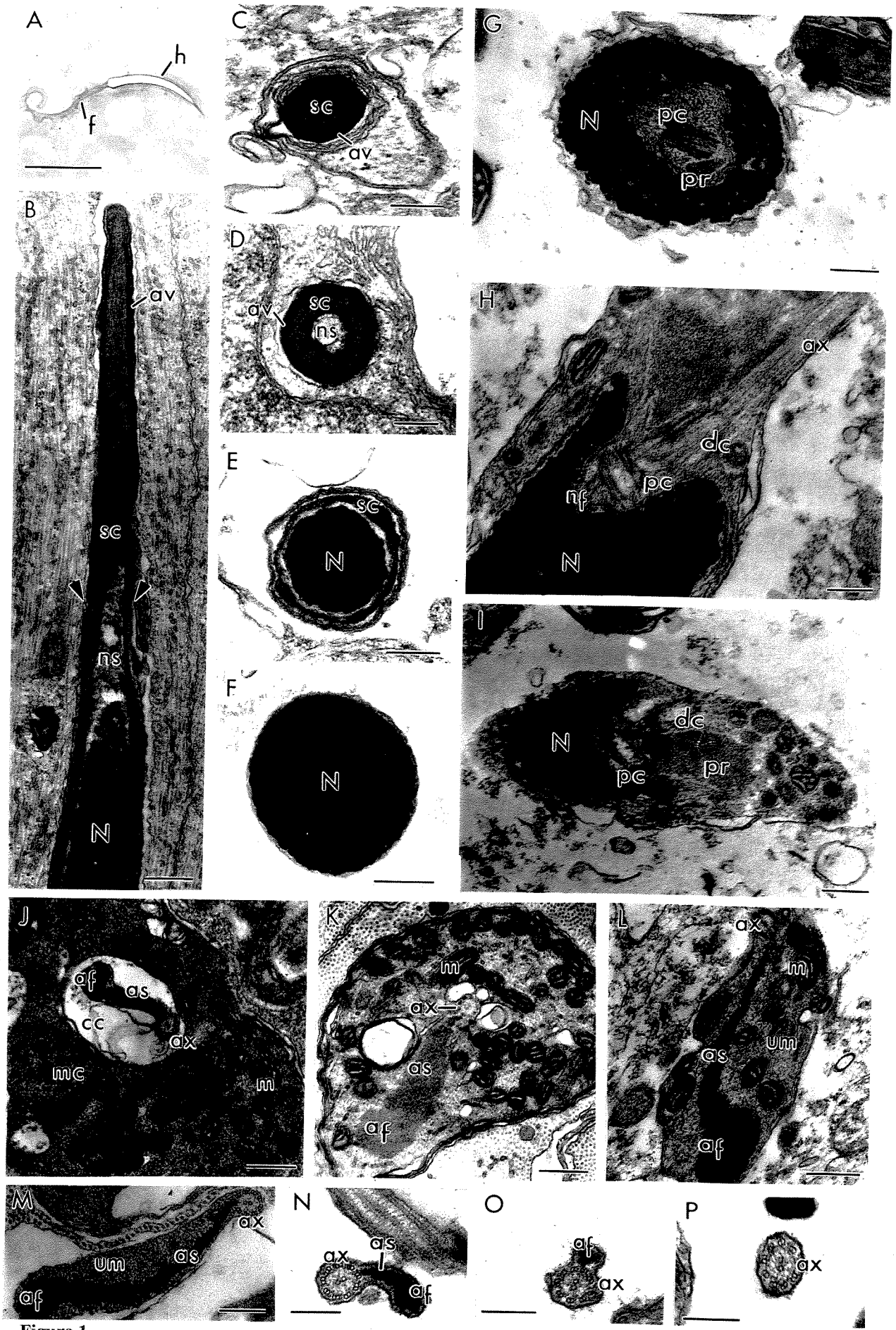
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Figure 1

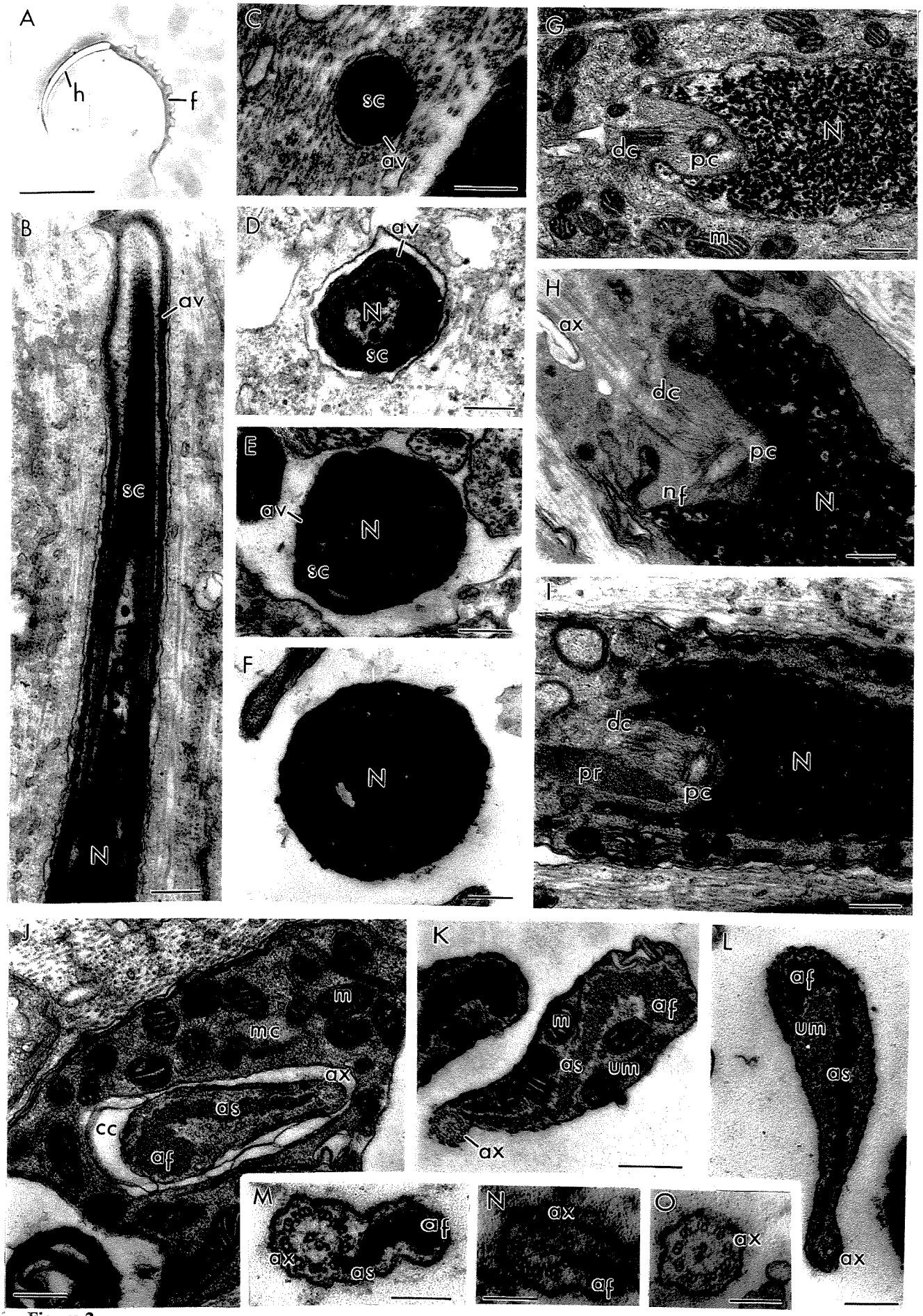
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Figure 2

Artigo VII

Ultrastructural characteristics of sperm in Hylodinae species (Anura, Leptodactylidae) and their relevance to taxonomic relationships of this group

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Key words: Hylodinae, sperm ultrastructure, *Hylodes*, *Crossodactylus*, *Megaelosia*

Running Title: Sperm ultrastructure in Hylodinae species

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Abstract

Hylodinae leptodactylids form a group of diurnal frogs assumed to be the basal group from which the dendrobatids arose. This assumption is based on classic analysis of morphological traits. In this work, we describe ultrastructural characteristics of sperm from three hylodine species (*Hylodes phyllodes*, *Crossodactylus* sp. n. and *Megaelosia massarti*), and reassess the intergeneric relationships within this group, as well as the possible Hylodinae/Dendrobatidae relationships. The sperm ultrastructure was very similar among the three species, and indicated the conserved nature of these gametes within the Hylodinae. The acrosomal complex was very similar to that of other leptodactylid, and so contributes little to our understanding of Hylodinae/Dendrobatidae relationships. The flagellar apparatus of *Crossodactylus* sp. n. was very similar to that of most leptodactylid. *Megaelosia massarti* and *Hylodes phyllodes* shared a distinctive condition in their axial and juxtaxonemal fibers which may serve to reinforce the affinities between these genera. This distinctive flagellar condition expands the already well-known variability in sperm structure within the Leptodactylidae.

INTRODUCTION

Leptodactylid frogs are restricted to the Americas from southern USA and the Antilles to southern South America (Frost, 2002). More than 1,000 species are recognized as belonging to this neotropical family (Frost, 2002), which represents about a quarter of the anuran species described so far. Within the Leptodactylidae, the subfamily Hylodinae (*sensu* Savage, 1973) encompasses three genera (*Hylodes*, *Crossodactylus* and *Megaelosia*) and 34 species, distributed from northwestern to southern Brazil and northern Argentina (Frost, 2002).

The monophyly of the Hylodinae frogs was proposed by Heyer (1975), who emphasized some peculiarities of *Megaelosia* not shared with the remaining two genera. The extensive similarities between *Hylodes* and *Crossodactylus*, as well as the notable differences involving *Megaelosia*, had already been noted by Lutz (1930). Based on morphological and karyotypical data, *Hylodes* and *Crossodactylus* have been tentatively considered as the closest relatives of the neotropical frogs belonging to the family Dendrobatidae (Noble, 1931; Lynch, 1971; Bogart, 1991; reviewed by Ford, 1993). On the other hand, according to Griffths (1959, 1963) and Ford (1993), the ancestral of the Dendrobatidae may have been an African ranoid frog.

Since the morphological (Ford,1989, *apud* Ford, 1993) and karyotypical (Bogart, 1991) analyses, few studies have addressed the question of dendrobatid affiliations. Recently, Aguiar-Jr. et al. (papers I and III, this thesis) and Rosa et al. (*in press*, paper II, this thesis) have used a cytogenetic approach to examine the Hylodinae/Dendrobatidae relationships. These investigations have pointed out that no unambiguous homeologies can be found between the karyotypes of these two groups, even with the employment of

banding techniques. In addition, according to Rosa et al. (*in press*, paper II, this thesis), the karyotypic peculiarities of *Megaelosia* meant that this genus contributed little to the understanding of the relationships between these two taxa.

In anurans, a variety of alternative approaches has been used to provide new data to supplement the traditional morphological parameters when reevaluating taxonomic and phylogenetic problems. As an example, the analyses of sperm ultrastructure has been used in many anuran groups belonging to both, Archaeo- and Neobatrachia, and has been helpful in suggesting evolutionary trends and in elucidating taxonomic relationships (Pugin-Rios, 1980; Jamieson et al., 1993; Kwon and Lee, 1995; Meyer et al., 1997; Aguiar-Jr. et al., 2003, paper V, this thesis; revision by Garda, 2002). It is generally agreed, however, that an expansion in the number of species analyzed is essential for further phylogenetic reconstruction based on such spermatological characteristics.

To date, in only 16 species belonging to three leptodactylid subfamilies has the sperm ultrastructure been studied, and none of these has included representatives of the Hylodinae (Pugin-Rios, 1980; Pugin and Garrido, 1981; Garrido et al., 1989; Báo et al., 1991; Amaral et al., 1999, 2000). Thus, the main purposes of this work were to examine the sperm ultrastructure of hylodine species in order to contribute with the elucidation of the evolutionary trends in sperm ultrastructural traits in the Leptodactylidae family and provide new data for assessing intergeneric relationships as well as the possible relationships with dendrobatid species already studied (Garda et al., 2002, paper IV, this thesis, Aguiar-Jr. et al. 2003, paper V, this thesis, Aguiar-Jr. et al, paper VI, this thesis).

MATERIALS AND METHODS

Adult males of *Hylodes phyllodes* and *Megaelosia massarti* were collected at Paranapiacaba, in themunicipality of Santo André, São Paulo State. One adult specimen of *Crossodactylus* sp. n. was collected in the "Parque Florestal de Itapetinga", in the municipality of Atibaia, São Paulo State. The specimens were collected under a permit from the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA) (20235/98-78 SP). Voucher specimens were deposited in the Museu de História Natural "Prof. Adão José Cardoso" (ZUEC) of the Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil. The specimens were assigned the following accession numbers: *H. phyllodes* (ZUEC 11554), *M. massarti* (ZUEC 11396), and *Crossodactylus* sp. n. (ZUEC 11437).

The testes were removed by dissection, cut into small pieces and fixed overnight at 4°C in 0.1 M sodium cacodylate buffer, pH 7.2, containing 2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose and 5 mM $CaCl_2$. Postfixation was done for 1 h in the same buffer containing 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM $CaCl_2$. The tissues were subsequently rinsed in sodium cacodylate buffer and incubated for 2 h in 0.5% uranyl acetate. After rinsing in buffer, the material was dehydrated in an increasing acetone series and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a LEO 906 transmission electron microscope.

RESULTS

The basic structure of the spermatozoa of the three hylodines showed great uniformity, especially in structure of the acrosomal complex and midpiece. Differences were observed in the shape of the tail accessories (juxtaxonemal and axial fibers).

Acrosomal complex

In the three species, a conical acrosomal vesicle covered the anterior portion of the nucleus. This vesicle was filled with material of variable electrondensity (Figs. 1A, 2A and 3A). Figure 2A shows only a very thin vesicle in *Crossodactylus* sp. n. . A subacrosomal cone was observed below the acrosomal vesicle. This cone showed a homogeneous electrondensity in all species (Figs. 1A, 2A and 3A) and extended beyond the limit of the acrosome (seen only in Figs. 2A and 3A). In *M. massarti* and *H. phyllodes*, the subacrosomal cone had a granular aspect, in contrast to *Crossodactylus* sp. n. This characteristic was more obvious in transversal sections of the acrosomal complex (Figs. 1B-D, 2B, C, and 3B-D). A subacrosomal space was observed between the subacrosomal cone and the nucleus, and was particularly large in *M. massarti* (Figs. 1A, 2A and 3A).

Nucleus

The chromatin did not completely fill the nucleus, and this created a nuclear space which was not observed in *Megaelosia massarti*. The chromatin was not fully compacted in the three species, particularly in *M. massarti* spermatozoa (Figs. 1A, E, F, 2A, C, D-F and 3A, D-F). However, highly compacted chromatin was seen in a transverse sections of the nucleus in *H. phyllodes* (Fig. 1D). The nucleus of *Crossodactylus* sp. n. spermatids contained very loosely condensed chromatin which bordered a more condensed central region (Fig. 2 D, E). Numerous mitochondria were associated with the posterior region of the nucleus in all species, and formed a "mitochondrial belt" in this region (Figs. 1E, 2D

and 3F). This belt was absent in some of the sperm examined, as shown for *M. massarti* (Fig. 3E, F).

Midpiece

The midpiece was short and enclosed the proximal and distal centrioles, which were arranged obliquely to each other. The distal centriole gave rise to the axoneme (Figs. 1F, 2E, F, H and 3G). Parallel to the distal centriole, a paraxonemal rod (*sensu* Jamieson et al., 1993) was observed in the spermatozoa of *H. phyllodes* and *Crossodactylus* sp. n. (Figs. 1F and 2E). In this region, the posteriormost portion of the nucleus showed a concavity termed nuclear fossa (Figs. 1F, 2F and 3G).

Tail piece

At the anteriormost portion, the flagellum was surrounded by the mitochondrial collar. This structure was poorly developed in the spermatozoa of *H. phyllodes*, which contained only a few mitochondria or only a cytoplasmic sheath (Figs 1F, G, 2G and 3H). The flagellum consisted of an axoneme with the usual 9+2 pattern, a juxtaxonemal fiber (connected to the axoneme on doublet number 3), a very thin undulating membrane and an axial fiber. The juxtaxonemal and axial fibers were connected through an electrondense material within the undulating membrane (Figs. 1H, I, 2H, I and 3I, J). *H. phyllodes* and *M. massarti* shared a very similar flagellar structure in which the juxtaxonemal and axial fibers had a "baseball bat" shape, although both were quite extensive in *H. phyllodes* (Figs. 1H, I and 3 I, J). This pattern was different from that of *Crossodactylus* sp. n. in which the

juxtaxonemal fiber was very short and the axial fiber had a globular shape (Fig. 2H, I). In more distal portions along the flagellum, the undulating membrane shrank and the accessory fibers moved closer to each other. Further down, the fibers fused and, in the most distal portion, only the axoneme was observed (Figs 1J-L, 2I-M and 3J-M).

DISCUSSION

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

The acrosomal complex was similar to that of other leptodactylid species (Báo et al, 1991; Amaral et al., 2000), as well as to most of the remaining species included in the Bufonoidea lineage (Pugin-Rios, 1980; Lee and Jamieson, 1993; Kwon and Lee, 1995; Báo et al., 2001), and also to that observed in the dendrobatid species examined so far (Garda et al., 2002, paper IV, this thesis; Aguiar-Jr. et al., 2003, paper V, this thesis, Aguiar-Jr. et al., paper VI, this thesis). However, this similarity between the Hylodinae and Dendrobatidae does not contribute to support the assumed evolutionary affinities between these two groups since it has been argued that the presence of a subacrosomal cone under a conical acrosomal vesicle is plesiomorphic (see Jamieson, 1995; Garda et al., 2002, paper IV, this thesis). According to Aguiar-Jr. et al. (paper VI, this thesis), until unambiguous spermatological synapomorphies are found, no relationships can be assumed based on the characteristics of the acrosomal complex in these groups. In any case, this condition differ considerably from that observed in ranoid frogs studied so far, including members of the Ranidae (genus *Rana*).

Although the degree of chromatin condensation has already been used to indicate affinities among anuran groups (Kwon and Lee, 1995), this characteristic is a poor indicator of relationships. In all of the species studied here, the low degree of chromatin condensation seen in most of the cells examined probably reflected the stage of maturation of these gametes rather than real affinities among them.

The number and distribution of mitochondria are very variable in lissamphibian sperm (Scheltinga et al., 2001). The mitochondrial belt observed around the base of the nucleus is probably a transient structure that is lost during spermiogenesis, as also suggested to occur with the mitochondrial collar in late testicular spermatozoa or in the seminal vesicle (Pugin-Rios, 1980; Garrido et al., 1989). The evolutionary significance of the mitochondrial collar is still unclear, being considered a synapomorphy uniting the Eubufonoidea (bufonoids, excluding myobatrachids) (Lee and Jamieson, 1992, 1993), or possibly a plesiomorphic characteristic (Scheltinga et al., 2001) (see also discussion in Aguiar-Jr. et al., paper VI, this thesis). The well-developed mitochondrial collar seen in *Crossodactylus* sp. n. and *M. massarti* is common in bufonoid and dendrobatid species (Jamieson et al., 1993; Kwon and Lee, 1995; Garda et al., 2002, paper IV; this thesis; Aguiar-Jr. et al., 2003, paper V, this thesis; Aguiar-Jr. et al., paper VI, this thesis). In contrast, in *H. phyllodes* this structure is poorly developed and resembles the condition found in *Leiopelma hochstetteri* by Scheltinga et al. (2001). These authors considered this structure to be plesiomorphic in anurans. However, any suggestion as to the significance of this condition in *H. phyllodes* would be very speculative at the moment.

Crossodactylus sp. n. had a flagellar apparatus very similar to that of other leptodactylids such as *Odontophrynus cultipes* (Báo et al, 1991) and *Pseudopaludicola falcipes* (Amaral et al., 2000), in which the axial fiber had a "globular" shape, although the

juxtaxonemal fiber seen in *Crossodactylus* was somewhat reduced. In contrast, the juxtaxonemal and axial fibers of *H. phyllodes* and *M. massarti* shared a distinctive "baseball bat" shape. A similar shape in the juxtaxonemal fiber observed in *Megaelosia* has been describe to *Physalaemus* spp. (Amaral et al., 1999), which surprisingly do not possess an axial fiber.

Despite of some differences in skull morphology, Lynch (1971) retained *Megaelosia* in the Hylodinae due to similarities shared with *Hylodes*, including external morphology (which could also be the result of convergence) and the presence of paired vocal sacs (see also Giaretta et al., 1993). Maxson and Heyer (1982) pointed out the close relationships between these genera based on comparative albumin data. The spermatological features described here concerning the sperm flagellar apparatus also seem to suggest a possible relationship between these genera, supporting the retention of *Megaelosia* in the Hylodinae.

Based on cytogenetics, Rosa et al. (*in press*, paper II, this thesis) and Aguiar-Jr. et al (paper III, this thesis) observed that *Hylodes* and *Crossodactylus* were karyotypically more similar to each other than to *Megaelosia*, which had a distinctive karyotype and considerable intrageneric variation in chromosome number and morphology. Thus, karyotypical and spermatological characteristics apparently have evolved at different rates, and suggest different evolutionary histories in the Hylodinae. Such differences among data sets are to be expected (see Futuyma, 1992), and no definite answer on the intergeneric relationships within the Hylodinae will be obtained until such data sets are examined within a phylogenetic framework.

The ultrastructural features of the flagellar apparatus of the hylodine species examined here were somewhat different from the pattern in dendrobatids (Garda et al.,

2002, paper IV, this thesis; Aguiar-Jr. et al., 2003, paper V, this thesis; Aguiar-Jr. et al. paper VI, this thesis), which show an apomorphic condition in which the juxtaxonemal fiber is absent and the undulating membrane is short and highly thickened. However, both groups conserves a bufonoid-like structure in which an axial fiber is connected to the axoneme through an axial sheath within an undulating membrane, in contrast to the condition found in Ranids (see Kwon and Lee, 1995).

For the family Leptodactylidae, the data presented here on the distinctive flagellar apparatus in *H. phyllodes* and *M. massarti* increases the already known variability in sperm ultrastructure within the family. This variability includes some of the evolutionary trends proposed by Lee and Jamieson (1993), distributed through the 16 species already analyzed (Pugin-Rios, 1980; Pugin and Garrido, 1981; Garrido et al., 1989; Báo et al., 1991; Amaral et al., 1999, 2000). As previously emphasized by Amaral et al. (2000), this variation endorses the view that Leptodactylidae may be a polyphyletic taxon, as suggested by Duellman and Trueb (1994).

Acknowledgements

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Legends:

Figure 1: Electron micrographs of *H. phyllodes* spermatozoa. **A:** Longitudinal section through the head region showing the acrosomal complex (acrosomal vesicle and subacrosomal cone). Note the subacrosomal space under the subacrosomal cone and the nuclear space at the tip of the nucleus. **B-E:** Cross sections of the head. In D, note the high degree of chromatin condensation. In E, note the mitochondrial belt around the basal portion of the nucleus. **F:** Longitudinal section through the posteriormost region of the nucleus, midpiece and proximal portion of the flagellum, showing the nuclear fossa and the axoneme with an adjacent accessory fiber (paraxonemal rod). Note the presence of a discrete cytoplasmic sheet without mitochondria. **G:** Transversal section of the anterior portion of the flagellum showing a poorly-developed mitochondrial collar separated from the flagellum by a cytoplasmic canal. **H and I:** Longitudinal section of the tail showing "baseball bat" shaped axial and juxtaxonemal fibers. Note the very thin undulating membrane connecting the accessory fibers and the axoneme. **J-L:** Progressive narrowing of the undulating membrane and disappearance of the axial and juxtaxonemal fibers. Note that only the axoneme is seen at the tip of the tail (L). **Scale Bars:** A, G and I = 0,35μm; B-D and J = 0,20μm; E = 0,55μm; F and L = 0,45μm; H = 0,9μm and K = 0,30μm.

Figure 2: Electron micrographs of *Crossodactylus* sp. n. spermatozoa. **A:** Longitudinal section of the head. Note the thin acrosomal vesicle, the sucabacrosomal cone, the subacrosomal and nuclear space. **B-D:** Cross section through different portions of the head. Note that, in contrast to the longitudinal section, a well-developed acrosomal vesicle is observed. In D, note the different degrees of chromatin condensation between the central

and peripheral portions (an aspect also seen in E), and the "mitochondrial belt" surrounding the basal portion of the nucleus. **E and F:** Longitudinal sections through an early spermatid. Note the oblique arrangement between the proximal and distal centrioles, the paraxonemal rod adjacent to the distal centriole, and the low degree of chromatin compactation. **G:** Transversal section of the anterior portion of the flagellum showing a well-developed mitochondrial collar separated from the flagellum by the cytoplasmic canal. **H:** Cross section of the tail. Note the globular shape of the axial fiber and the short extension of the juxtaxonemal fiber. **I-L:** Progressive narrowing of the undulating membrane and approximation of the accessory fibers. **M:** The tail lacks accessory fibers at its tip end, where only the axoneme is seen. **Scale Bars:** A, E, G and H = 0,6μm; B, C, F and I-K = 0,30μm; D = 0.7μm; L and M = 0,20μm.

Figure 3: Electron micrographs of *M. massarti* spermatozoa. **A:** Longitudinal section through the head showing the poorly compacted chromatin, the acrosomal vesicle, the subacrosomal cone and a very large subacrosomal space. **B-E:** Cross section through the acrosomal complex and nucleus. Note the progressive enlargement of the nucleus. **F:** Basal portion of the nucleus showing the facultative presence of the "mitochondrial belt". **G:** Longitudinal section through the final portion of the flagellum and midpiece. Note the oblique arrangement of the centrioles. **H.** Well-developed mitochondrial collar around the anteriormost portion of the flagellum. **I.** Cross section of the tail showing the "baseball bat" axial and juxtaxomeal fibers. Note the thin undulating membrane connecting the axoneme to accessory fibers. **J-L:** Progressive narrowing of the undulating membrane and approximation of accessory fibers. **M:** Only the axoneme was seen at the tip of the tail.

Scale Bars: A and E = 0.40μm; B and D = 0,25μm; C, J and L = 0,20μm; F = 0,5μm; G = 0,35μm; H = 0,60μm; I, K and M = 0,30μm.

Abbreviations: af = axial fiber; av = acrosomal vesicle; ax = axoneme; cc = cytoplasmic canal; jf = juxtaxonemal fiber; m = mitochondria; mc = mitochondrial collar; N = nucleus; ns = nuclear space; pr = paraxonemal rod; sc = subacrosomal cone; ss = subacrosomal space; um = undulating membrane.

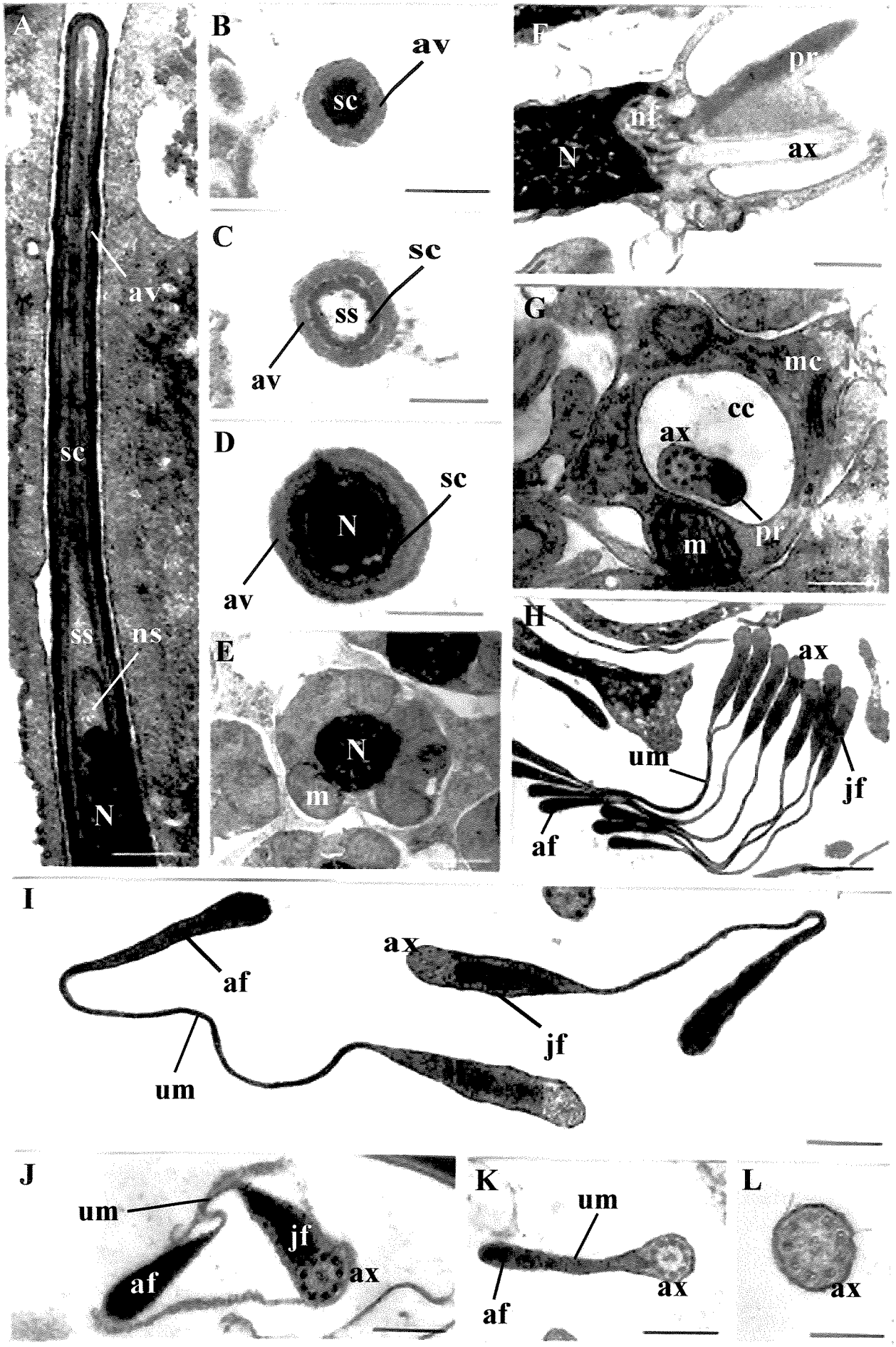
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Figure 1

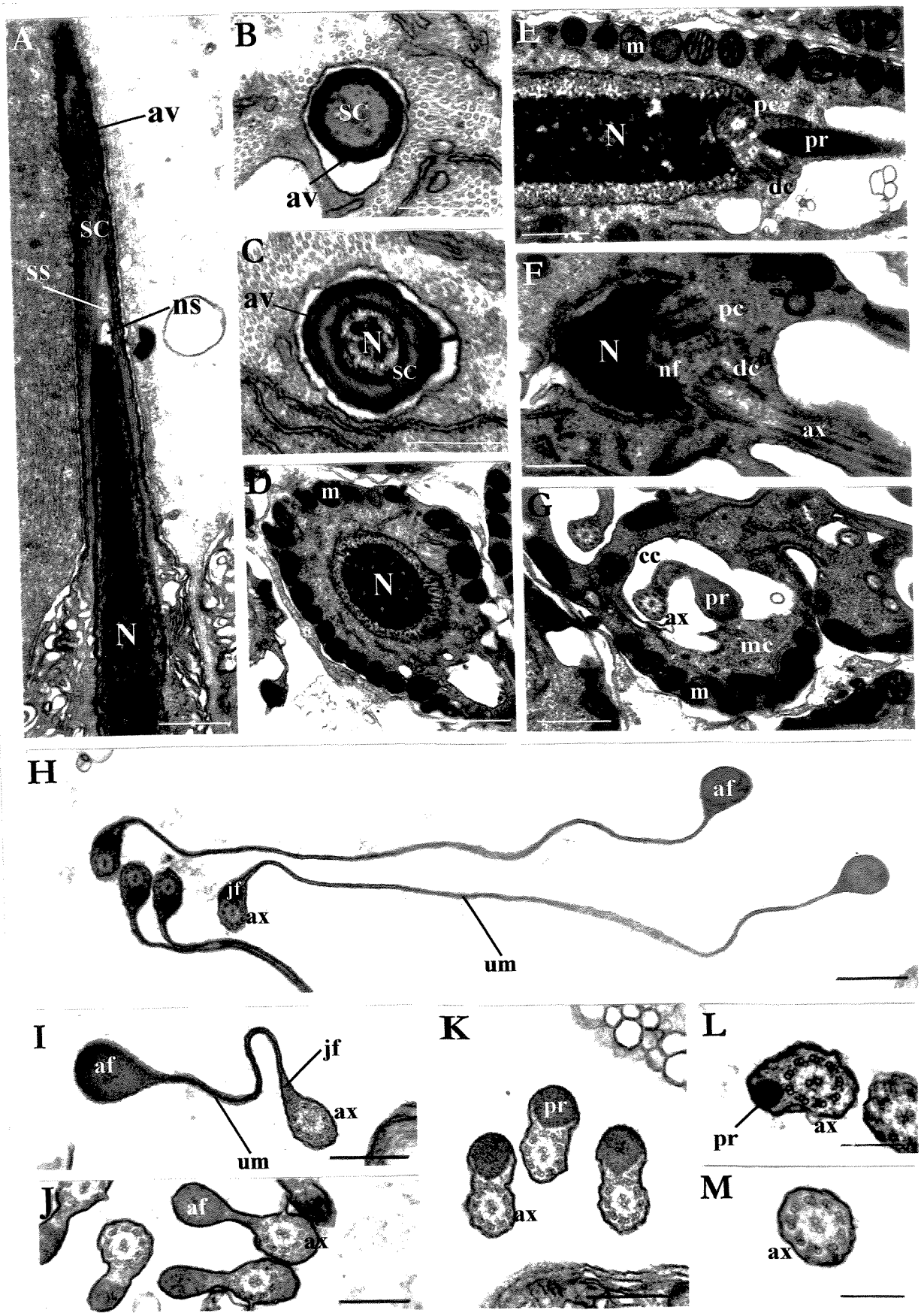
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Figure 2

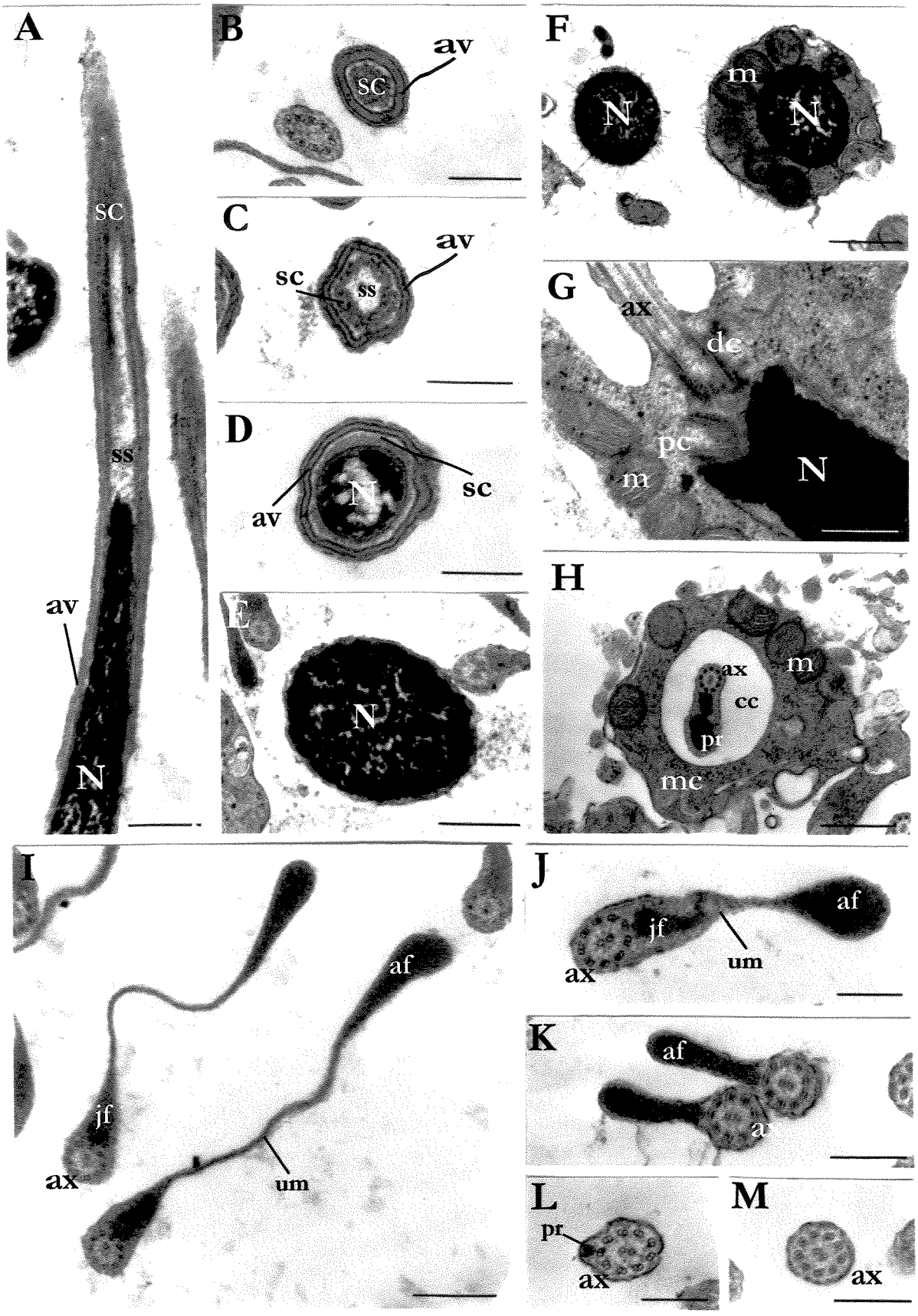
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Figure 3

4. Conclusões

4. Conclusões

O estudo citogenético e ultra-estrutural de espécies da subfamília Hylodinae e da família Dendrobatidae contribuíram de modo significativo para um melhor entendimento de aspectos intra- e intergenéricos dentro de cada grupo. Além disso, essas análises também forneceram elementos capazes de suportar uma maior afinidade de Dendrobatidae com a linhagem Bufonoidea dos neobatráquios, bem como elementos que, de forma clara, evidenciaram que a análise citogenética ainda não é capaz de reforçar as supostas relações entre esses táxons.

Do ponto de vista citogenético, mesmo com a utilização de técnicas de bandamento cromossômico (inéditas para ambos os grupos), não foram encontradas homeologias seguras entre os cariótipos. A falta de homeologias reconhecíveis pode ser reflexo de um longo tempo de divergência entre os hilodíneos e dendrobatídeos, que teria permitido a diversificação cariotípica observada atualmente, ou mesmo de histórias evolutivas totalmente independentes. Se de fato Hylodinae foi o grupo basal a partir do qual os Dendrobatidae se originaram, esta história parece não estar refletida nas características cariotípicas desses grupos, ou pelos menos de seus representantes aqui estudados.

Bogart (1991), na tentativa de correlacionar variação cariotípica e estratégia de vida em grupos de anuros, argumentou que na maioria dos gêneros que apresentavam desova de pequeno tamanho (número reduzido de ovos), cuidado parental, desenvolvimento terrestre e/ou desenvolvimento direto, grande variação cariotípica também era observada. Segundo ele, espécies que incluíam estas características especializadas em seu comportamento reprodutivo teriam maior tendência a se reproduzir em demes isoladas, o que portanto facilitaria a fixação de mudanças carotípicas. Estudando espécies de dendrobatídeos pertencentes aos gêneros *Dendrobates*, *Minyobates*, *Epipedobates* e *Colostethus*, Bogart concluiu que este modelo de correlação entre variação cariotípica e estratégia de vida aparentemente também se aplicava a essa família, uma vez que grande parte da variação cromossômica era encontrada em *Dendrobates* (2n = 18 a 20), gênero tido como mais especializado em termos reprodutivos. Bogart atribuiu uma tendência de redução do número de cromossomos em Dendrobatidae, de 2n = 24, observado nos gêneros menos especializados (*Colostethus* e *Epipedobates*), para 2n = 20 e 18 em *Dendrobates*. Adicionalmente, Bogart ponderou que se a evolução cromossômica e a variação cariotípica

fossem realmente influenciadas por fatores ecológicos e etológicos, espécies de *Colostethus* (gênero menos especializado) deveriam apresentar os cariótipos menos variáveis dentre os demais gêneros. Foi neste contexto que o autor, ao tratar da problemática envolvendo o possível ancestral que teria dado origem aos dendrobatídeos, supôs que do ponto de vista cariotípico tal ancestral estaria entre os leptodactilídeos hilodíneos.

A hipótese de relacionamento formulada por Bogart (1991) levava em conta, no entanto, aspectos cariotípicos pouco conclusivos. A presença de 2n=24 cromossomos em *Hylodes nasus*, número diplóide maior e mais comum (modal) encontrado para os dendrobatídeos, a presença de cromossomos telocêntricos em *H. nasus* e *H. glabrus* e o par cromossômico no. 1 proporcionalmente maior que os demais do complemento, foram os principais aspectos a convencerem o autor de que *Hylodes* e os demais hilodíneos e não os ranídeos africanos seriam possivelmente o grupo ancestral de Dendrobatidae.

Não há, no entanto, como supor que o número modal dos dendrobatídeos, compartilhado com *Hylodes nasus*, seja plesiomórfico. Para tal teríamos que admitir a hipótese do "mais comum = primitivo", o que não corresponde em grande parte dos casos (veja Qumsiyeh & Baker, 1988; Amorim, 1997). Além disso, naquele mesmo trabalho Bogart sugeriu que a presença de cromossomos telocêntricos nos quatro gêneros analisados era indicativa de que tanto fusão quanto fissão cêntrica poderiam ser os mecanismos evolutivos responsáveis pelas mudanças no número cromossômico observadas na família. A possibilidade de ocorrência de ambos os eventos dificulta ainda mais a tentativa de proposição de um número cromossômico ancestral para Dendrobatidae. Adicionalmente, dentro dos Dendrobatidae a expectativa expressa por Bogart com relação ao gênero *Colostethus* não obteve respaldo, uma vez que significativa variação na morfologia cromossômica foi encontrada no gênero e variação numérica foi também recentemente descrita por Veiga-Menoncello *et al.* (2001), que encontraram um número diplóide de 22 cromossomos para espécies do grupo *brunneus* próximas de *Colostethus marchesianus*. É possível, portanto, que nos dendrobatídeos a evolução cromossômica seja independente da evolução de características do comportamento reprodutivo e que a presença de 2n=24 cromossomos em *H. nasus* e em grande parte dos dendrobatídeos seja resultado de convergência.

A presença do par cromossômico no. 1 notavelmente grande nas espécies de *Dendrobates* com 20 cromossomos fez desta característica uma aliada para a suposição de

Bogart (1991). Entretanto, esse parâmetro parece ser altamente variável e essa característica não se destaca mesmo em outras espécies de *Hylodes* (veja *H. asper*, artigo III, tabela I) e outros dendrobatídeos (veja *Epipedobates femoralis* e *E. trivittatus*, artigo I, tabela I).

Portanto, as semelhanças cariotípicas entre leptodactilídeos e dendrobatídeos, apontadas por Morescalchi (1973) e Rasotto *et al.* (1987) e endossadas por Bogart (1991), embora se restringindo aos hilodíneos, parecem, até o momento, não implicar necessariamente em afinidades evolutivas entre esses grupos. De acordo com King (1990), o simples compartilhamento de "cariomorfos" semelhantes entre dois grupos realmente não é um bom indicador de relacionamento entre eles.

Se pela óptica da citogenética não foi possível estabelecer relações entre Hylodinae e Dendrobatidae, do ponto de vista da ultra-estrutura dos espermatozóides as análises aqui conduzidas ajudaram a lançar alguma luz à questão do posicionamento dos dendrobatídeos dentro dos Anura. Os espermatozóides de Dendrobatidae podem ser considerados como sendo "do tipo bufonóideo", o que significa que, embora com algumas particularidades, o padrão espermatológico observado nesse grupo se assemelha muito ao observado na linhagem Bufonoidea dos neobatráquios (incluindo os leptodactilídeos hilodíneos), dando indícios de sua alocação nesta linhagem e afastando (embora não excluindo) a possibilidade de uma origem a partir da linhagem Ranoidea. As particularidades mencionadas acima dizem respeito a possíveis autoapomorfias, tais como membrana ondulante curta e extremamente dilatada e ausência de fibra juxta-axonemal.

As duas ferramentas aqui utilizadas divergiram na maioria dos casos, mas também foram capazes de apontar na mesma direção para alguns outros. A exemplo de convergência, tanto os dados citogenéticos quanto ultra-estruturais apontaram para a singularidade de *Epipedobates femoralis* dentro do gênero. A citogenética mostrou um cariótipo que, de forma peculiar ao das demais espécies analisadas, não apresentou cromossomos telocêntricos, a NOR foi localizada numa região de conspícua constrição secundária e nenhum cromossomo sub-telocêntrico foi observado entre os seis pares de cromossomos grandes (Aguiar-Jr. *et al.*, 2002, artigo I). Já a análise ultra-estrutural revelou a presença de espermatozóide biflagelado nesta espécie, sendo uma característica compartilhada com membros do gênero *Colostethus*, com o qual já se supunha relações, e diferindo das demais espécies congenéricas (Vences *et al.*, 2000; Garda *et al.*, 2002, artigo IV; Aguiar-Jr. *et al.*, 2003, artigo V; Aguiar-Jr. *et al.*, artigo VI).

Quanto ao posicionamento de *Megaelosia* dentro da subfamília Hylodinae, os dados cariotípicos apontaram uma singularidade deste gênero tanto em termos de variação intragenérica no número diplóide (2n = 28 a 32) quanto na morfologia dos cromossomos (Melo *et al.*, 1995; Giaretta & Aguiar-Jr., 1998; Rosa *et al.*, *in press*, artigo II). Por outro lado, os dados ultra-estruturais mostraram uma grande uniformidade dentro da subfamília, com *Megaelosia* e *Hylodes* compartilhando a presença singular dentro dos leptodactilídeos de demais bufonóideos de uma fibra axial em forma de "taco de *baseball*", diferindo da forma globular normalmente observada (Aguiar-Jr. *et al.*, artigo VII). As características ultra-estruturais parecem reafirmar *Megaelosia* como membro de Hylodinae quando aliada às características derivadas do aparato vocal também compartilhadas com *Hylodes*, características estas que segundo Lynch (1971) eram as únicas que retinham o gênero na subfamília, a despeito de grandes diferenças no padrão de musculatura.

De uma maneira geral, os dados ultra-estruturais também se revelaram bastante conservativos nos dendrobatídeos. Mesmo o espermatozóide biflagelado encontrado em *Epipedobates femoralis* e *Colostethus* sp. (Aguiar-Jr. *et al.*, 2003, artigo V) conserva muita semelhança com as demais espécies estudadas, com exceção da presença de mitocôndrias no interior da membrana ondulante, característica não observada nas espécies biflageladas.

Os dados cariotípicos, no entanto, se mostraram mais variáveis dentro de ambos os grupos. Em Hylodinae, as diferenças encontradas entre *H. asper* e *H. phyllodes*, na morfologia de alguns cromossomos e padrão de banda C (Aguiar-Jr. *et al.*, artigo III), podem reforçar sua alocação em grupos distintos dentro do gênero (Frost, 2002). A interpretação do possível caminho evolutivo que tenha levado à grande variabilidade dentro do gênero *Megaelosia* (Rosa *et al.*, *in press*, artigo II) é difícil dada a ausência de homeologias fortemente respaldadas.

Para os Dendrobatidae, as diferenças morfológicas, de banda C e de localização da NOR entre os cariótipos das espécies de *Epipedobates* (Aguiar-Jr. *et al.*, 2002, artigo I) pode ser reflexo do fato de o gênero possivelmente tratar-se de um grupo não-natural, como aventado por Myers (1987). No entanto, levando-se em conta o provável monofiletismo dos Dendrobatidae (Ford & Cannatella, 1993; Vences *et al.*, 2000), quando mais espécies de *Epipedobates* forem analisadas, juntamente com membros dos demais gêneros, homeologias capazes de definir grandes grupos podem surgir. O mesmo pode ser considerado para os Hylodinae, também tidos como um grupo monofilético.

De um modo geral, os dados apresentados na presente tese, embora incipientes, servem de incentivo para que novas espécies sejam estudadas na busca de possíveis homeologias capazes de definir grupos e histórias evolutivas com a ferramenta da análise citogenética, e também para a busca de novos caracteres espermatológicos capazes de elucidar os questionamentos acerca desses grupos. Com a reunião de um maior número de dados obtidos de mais espécies de cada táxon, as duas ferramentas poderão ser integradas e reinterpretadas numa única análise de escopo filogenético.

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