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Estudo citogenético e da ultra-estrutura do espermatozóide de espécies do gênero *Colostethus* (Anura – Dendrobatidae)

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Aprender é a única coisa de que a mente nunca se cansa, nunca tem medo e nunca se arrepende.
(Leonardo da Vinci)

Dedico este trabalho
ao meu marido Roger
e aos meus queridos
pais, Nelson e Rosali.

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SUMÁRIO

ABSTRACT

The family Dendrobatidae consists of approximately 230 species that are grouped in nine genera. *Colostethus* is the largest genus with 128 nominal species. Some *Colostethus* species groups have been proposed and, although used, have been criticized because of the lack of apomorphic characters to support the species groupings. In this work, we examined the cytogenetics and sperm ultrastructure of *Colostethus* as part of an investigation into the taxonomic and intrageneric relationships of this genus. The karyotypes of *Colostethus brunneus, C. marchesianus, C. caeruleodactylus, Colostethus* sp. 1 (aff. *marchesianus*), *Colostethus* sp. 2 (aff. *marchesianus*), *Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp. were analyzed using conventional Giemsa staining, C-banding and Ag-NOR techniques. Fluorescence *in situ* hybridization (FISH) with an rDNA probe was also used when necessary. The ultrastructure of spermatozoa from all species, except for *Colostethus caeruleodactylus* and *Colostethus* sp. 2 (aff. *marchesianus*) and including *C. nidicola* and *C. stepheni,* were also examined. Cytogenetic analysis showed that *C. brunneus* had 24 chromosomes whereas the other species had 2n = 22 chromosomes. Variations in the C-banding patterns of the karyotypes were seen in all of the species. However, the *Colostethus* species with 2n = 22 showed a faintly stained band on the long arm of pair 7, which probably represent a homeology among these species. The nucleolus organizer region (NOR) was located on the long arm of pair 4 of *C. marchesianus, Colostethus* sp. 1 (aff. *marchesianus*) and *C. caeruleodactylus* and on the short arm of the same pair of the *Colostethus* sp. (aff. *trilineatus*). In *C. brunneus*, the NOR was located on the long arm of pair 3, which was morphologically similar to pair 4 in the other species. In *Colostethus* sp. 2 (aff. *marchesianus*) and *Colostethus* sp. the NOR was located on different chromosomal pairs. Additional NOR sites and size heteromorphism were also seen in some species, and were confirmed by FISH with an rDNA probe. However, in one specimen of *Colostethus* sp. 2 (aff. *marchesianus*), FISH revealed an additional site that was never seen by silver-staining. The difference in chromosomal number, heterochromatin distribution and NOR localization, made it possible to distinguish among the species. Ultrastructural analysis of

the spermatozoa showed the presence of biflagellated spermatozoa in all species, except for *C. stepheni*. The acrosomal complex structure and flagellar apparatus of the spermatozoa of the species examined resembled those of bufonoids. In contrast to the flagella of the other species, the single flagellum of *C. stepheni* spermatozoa contained mitochondria in the undulating membrane and a very thickened axial sheath. The results of the cytogenetic analysis were useful for distinguishing among these morphologically similar species. Similarly, ultrastructural analysis of the spermatozoa helped to elucidate intrageneric relationships since *C. brunneus*, *C. marchesianus, Colostethus* sp. 1 (aff. *marchesianus*) and *Colostethus* sp. (aff. *trilineatus*), which belonged to the "brunneus" group, had biflagellate spermatozoa, whereas *C. stepheni* ("alagoanus" group) had a spermatozoa with a single flagellum. The species in the "brunneus" group, were probably closely related to *Allobates femoralis*, which has biflagellate spermatozoa, whereas *C. stepheni* was not closely related, in agreement with molecular phylogenetic studies. The ultrastructural data obtained so far do not support the creation of the "trilineatus" group recently proposed based on the unification of the "brunneus" and "alagoanus" groups.

RESUMO

A família Dendrobatidae é formada por cerca de 230 espécies agrupadas em 9 gêneros. *Colostethus* é o maior deles com 128 espécies descritas. Alguns grupos de espécies foram propostos para o gênero, os quais, embora utilizados, foram bastante criticados devido à ausência de apomorfias que suportassem tais agrupamentos. Assim, as relações de parentesco intragenéricas continuam incertas. Neste trabalho, algumas espécies do gênero *Colostethus* foram estudadas através da análise citogenética e da ultra-estrutura do espermatozóide, a fim de contribuir para um melhor entendimento das relações de parentesco intragenéricas, bem como taxonômicas. Os cariótipos das espécies *Colostethus brunneus, C. marchesianus, C. caeruleodactylus, Colostethus* sp. 1 (aff. *marchesianus*), *Colostethus* sp. 2 (aff. *marchesianus*), *Colostethus* sp.(aff. *trilineatus*) e *Colostethus* sp. foram analisados pelos métodos de coloração convencional, bandamento C e impregnação por prata. Quando necessário, a técnica de hibridação *in situ* com sonda de DNAr também foi empregada. A análise da ultra-estrutura do espermatozóide foi realizada em *C. nidicola*, *C. stepheni* e em todas as espécies mencionadas acima, com exceção de *Colostethus caeruleodactylus* e *Colostethus* sp. 2 (aff. *marchesianus*). Os dados citogenéticos revelaram a presença de 24 cromossomos em *C. brunneus,* enquanto as outras espécies apresentaram 22 cromossomos. Diferenças no padrão de heterocromatina foram observadas nos cariótipos das espécies analisadas. No entanto, as espécies de *Colostethus* com 2n = 22 apresentaram uma banda fracamente corada no braço longo do par 7, a qual possivelmente seja uma homeologia entre estas espécies. A região organizadora do nucléolo (NOR) foi localizada no braço longo do par 4 das espécies *C. marchesianus, Colostethus* sp. 1 (aff. *marchesianus*), *C. caeruleodactylus* e no braço curto de *Colostethus* sp. (aff. *trilineatus*). Em *C. brunneus*, foi encontrada no braço longo do par 3, o qual possui a mesma morfologia do par 4 das referidas espécies. *Colostethus* sp. 2 (aff. *marchesianus*) e *Colostethus* sp., apresentaram a NOR em pares distintos. NORs adicionais e heteromorfismo de tamanho também foram observados em algumas das espécies, os quais puderam ser confirmados após a hibridação *in situ* com sonda de DNAr. Porém, em um indivíduo de *Colostethus* sp.

2 (aff. *marchesianus*) esta técnica evidenciou um sítio adicional, homólogo à sonda de DNAr, nunca observado pelo método AgNOR. As diferenças no número de cromossomos, distribuição de heterocromatina e localização da NOR, possibilitaram a distinção entre as espécies estudadas. A análise da ultra-estrutura do espermatozóide revelou a presença de espermatozóides biflagelados em todas espécies, com exceção de *C. stepheni*. Considerando-se as estruturas do complexo acrossomal e do aparato flagelar, os espermatozóides das espécies analisadas assemelham-se aos dos bufonóides. Além da presença de um único flagelo, o espermatozóide de *C. stepheni* também diferiu do padrão observado nas outras espécies, por apresentar mitocôndrias dentro da membrana ondulante, a qual juntamente com a bainha axial mostraram-se bastante dilatadas. Os resultados obtidos através da análise citogenética foram úteis na distinção entre as espécies estudadas, uma vez que estas possuem grande similaridade morfológica, o que dificulta a identificação. Por outro lado, a análise ultra-estrutural contribuiu para o esclarecimento de questões intragenéricas, pois *C. brunneus. C. marchesianus, Colostethus* sp. 1 (aff. *marchesianus*) e *Colostethus* sp. (aff. *trilineatus*), pertencentes ao grupo "brunneus" possuem espermatozóides biflagelados, enquanto que em *C. stepheni* (grupo "alagoanus") é uniflagelado. Essas espécies estudadas do grupo "brunneus", são, possivelmente, proximamente relacionadas à *Allobates femoralis*, cujo espermatozóide também possui dois flagelos, enquanto *C. stepheni* está mais distante, o que está de acordo com estudos de filogenia molecular. Portanto, dados ultra-estruturais obtidos até o momento não concordam com a criação do grupo "trilineatus", recentemente proposto, a partir da união dos grupos "brunneus" e "alagoanus".

1. INTRODUÇÃO

1.1. A Ordem Anura: aspectos gerais e filogenéticos

A Classe Amphibia compreende três Ordens, Anura, Gymnophiona e Urodela. A grande maioria dos anfíbios viventes, pertence à Ordem Anura, a qual é composta atualmente por 33 famílias com mais de 5000 espécies descritas. Os anfíbios anuros habitam todos os continentes, com exceção dos pólos, algumas ilhas oceânicas e regiões desérticas (Duellman & Trueb, 1986; Frost, 2004).

Apesar do grande número de espécies e da diversidade de hábitats ocupados, os anuros são morfologicamente conservados, o que torna as investigações filogenéticas baseadas nestes caracteres extremamente difíceis. Com exceção das famílias basais (Archeobatrachia), as relações de parentesco entre os anuros permanecem pouco esclarecidas. Parte deste problema pode ser devido ao fato de que muitas famílias de Neobatrachia derivaram de um ancestral há um curto período de tempo na escala evolutiva (Hillis, 1991). Além disso, outras razões parecem estar envolvidas na difícil elucidação das relações de parentesco deste grupo. Dentre estas razões Hillis *et al.* (1993) e Emerson *et al.* (2000) mencionaram a utilização de poucos exemplares como representantes de grandes grupos, ou a utilização de poucas seqüências de DNA potencialmente informativas em estudos moleculares, o que resultou em árvores filogenéticas com topologias inconclusivas. No entanto, segundo Hillis *et al.* (1993) este problema pode ser minimizado ampliando-se o número de táxons e o número de caracteres das seqüências analisadas.

A reconstrução filogenética baseada somente em um conjunto de dados, não é simultaneamente informativa para todos os níveis taxonômicos. Algumas técnicas de estudo são úteis para resolver questões entre espécies mais aparentadas, enquanto outras elucidam relações entre taxa mais elevados (Hillis, 1987). A escassez de dados morfológicos anatômicos informativos disponíveis, indica que outras fontes de dados são necessárias para maximizar a resolução filogenética dos Anura.

De acordo com Futuyma (1992), diferentes caracteres podem ter taxas evolutivas distintas. Desta forma, os estudos citogenéticos e da ultra-estrutura do espermatozóide podem fornecer novos dados que aliados aos moleculares, bioquímicos e morfológicos

clássicos, poderão contribuir para uma melhor compreensão das relações de parentesco dos anfíbios anuros.

1.2. A citogenética nos Anura

Até o final da década de 70, os estudos citogenéticos nos anuros eram baseados principalmente na descrição do número e da morfologia cromossômica de cariótipos submetidos à coloração convencional. Apesar de simples, estes estudos revelaram a existência de grupos cariotipicamente conservados, como *Bufo* (Bogart, 1973) e com uma considerável variabilidade na morfologia e no número de cromossomos, como em *Eleutherodactylus* (ver Bogart & Hedges, 1995, para referências). Além disso, mecanismos de evolução cromossômica, tais como fusão/fissão cêntrica e poliploidia, também foram sugeridos a partir de comparações entre cariótipos corados convencionalmente por Giemsa.

Os estudos de Morescalchi (1968, 1973) e Lynch (1971) apontaram que nas famílias mais primitivas o alto número cromossômico e a presença de microcromossomos e cromossomos telocêntricos eram freqüentes, enquanto que as espécies mais derivadas apresentavam um menor número de cromossomos em seus cariótipos. Entretanto, Bogart (1973) em sua revisão sobre a evolução cariotípica nos anuros, contestou a proposta de Morescalchi (1968), uma vez que em famílias primitivas como Ascaphidae, observou-se uma variação no número de cromossomos, o que segundo Bogart (1973) dificultaria a comparação com outras espécies de anuros. Além disso, o cariótipo de *Rhinophrynus dorsalis*, membro de uma família considerada primitiva, apresentou um baixo número cromossômico (2n = 22) e seria similar ao de espécies de microhilídeos. Baseado nestes argumentos, Bogart (1973) especulou que cariótipos de espécies pertencentes a famílias consideradas mais derivadas poderiam ter originado a partir de um cariótipo ancestral com 2n = 26 cromossomos do tipo de pelobatídeo. Este cariótipo "tipo-pelobatídeo" é encontrado em gêneros considerados primitivos, como *Telmatobius* e *Thoropa* (Leptodactylidae). Segundo Bogart (1973), a família Dendrobatidae também poderia ser derivada de um ancestral leptodactilídeo com 26 cromossomos, como *Elosia* (= *Hylodes*).

Beçak (1968) e Batistic (1970), analisando diferentes espécies de Hylidae e Leptodactylidae, sugeriram a fusão cêntrica como um dos principais mecanismos envolvidos no processo de redução no número de cromossomos. No entanto, em algumas espécies outras alterações como translocações seguida de deleções, também foram consideradas para explicar a diminuição no número diplóide, as quais poderiam ter mascarado o processo de fusão. Embora rearranjos cromossômicos tenham sido sugeridos para explicar a variação cromossômica, análises adicionais não foram realizadas posteriormente.

Com o advento das técnicas de bandamento cromossômico, a citogenética comparativa contribuiu para um melhor entendimento da evolução cariotípica nos anuros.

Miura *et al.* (1995) analisaram diferentes espécies de rãs-marrons (brown frogs) e descreveram um evidente caso de redução no número de cromossomos, de 2n = 26 para 2n = 24. Através de uma análise cuidadosa dos padrões de banda C e bandas de replicação tardia, foi possível evidenciar um evento de fusão *in tandem* entre os cromossomos 11 e 13 de um cariótipo tido como ancestral com 2n = 26, originando o par 6 de tamanho intermediário das espécies com 2n = 24 cromossomos.

Além dos eventos de fusão cromossômica promovendo a redução do complemento diplóide, o aumento do número de cromossomos originados por fissão também já foi relatado em algumas espécies de anuros, conforme descrito por Kuramoto e Allison (1989) para *Barigenys flavigularis* (Michrohylidae) e Busin *et al.* (2001) para *Pseudis cardosoi* (Hylinae). Por outro lado, a presença de 31 cromossomos em espécimes de *Eleutherodactylus glandulifer* não permitiu apontar uma única direção para a transformação cariotípica ocorrida, podendo ter sido originada a partir de fissão ou fusão cromossômica (Bogart, 1991).

A poliploidização é também um dos eventos envolvidos na especiação dos anfíbios anuros. A ocorrência de poliploidia natural já foi reportada em pelo menos 38 espécies de diferentes famílias (veja revisão de Tymowska, 1991). O aumento do lote cromossômico pode ocorrer por duas vias diferentes, autoploidia ou aloploidia. De acordo com Tymowska (1991), a autopoliploidização é o mecanismo mais comum em anuros. Entretanto, em

Xenopus há evidências da ocorrência de alopoliploidização, fenômeno que envolve a formação de híbridos e eventos de não-disjunção cromossômica.

Em anuros, a presença de cromossomos sexuais heteromórficos é incomum e aparentemente representa um estado derivado. Das muitas espécies estudadas citogeneticamente, pouco mais de 20 casos foram relatados na literatura e muitas vezes, a identificação destes cromossomos no cariótipo só pôde ser feita após a utilização de alguma técnica de bandamento cromossômico (Schmid 1980; Schmid *et al.*, 1983, Nishioka *et al.*, 1993; Cuevas & Formas, 1996; Lourenço *et al.*, 1999).

O emprego das diferentes técnicas citogenéticas, entre elas, bandamento C, impregnação por prata (AgNOR), bandamento de replicação tardia, fluorocromos e hibridação *in situ*, tem possibilitado um melhor entendimento das questões taxonômicas e filogenéticas, assim como da evolução cariotípica desses animais (Schmid, 1978a, b; 1980, 1982; Schmid *et al.*, 1990, 1995; King, 1980; Lourenço *et al.*, 1999, 2000, 2003; Rosa *et al.*, 2003; Aguiar-Jr *et al.*, 2002, 2003a, entre outros).

1.2.1. Heterocromatina

O termo heterocromatina foi criado pelo botânico Emil Heitz em 1928, para denominar e descrever segmentos cromossômicos ou, em alguns casos, cromossomos inteiros que se mantinham em estado condensado durante toda a intérfase. A definição proposta por Heitz foi inteiramente baseada em análises morfológicas (John, 1988).

Ris & Korenberg (1979) mostraram, através de estudos autoradiográficos, que a síntese de RNA é possível apenas na cromatina difusa, sendo então a cromatina condensada transcricionalmente inativa.

Há alguns anos a heterocromatina era discriminada em facultativa e constitutiva. Porém, atualmente se sabe que isto não é necessário, uma vez que a organização de ambos os tipos de cromatina é baseada no mesmo modelo de interação da cromatina nucleossomal com complexos de proteínas (Henning, 1999). Esta proposta de generalização para o termo heterocromatina baseia-se na identificação de uma proteína denominada Mr 51,000 (p51),

presente nos cromossomos sexuais meióticos, os quais durante a prófase desta divisão formam uma estrutura chamada de corpúsculo XY ou vesícula sexual. Através de experimentos imunicitoquímicos foi possível detectar que esta proteína está homogeneamente distribuída no corpúsculo XY de roedores e na região centromérica dos autossomos em células germinativas. Smith e Benavente (1995) ainda especularam que a p51 pode ser um componente do mecanismo que torna as regiões heterocromáticas inacessíveis para a transcrição e/ou eventos de recombinação.

Pardue e Henning (1990) já se referiam à heterocromatina como "item de colecionadores", tendo valor apenas para quem entende e conhece suas funções, uma vez que muitos pesquisadores a consideravam como "DNA lixo", devido à inexistência de genes. Entretanto, muitos genes funcionais têm sido descritos na heterocromatina de *Drosophila melanogaster*, embora a densidade destes genes seja muito menor do que na eucromatina (Hilliker *et al.*, 1980). Porém, Sumner (1990) sugere que estes genes possam estar contidos em pequenos segmentos de eucromatina, dentro de regiões heterocromáticas.

Segundo King (1991), a heterocromatina desempenha um importante papel, protegendo os sítios eucromáticos adjacentes de modificações estruturais por recombinação, uma vez que diminui a formação de quiasmas. Outros papéis já foram propostos para a heterocromatina, como a inativação de genes localizados muito próximos (Reuter & Spierer, 1992).

O fenômeno heterocromático depende de dois componentes, seqüências de DNA e proteínas DNA-específicas. As proteínas envolvidas com a heterocromatina não são somente proteínas histônicas. Um dos fatores de condensação da heterocromatina é uma proteína denominada HP1, descrita pela primeira vez em *Drosophila* (Eissenberg & Elgin, 2000). Embora a heterocromatina seja predominantemente constituída por seqüências de DNA altamente repetitivas e não codificadoras (John, 1988; Sumner, 1994), há evidências de que a heterocromatina interfere com a replicação do DNA e contribui na estrutura cromossômica, expressão gênica, organização do genoma e evolução (veja revisão de Redi *et al.*, 2001).

Em estudos citogenéticos, a heterocromatina tem sido comumente evidenciada através da técnica de bandamento C. Além disso, técnicas de citogenética molecular, como

bandamento por enzimas de restrição, fluorocromos e hibridação *in situ,* também podem ser utilizadas.

O bandamento C foi descoberto acidentalmente por Pardue e Gall em 1970. Estes autores sugeriram que as regiões de banda C eram quase que exclusivamente sítios de DNA altamente repetitivo (DNA satélite). Seqüências de DNA altamente repetitivo têm sido localizadas nas regiões de banda C em uma grande variedade de espécies. Embora exista uma associação entre DNA altamente repetitivo e banda C, há situações em que este tipo de DNA não é encontrado em regiões evidenciadas pela técnica de bandamento C (Sumner, 1990).

O processo bioquímico que explica o bandamento C foi inicialmente descrito por Holmquist (1979), o qual envolve três passos: um tratamento preliminar com ácido hidroclorídrico, que causa uma despurinação do DNA; um tratamento alcalino que causa quebra dos sítios apurínicos, assim como a desnaturação irreversível do DNA; e um tratamento salino que promove a remoção do DNA despurinado. Acredita-se que a extração do DNA das regiões de heterocromatina ocorra de forma mais lenta, porém não se conhece a razão pela qual as regiões não banda C são preferencialmente extraídas.

O bandamento C é um importante método para a identificação de cromossomos de Anura, uma vez que os bandamentos G, R e Q são empregados satisfatoriamente apenas em vertebrados superiores. As características do bandamento C que auxiliam na identificação dos cromossomos são o tamanho e a localização das bandas, as quais são normalmente encontradas nas regiões centroméricas, podendo também serem localizadas nas regiões teloméricas, adjacentes às regiões organizadoras do nucléolo e intersticiais dos cromossomos (Sumner, 1990; 1994). De acordo com Sumner (1990), cromossomos que não apresentam bandas C são raros e, provavelmente, não ocorram em cariótipos normais.

Após a utilização do bandamento C em várias espécies de diferentes famílias de Archaeobatrachia, verificou-se uma quantidade relativamente pequena de heterocromatina presente no cariótipo deste grupo de anuros. Por outro lado, espécies pertencentes a algumas famílias de Neobatrachia, quando analisadas através desta técnica, revelaram uma enorme diversidade interespecífica, tanto em quantidade, quanto em distribuição de

heterocromatina, sendo, portanto, muito diferente do padrão encontrado para os Archaeobatrachia (King, 1991).

Além de variações interespecíficas, variações intra-específicas no padrão de banda C, embora pouco freqüentes, já foram reportadas em anuros, indicando principalmente polimorfismos de tamanho dessas regiões (Schmid, 1978a, b; Schmid, 1980; Schmid *et al,*. 1990; Miura *et al,*. 1995). Por outro lado, Kasahara *et al.* (1996) sugeriram que a regularidade na distribuição de heterocromatina encontrada em diferentes espécies de *Bufo* possa caracterizar um grupo dentro deste gênero.

A variação no padrão de heterocromatina observada em Amphibia levou King (1991) a sugerir três processos gerais para indicar a evolução da heterocromatina no genoma deste grupo, sendo eles: a adição de heterocromatina a sítios cromossômicos específicos através da amplificação de segmentos de DNA repetitivo, a transformação de regiões cromossômicas eucromáticas em regiões heterocromáticas e a evolução combinada de sítios heterocromáticos múltiplos. De acordo com o autor, o aumento na quantidade de heterocromatina é uma tendência evolutiva em Amphibia, embora, a possibilidade de diminuição dessa cromatina em alguns grupos não fora descartada. Formas e Cuevas (2000) sugeriram que a perda de heterocromatina centromérica no par 1 de *Telmatobufo bullocki,* seja uma condição derivada, devido a sua presença em *Caudiverbera* (gênero monotípico, tido como grupo-irmão de *Telmatobufo*)

O padrão de distribuição e quantidade de heterocromatina detectado através da técnica de bandamento C, assim como a caracterização da composição das bandas heterocromáticas obtidas a partir do uso de fluorocromos, têm sido utilizados como ferramentas importantes na busca de uma melhor compreensão das questões sistemáticas, bem como da evolução cariotípica na Ordem Anura (Schmid, 1978a, b, 1980; king, 1980; Miura *et al.*, 1995; Lourenço *et al.*, 1999; Busin *et al.*, 2001; Odierna *et al.*, 2001; Kasahara *et al.*, 2003).

1.2.2. Região organizadora do nucléolo (NOR)

Os organizadores nucleolares são sítios cromossômicos formados por várias cópias de genes que codificam os RNA ribossomais (RNAr) 18S, 5,8S e 28S, organizadas repetidamente (*in tandem*) e separadas por seqüências espaçadoras, as quais podem ser descritas como internas ou externas, dependendo da posição que ocupam. O tamanho total da região transcrita pode ser variável devido à variação no tamanho das regiões espaçadoras. A região espaçadora não-transcrita (IGS) geralmente varia em comprimento entre e dentro das espécies (Miller, 1981).

Os RNAs ribossomais são sintetizados e processados formando pré-ribossomos no nucléolo, e posteriormente tornam-se parte de ribossomos maduros no citoplasma. Durante o processo de maturação do RNAr, os espaçadores são removidos do transcrito primário (Long & Dawid, 1980). Em suma, os organizadores nucleolares são segmentos cromossômicos de cuja atividade se origina o nucléolo (Miller, 1981; Sumner, 1990).

Em geral, os sítios de RNAr aparecem como constrições secundárias. Porém, algumas vezes estas constrições contêm heterocromatina (King, 1980; Schmid, 1982; Goessens, 1984; Schwarzacher & Wachtler, 1993).

Em 1973, Matsui e Sasaki demonstraram pela primeira vez a localização das NORs através do bandamento N, o qual envolve a coloração com Giemsa após a extração de histonas e ácidos nucléicos dos cromossomos. A partir de então os métodos de coloração para a NOR evoluiram da complexidade para a simplicidade. A técnica original de Howell *et al.* (1975) envolve diversos estágios como: fixação dos cromossomos com formalina, tratamento com uma solução pré-corante de prata amoniacal, um tratamento com hidróxido de sódio diluído e, finalmente, a coloração com uma mistura de formalina e prata amoniacal. Goodpasture & Bloom (1975) publicaram, no mesmo ano, uma técnica que envolve a utilização de nitrato de prata a 50%, colocado sobre a preparação cromossômica, coberto com lamínula e exposto ao calor de uma lâmpada. Em seguida, os cromossomos recebem uma mistura de prata amoniacal e formalina. Variações desta técnica foram publicadas posteriormente por outros pesquisadores. Uma alternativa proposta para a coloração de prata foi dada por Olert (1979), que misturou uma solução de prata a 50%

com ácido fórmico diluído e incubou as lâminas nesta solução. Este procedimento foi modificado por Howell & Black (1980), que fizeram uma solução coloidal reveladora ao adicionar ácido fórmico a uma solução de gelatina diluída. Este revelador coloidal foi misturado com uma solução de nitrato de prata imediatamente antes do uso, sendo, então, as lâminas incubadas em uma estufa aquecida.

A especificidade pela prata dá-se em razão da reação desta com as proteínas acídicas, denominadas argirofílicas ou proteínas Ag-NOR, que permanecem associadas ao DNAr mesmo durante a mitose e pelo menos até o paquíteno da meiose (Howell, 1977; Schwarzacher *et al.*, 1978; Roussel *et al.*, 1996). Desta forma, apenas as NORs que estiverem ativas no ciclo celular anterior serão evidenciadas pelo método. Algumas proteínas Ag-NOR já foram identificadas, entre elas, a nucleolina, RNA polimerase I, DNA topoisomerase I e UBF (Howell, 1977; Schwarzacher *et al,*. 1978; Hernandez-Verdun, 1993). No entanto, o grupamento que interage com o precipitado metálico derivado da redução do íon prata permanece incerto, podendo ser este sulfidril (SH), dissulfeto (S-S), fosfato ou carboxila (Olert *et al.*, 1979; De Capoa *et al.*, 1982; Buys & Osing, 1984; Hubbel, 1985).

Além da coloração usual com nitrato de prata, a qual pode marcar também outras regiões cromossômicas além da NOR (Sumner, 1990), técnicas citoquímicas como coloração com fluorocromos (mitramicina ou cromomicina) específicos para regiões ricas em CG a e hibridação *in situ* com sondas de DNAr são amplamente utilizadas na detecção das regiões organizadoras do nucléolo. Uma característica da NOR é a sua riqueza em CG, principalmente as seqüências dos genes 18S e 28S (Sumner, 1990). Entretanto, o uso de fluorocromos pode evidenciar outras porções ricas em CG, como regiões de heterocromatina. Desta forma, a hibridação *in situ* com sonda de DNAr é o método mais eficiente e específico, uma vez que todas as seqüências de DNAr são detectadas.

Em anuros a localização da NOR é essencial para estudos citogenéticos comparativos. De acordo com Schmid (1978a, b) e Schmid *et al.* (1990) a localização e o número de NORs tendem a ser característicos de cada espécie, sendo que em espécies ou grupos de espécies relacionados, as NORs estão geralmente localizadas nas mesmas regiões cromossômicas.

Heteromorfismos de tamanho de NORs são freqüentemente encontrados entre indivíduos da mesma espécie, em função da diferença no número de cópias do gene ribossomal presente em cada NOR (Schmid *et al.*, 1978a, b; Schmid, 1982, Silva *et al.*, 1999; Lourenço *et al.*, 2000, 2003). Variações intra- e interpopulacionais no número e localização das NORs foram relatadas para *Hyla versicolor* e *Hyla chrysoscelis* (Wiley *et al.*, 1989), *Bufo terrestris* (Foote *et al.*, 1991), *Agalychnis callidryas* (Schmid *et al.*, 1995), *Hyla ebraccata* (Kaiser *et al.*, 1996), *Physalaemus petersi* (Lourenço *et al.*, 1998), *Physalaemus cuvieri* (Silva *et al.*, 1999), *Hyla nana* (Medeiros *et al.*, 2003).

De acordo com King (1990), a presença de apenas um par de NORs no genoma dos anuros é uma condição encontrada tanto em famílias primitivas quanto derivadas. Porém, a ocorrência de mais de um par (NORs múltiplas) sugere uma condição apomórfica em relação à presença de NORs únicas.

Uma vez que a impregnação pela prata pode marcar regiões de heterocromatina (Sumner, 1990), a utilização da técnica de hibridação *in situ* com sonda de DNAr é o método mais eficaz, pois detecta as seqüências de DNAr. Em anuros, até o momento todas as NORs evidenciadas pela prata foram também observadas por hibridação *in situ* (Schmid *et al.*, 1986, 1993, 1995; King *et al.*, 1990; Foote *et al.*, 1991; Lourenço *et al.*, 1998). Porém, em *Hyla versicolor* e *Hyla chrysoscelis* (Wiley *et al.*, 1989) e *Hyla nana* (Medeiros *et al.*, 2003), marcações adicionais não evidenciadas pelo método Ag-NOR foram detectadas pela hibridação *in situ*. Tais marcações nunca foram evidenciadas como NORs ativas, o que afasta a possibilidade de ocorrência de mecanismo de regulação de expressão dessa região em anuros.

De acordo com dados de Andreone *et al.* (2003) a posição da NOR é um caracter filogeneticamente informativo. Desta maneira, a análise do número e localização das NORs no genoma é de grande importância para o entendimento da evolução cariotípica deste grupo, bem como para o reconhecimento de homeologias cromossômicas entre diferentes espécies e populações.

1.3. A ultra-estrutura do espermatozóide em anuros

A análise da ultra-estrutura dos espermatozóides tem sido o objeto de estudo há cerca de vinte anos em muitos grupos animais e é reconhecida como um importante indicador de relações filogenéticas (Baccetti, 1979; Dallai & Mazzini, 1983; Jamieson, 1987; Mattei, 1988).

Das 33 famílias de Anura atualmente reconhecidas (Frost, 2004), o estudo do espermatozóide foi realizado em algumas espécies de 21 delas, incluindo a família Dendrobatidae (veja revisão de Scheltinga & Jamieson, 2003; Garda *et al.*, 2002; Aguiar-Jr *et al.*, 2003b, 2004).

Os espermatozóides dos Anura fornecem um conjunto de caracteres taxonômicos bastante úteis, uma vez que apresentam variações na estrutura da cabeça (principalmente no formato do acrossomo e perforatório), além da disposição dos centríolos e constituintes da cauda (Kwon & Lee, 1995).

A vesícula acrossomal pode apresentar um formato cônico e recobrir a porção anterior do núcleo, porém não estando diretamente associada a ele. Esta condição (tida como plesiomórfica) é observada na maioria das famílias dos bufonóides (Pugin-Rios, 1980; Lee & Jamieson, 1993; Scheltinga & Jamieson, 2003). O formato sacular ocupando apenas o topo do núcleo, ou ainda íntima associação da vesícula acrossomal com núcleo parece ser uma condição apomórfica presente em Ranidae, Pipidae e em alguns membros de Leptodactylidae (Pugin-Rios, 1980; Scheltinga & Jamieson, 2003).

Abaixo da vesícula acrossomal, o cone subacrosomal pode ser observado em algumas espécies. Esta estrutura foi descrita inicialmente em *Ascaphus truei* (Archeobatrachia) por Jamieson *et al.* (1993). A homologia entre o cone subacrossomal e o perforatório cônico presente na maioria dos bufonóides estudados (Jamieson, 1999) foi sugerida por Garda *et al.* (2002). Por outro lado, a ausência de qualquer estrutura perforatória mencionada até o momento em Pipidae, Ranidae e em alguns membros de Leptodactylidae é uma condição apomórfica (Scheltinga & Jamieson, 2003).

De acordo com Kown e Lee (1995), o posicionamento angular dos centríolos também pode ser informativo. Nos espermatozóides com um único flagelo, os centríolos

podem estar dispostos de forma paralela, oblíqua ou mutuamente perpendicular. Enquanto que nos espermatozóides biflagelados, os centríolos são adjacentes e paralelos (veja revisão de Scheltinga & Jamieson, 2003). De acordo com estes autores, a organização perpendicular dos centríolos em gimnofionas e urodelos parece ser plesiomórfica, portanto o posicionamento paralelo e oblíquo observado em alguns anuros parece indicar uma derivação desta condição.

Após a descrição do espermatozóide de uma espécie tida como basal, *Leiopelma hochstetteri,* o significado evolutivo de algumas estruturas foi revisto (Scheltinga *et al.,* 2001). De acordo com Lee e Jamieson (1993), a presença de um colar de mitocôndrias encontrado nos bufonóides poderia ser considerada uma sinapomorfia deste grupo. No entanto, Scheltinga *et al.* (2001) observaram a presença de um colar rudimentar em *L. hochstetteri*, o qual é similar a condição vista no peixe pulmonado, *Neoceratodus forsteri.* Por outro lado, o bem desenvolvido colar de mitocôndrias dos bufonóides, relembra a condição vista em peixes acantopterígios e, por esta razão, parece ser uma condição plesiomórfica. Porém, a possibilidade de uma reversão não foi descartada (Scheltinga *et al.,* 2001).

Embora muitos dos caracteres espermatológicos careçam de um significado evolutivo consistente, a ultra-estrutura do espermatozóide tem auxiliado no entendimento das relações de afinidade entre os anuros.

Com base na ultra-estrutura do espermatozóide, em especial as estruturas da cauda Meyer *et al.* (1997) distinguiram três linhagens dentro do gênero *Cyclorana* e encontraram evidências para a alocação de *Litoria alboguttata* dentro do gênero *Cyclorana*, como previamente sugerido pelos dados moleculares, morfológicos e cariotípicos.

Aguiar-Jr *et al.* (2003b) relataram a presença de espermatozóides biflagelares em duas espécies da família Dendrobatidae, *Epipedobates femoralis* (*Allobates femoralis*) e *Colostethus* sp. Embora o significado filogenético da biflagelaridade permaneça desconhecido, esta característica suportou a alocação de *E. femoralis* dentro do gênero *Allobates*, como anteriormente proposto por Zimmermann & Zimmermann (1988).

A utilização de dados de ultra-estrutura do espermatozóide juntamente com os morfológicos anatômicos, citogenéticos e moleculares parecem promissores na busca do entendimento das relações de parentesco entre os diferentes níveis taxonômicos de anuros.

1.4. A Família Dendrobatidae e o gênero *Colostethus* – aspectos gerais

A Família Dendrobatidae é composta pelos gêneros *Allobates, Aromobates, Colostethus, Cryptophyllobates, Dendrobates, Epipedobates, Mannophryne, Nephelobates* e *Phyllobates*, os quais agrupam cerca de 230 espécies (Frost, 2004), distribuídas nas regiões tropicais e subtropicais da Nicarágua até a Bolívia, nas Guianas e nas regiões amazônica e sudeste do Brasil (Duellman & Trueb, 1986; Frost, 2004).

Os dendrobatídeos são conhecidos por suas brilhantes cores de advertência (aposemáticas) e pela presença de alcalóides tóxicos na pele de alguns de seus membros (Ford, 1993). Estes alcalóides têm atraído a atenção de pesquisas biomédicas, devido aos seus diversos efeitos nos tecidos muscular e nervoso (Myers & Daly, 1983). Recentemente, foi descoberta a existência de uma substância anestésica mais potente que a morfina, denominada epibatidine presente na pele de *Epipedobates tricolor* (Spande *et al.*, 1992). Além disso, algumas tribos indígenas da América do Sul utilizam as toxinas liberadas pelas glândulas da pele destes animais, com a finalidade de preparar suas flechas e zarabatanas para a caça.

A posição da família Dendrobatidae dentro dos Neobatrachia é ainda debatida. Alguns autores consideram a família mais próxima aos Leptodactylidae da América do Sul relacionada ao gênero *Hylodes* (Noble, 1931; Lynch, 1971 e Ardila-Robayo, 1979) e outros aos Ranidae africanos (Griffths, 1959, 1963). O relacionamento dos dendrobatídeos com os ranídeos ou leptodactilídeos depende das características analisadas, uma vez que Ford (1993), através de análises baseadas em caracteres osteológicos, afirmou que os dendrobatídeos são uma unidade monofilética e que estão estritamente relacionados com os ranídeos. No entanto, Morescalchi (1973) descreveu os cromossomos dos dendrobatídeos

como sendo do tipo dos leptodactilídeos, e também levantou a hipótese de que todos os neobatráquios, incluindo os ranídeos, sejam derivados de um "estoque" leptodactilídeo. Weygoldt (1987), baseado em dados comportamentais, corrobora a hipótese de Lynch (1971), uma vez que as espécies pertencentes ao gênero *Hylodes* (*sensu* – Lynch, 1971), são muito ágeis, diurnas e possuem um comportamento territorial e agressivo, além de lembrarem a morfologia externa de alguns dendrobatídeos, em especial, as espécies de coloração críptica. Recentemente, as análises filogenéticas baseadas em caracteres moleculares mostraram que os dendrobatídeos estão associados com os leptodactilídeos e excluídos do grupo dos ranídeos (Hay *et al.*, 1995; Vences *et al.*, 2000, 2003).

A grande maioria dos dendrobatídeos é de hábito diurno, com exceção de *Aromobates nocturnos* (Myers *et al.*, 1991), e possuem uma complexa história de vida. Os ovos são colocados em ninhos terrestres e as larvas da maioria das espécies são transportadas no dorso de um dos parentais até um ambiente aquático, onde o desenvolvimento é completado (Savage, 1968; Praderio & Robinson, 1990). Em muitas espécies, o comportamento é conhecido e inclui cuidado parental e territorialidade (Weygoldt, 1987; Bogart, 1991).

De acordo com Savage (1968) e Bogart (1991), nesta família há uma aparente progressão de espécies menos especializadas para mais especializadas, especialização esta correlacionada com cuidado parental e tamanho da desova. O gênero *Colostethus* possui características menos especializadas quando comparado a outros gêneros, pois apresenta um maior número de ovos, menor territorialidade e menor cuidado com a prole (ver Bogart, 1991, para referências).

Segundo Frost (2004), *Colostethus* é o maior gênero com 128 espécies descritas, distribuídas principalmente na baixa América Central, nordeste da América do Sul e com algumas representações no leste dos Andes (Myers *et al.*, 1991; Frost, 2004).

As espécies de *Colostethus* apresentam coloração parda e em geral não possuem toxinas na pele (Duellman & Simmons, 1988), com exceção de *Colostethus inguinalis*, uma espécie do Panamá, na qual Daly *et al.* (1994) detectou a presença de uma toxina denominada tetrodotoxina. Membros deste gênero parecem não produzir nem seqüestrar

nenhum dos alcalóides lipofílicos, característica esta que define as linhagens dos gêneros *Epipedobates*, *Dendrobates* e *Phyllobates*.

A ausência de uma defesa química tem sido considerada uma característica basal em *Colostethus*, mas após a descoberta de um dendrobatídeo basal (*Aromobates nocturnos*), o qual possui uma secreção volátil defensiva, levantou-se a possibilidade que a perda de alguma defesa química primitiva pode ser uma característica derivada de *Colostethus* (Myers *et al.*, 1991).

Devido ao grande número de espécies pertencentes ao gênero *Colostethus,* as relações inter- e intragenéricas são pobremente esclarecidas. Alguns grupos de espécies foram propostos na tentativa de elucidar relações internas deste gênero (Rivero,"1988" 1990; Rivero & Serna, "1988" 1989; Myers *et al.,* 1991). No entanto, o monofiletismo de cada grupo é bastante questionável, pois muitos dos caracteres utilizados podem representar simplésiomorfias ou homoplasias (Coloma, 1995; Grant *et al.,* 1997). Recentemente, uma análise filogenética molecular proposta por Vences *et al.* (2003), identificou alguns grupos bem defindos de espécies de *Colostethus*, os quais em parte, coincidiram com os agrupamentos propostos por Rivero ("1988" 1990). No entanto, muitas espécies de *Colostethus*, se mostraram mais aparentadas com membros de outros gêneros da família Dendrobatidae, o que sugere a necessidade de ampla revisão deste gênero, em função da identificação de seu *status* polifilético.

Os primeiros estudos citogenéticos realizados nesta família restringiram-se apenas à determinação do número e à análise da morfologia cromossômica (Duellman,1967; Rada de Martinez, 1976; Rasotto, 1987; Bogart, 1991). Recentemente, dados inéditos sobre a quantidade e distribuição de heterocromatina, assim como da localização das regiões organizadoras do nucléolo (NORs) foram descritos para algumas espécies de *Epipedobates, Allobates, Colostethus* e *Mannophryne* (Aguiar-Jr *et al.,* 2002; Kaiser *et al.,* 2003; Veiga-Menoncello *et al.,* 2003).

Bogart (1991) especula sobre uma tendência à diminuição do número cromossômico de 2n = 24 para 2n = 18 nos dendrobatídeos, sugerindo ainda que 2n = 24 seja o ancestral na família. De acordo com o autor, a variação cromossômica pode estar relacionada com a história de estratégia de vida, uma vez que as espécies do gênero menos

especializado (*Colostethus*) apresentaram maior número de cromossomos, enquanto que as espécies de *Dendrobates,* tido como o gênero mais especializado em termos de comportamento reprodutivo, apresentaram 2n = 20 ou 18 cromossomos.

A ocorrência de variabilidade intragenérica no número de cromossomos era conhecida apenas para *Dendrobates* (Bogart, 1991). Porém, recentemente foi descrito um novo número cromossômico (2n = 22) em espécies de *Colostethus* (Kaiser *et al.*, 2003; Veiga-Menoncello *et al.*, 2003), o que indica que o número de cromossomos não é conservado nestes dois gêneros da família Dendrobatidae.

1.5. Justificativa

Colostethus é o maior gênero da Família Dendrobatidae, possuindo 128 espécies descritas (Frost, 2004), porém cerca de apenas 10 por cento dessas espécies foram estudadas citogeneticamente.

Este gênero tem sofrido ao longo do tempo algumas reestruturações internas. Rivero ("1988" 1990) revisou os agrupamentos fenéticos criados por Edwards (1974 - *appud* Rivero, "1988" 1990) e sugeriu alguns grupos baseados em características apomórficas e distribuição geográfica. Embora estes agrupamentos tenham sido bastante criticados, pois a grande maioria dos estados de caracteres representa simplesiomorfias (Coloma, 1995; Grant *et al.* 1997), pouco foi feito na tentativa de compreender a sistemática deste gênero, uma vez que muitas espécies foram posteriormente descritas. La Marca (1992, 1994), na tentativa de contribuir para a sistemática deste subgrupo de dendrobatídeo, elevou a condição genérica (*Mannophryne* e *Nephelobates* respectivamente), a maioria das espécies pertencentes aos grupos VII (grupo "collaris") e grupo VIII (grupo "alboguttatus") (*sensu* - Rivero, "1988" 1990). Posteriormente, Morales (2000) propôs também com base em caracteres morfológicos a criação de um novo grupo monofilético, denominado grupo "trilineatus", formado a partir da união das espécies do grupo II (grupo "brunneus") e do grupo III (grupo "alagoanus") (*sensu* - Rivero, "1988" 1990). No entanto, o

reconhecimento da validade deste novo grupo ainda não foi confirmado por outros pesquisadores.

Coloma (1995), em sua revisão do gênero *Colostethus*, relata que populações atualmente atribuídas à espécie *Colostethus marchesianus* não exibem grandes diferenças morfológicas, entretanto, diferenças no canto podem ser notadas, sugerindo que mais de uma espécie esteja inserida neste táxon. De acordo com as observações de A. P. Lima (com. pessoal), *Colostethus marchesianus* de Manaus (Reserva Florestal Adolfo Ducke), talvez esteja erronemante denominda, pois apresenta algumas diferenças morfológicas, quando comparado a *Colostethus marchesianus* da localidade-tipo (Missão de Taraquá – AM).

Dados cariotípicos de *C. brunneus* presentes na literatura (Bogart, 1991) mostraram a presença de 2n = 24 cromossomos em uma população do Peru. No entanto, em nossas análises prévias, observamos que *C. marchesianus* de Manaus possui 2n = 22 cromossomos, o que sugere a existência de uma variabilidade cariotípica dentro do grupo "brunneus".

Recentemente algumas espécies de *Colostethus* da Amazônia brasileira, morfologicamente semelhantes a *Colostethus marchesianus* foram analisadas citogeneticamente e revelaram um novo número cromossômico para o gênero (2n = 22). Apesar da semelhança morfológica e cariotípica, estas espécies puderam ser separadas entre si, através da localização da região organizadora de nucléolo (NOR) e do padrão de distribuição de heterocromatina. Além disso, estas espécies foram distingüidas das demais espécies do gênero já analisadas citogeneticamente, por apresentarem um par a menos de cromossomos (Veiga-Menoncello, 2000)

Portanto, a análise citogenética de populações de *C. marchesianus* de diferentes localidades, juntamente com *C. brunneus* da Chapada dos Guimarães (localidade-tipo) e de outras espécies morfologicamente semelhantes a estas, pode ser elucidativa para a compreensão da taxonomia do grupo "brunneus", uma vez que há dificuldade na identificação das espécies semelhantes à *C. marchesianus* e *C. brunneus.*

A análise ultra-estrutural do espermatozóide tem contribuído para a sistemática de Anura e, como este tipo de estudo para o gênero *Colostethus* restringe-se a único trabalho

na literatura (Aguiar-Jr *et al.*, 2003b), espera-se obter resultados que associados à citogenética possam contribuir para a sistemática do grupo "brunneus".

1.6. Objetivos

1.6.1. Caracterizar citogeneticamente as espécies *Colostethus brunneus* da Chapada dos Guimarães (localidade-tipo), diferentes populações de *C. marchesianus* e espécies morfologicamente semelhantes a estas.

1.6.2. Caracterizar a ultra-estrutura do espermatozóide de *C. brunneus*, *C. marchesianus* e de algumas espécies semelhantes a *C. marchesianus*, além de outras espécies do gênero que ocorrem no Brasil.

1.6.3. Analisar comparativamente os dados citogenéticos e ultraestruturais de *Colostethus*, contribuindo com dados para o entendimento da taxonomia e filogenia deste grupo.

1.7. Referências Bibliográficas

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2. ANÁLISE CITOGENÉTICA

ARTIGO I

Obs.: Os dados citogenéticos referentes às espécies *Colostethus caeruleodactylus* e *Colostethus* sp. 2 (aff. *marchesianus*) fizeram parte da tese de mestrado de Veiga-Menoncello, 2000. As outras duas espécies foram analisadas durante o doutorado.

Cytogenetic analysis of four central Amazonian species of *Colostethus* (Anura – Dendrobatidae) with a diploid complement of 22 chromosomes

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Colostethus marchesianus from the type locality and three related species had 2n = 22 chromosomes, which differed from most other *Colostethus* species that have 2n = 24 chromosomes. The species analyzed were morphologically similar and showed a conservative karyotype, although they could be distinguished from each other by their C-banding pattern. Additional NOR sites, heteromorphism in NOR size and heterochromatin, and an additional rDNA site detected by FISH, were observed. These data suggest that chromosomal rearrangements and hetrochromatin-related events may have contributed to the karyotype differentiation of these *Colostethus*.

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According to FROST (2002), the family Dendrobatidae consists of 207 species grouped in nine genera: *Allobates* (1 sp.), *Aromobates* (1 sp.), *Colostethus* (113 spp.), *Cryptophillobates* (1 sp.), *Dendrobates* (37 spp.), *Epipedobates* (28 spp.), *Mannophryne* (12 spp.), *Nephelobates* (9 spp.) and *Phyllobates* (5 spp.).

Colostethus is the largest dendrobatid genus and is considered to be a basal group within the dendrobatids (LYNCH 1982). The species of this genus are widespread in lower Central America, northwestern South America, the Amazon and in some areas of the eastern Andes (DUELLMAN and TRUEB 1986; MYERS et al. 1991). Many *Colostethus* species are very similar in morphology and color pattern and therefore difficult to distinguish from each other.

In a review of the *Colostethus* species from Ecuador, COLOMA (1995) mentioned that populations currently assigned to *C. marchesianus* do not show major morphological differences. However, differences in their announcement calls suggest that more than one species is included in this taxon. In the Brazilian Amazon, the name *C. marchesianus* has also been attributed to some populations of *Colostethus* (A. P. Lima, pers. obs.). Recently, LIMA and CALDWELL (2001) described a species of *Colostethus* with blue digits (*C. caeruleodactylus*) and observed that, in addition to this species, there are other undescribed species morphologically similar to *C. marchesianus* that occur near Manaus in the Amazon. Two of these species are analyzed in the present study and are referred to here as *Colostethus* sp. 1 and *Colostethus* sp. 2, the former frequently being called *C. marchesianus* (HERO 1990; GASCON 1991). Although these species are morphologically very similar, they have distinct calls.

In general, anurans show conserved morphological characteristics, which make the use of such characters difficult in taxonomic and phylogenetic investigations (HILLIS 1991) so that other methods are required. The identification of chromosomal numbers and morphology, and the availability of various staining techniques has provided new data for reassessing anuran systematics (KING 1980; MIURA 1995; LOURENÇO et al. 1999; BUSIN et al. 2001). The cytogenetic information available for the Dendrobatidae is restricted to the number of chromosomes and the morphology of the karayotype. However, chromosome banding studies recently published by AGUIAR JR et al. (2002), KAISER et al. (2003) and VEIGA-MENONCELLO et al. (2003), have helped to clarify some inter- and intrageneric relationships.

In view of the morphological similarity among *Colostethus marchesianus* and the three related species, we have examined the karyotype, NOR localization and C-banding pattern in these frogs in order to assess the usefulness of cytogenetic characteristics in distinguishing these species.

MATERIAL AND METHODS

The material examined consisted of six specimens of *C. marchesianus*, with two males and two females from the type locality at Missão de Taracuá (00°07′56″N, 68°33′03″W) in the state of Amazonas, Brazil, and two males from São Gabriel da Cachoeira, located 150 km east of the type locality, in Amazonas, four specimens of *Colostethus caeruleodactylus* (3 males and 1 female) from the municipality of Careiro, at km 12 on the road to Autazes, state of Amazonas, Brazil (03°,37′,10.4″S, 59°,86′,78.4″W), six specimens of *Colostethus* sp. 1 (5 males and 1 female) from the Reserva Florestal Adolfo Ducke (RFAD), located 25 km from Manaus, Amazonas, (03°,08′S, 60°,04′W) and 10 specimens of *Colostethus* sp. 2 (9 males and 1 female) collected in the same region as the *C. caeruleodactylus* individuals. All specimens were collected and identified by A. P. Lima from February to August 1988 and in February 1999 and 2000 under a permit issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (Proc. No. 02005.001367/99-58-AM). The animals were deposited in the Museu de História Natural "Professor Adão José Cardoso" (ZUEC) at the Universidade Estadual de Campinas or in the herpetological collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, under the following accession numbers: INPA 10192, 10198, 10201, 10206, 10211, and 10220 (*Colostethus marchesianus*), ZUEC 11633, 11634, 11637 and 11640 (*Colostethus caeruleodactylus*), ZUEC 11806, 11810, 11812, 11814, 11816 and 11818 (*Colostethus* sp. 1), and ZUEC 11707–09, 11711, 11712, 11716, 11719 and INPA 7264–66 (*Colostethus* sp. 2).

Metaphases were prepared from a suspension of intestinal epithelium and testicular cells from animals pre-treated with 2 % colchicine for at least 4 h, as described by KING and ROFE (1976) and SCHMID (1978a). The cells were fixed in methanol/acetic acid fixative (3:1) and the slides were stained with 10 % Giemsa solution for analysis of the chromosome number, or labeled with silver nitrate for nucleolar organizer region (AgNOR) detection, according to HOWELL and BLACK (1980) and for C-banding using the technique of SUMNER (1972), with modifications in the duration of treatment with $Ba(OH)_2$. Fluorescent in situ hybridization (FISH) to identify the ribosomal genes was done according to VIEGAS-PÉQUIGNOT (1992) using a recombinant plasmid (HM 123) containing fragments of *Xenopus laevis* rDNA (MEUNIER-ROTIVAL et al. 1979). This plasmid was biotin-labeled using the nick translation reaction described in the GIBCO protocol. After FISH, the slides were examined with an Olympus BX 60 microscope or a BioRad MRC 1024 UV confocal microscope. The number of metaphases analyzed is shown in Table 1 and the chromosomes were classified according to GREEN and SESSIONS (1991).

Table 1. *Number of silver-stained and C-banded metaphases analyzed from each specimen. ZUEC: Museu de História Natural "Prof. Dr. Adão José Cardoso". INPA: herpetological collections of the Instituto Nacional de Pesquisas da Amazonia.*

Specimen	Metaphases analyzed by		Specimen	Metaphases analyzed by	
	Ag-NOR	C-banding		Ag-NOR	C-banding
C. marchesianus			*Colostethus* sp. 1		
INPA 10192	03	04	ZUEC 11806	04	04
INPA 10198	01	06	ZUEC 11810	06	07
INPA 10201	09	07	ZUEC 11812	26	10
INPA 10206	09	13	ZUEC 11814	06	09
INPA 10211	01	03	ZUEC 11816	—	24
INPA 10220	10	06	ZUEC 11818	09	04
C. caeruleodactylus			*Colostethus* sp. 2		
ZUEC 11633	02	02	ZUEC 11707	10	05
ZUEC 11634	11	02	ZUEC 11708	11	05
ZUEC 11637	05	01	ZUEC 11709	05	06
ZUEC 11640	02	10	ZUEC 11711	02	05
			ZUEC 11712	22	43
			ZUEC 11716	14	06
			ZUEC 11719	03	07
			INPA 7264	01	01
			INPA 7265	01	02
			INPA 7266	04	02

RESULTS

The chromosomal complement in the four species examined was 2n = 22 (Fig. 1), and was confirmed by meiotic chromosome analysis. The karyotypes of *C. marchesianus* and *C. caeruleodactylus* consisted of eight pairs of metacentric chromosomes (1, 2, 5, 6, 8–11), two submetacentrics (3 and 4), and one subtelocentric pair (7). In one specimen of *C. marchesianus* (INPA 10206), a secondary constriction (AgNOR negative) was observed in one of the homologs of pair 7. The karyotypes of *Colostethus* sp. 1 and *Colostethus* sp. 2 were very similar to those of *C. marchesianus* and *C. caeruleodactylus*, differing only in the morphology of pair 3, which was metacentric in these species (Fig. 1 and 5, Table 2). All karyotypes had a bimodal structure (six large and five small pairs).

In *C. marchesianus*, *C. caeruleodactylus* and *Colostethus* sp. 1, the NOR site was located in the interstitial region on the long arm of pair 4. In *Colostethus* sp. 2, the NOR site was detected on the short arm of pair 8; in one specimen (ZUEC 11712), the NOR was heteromorphic between the homologs (Fig. 2). In two specimens of *C. caeruleodactylus*, an

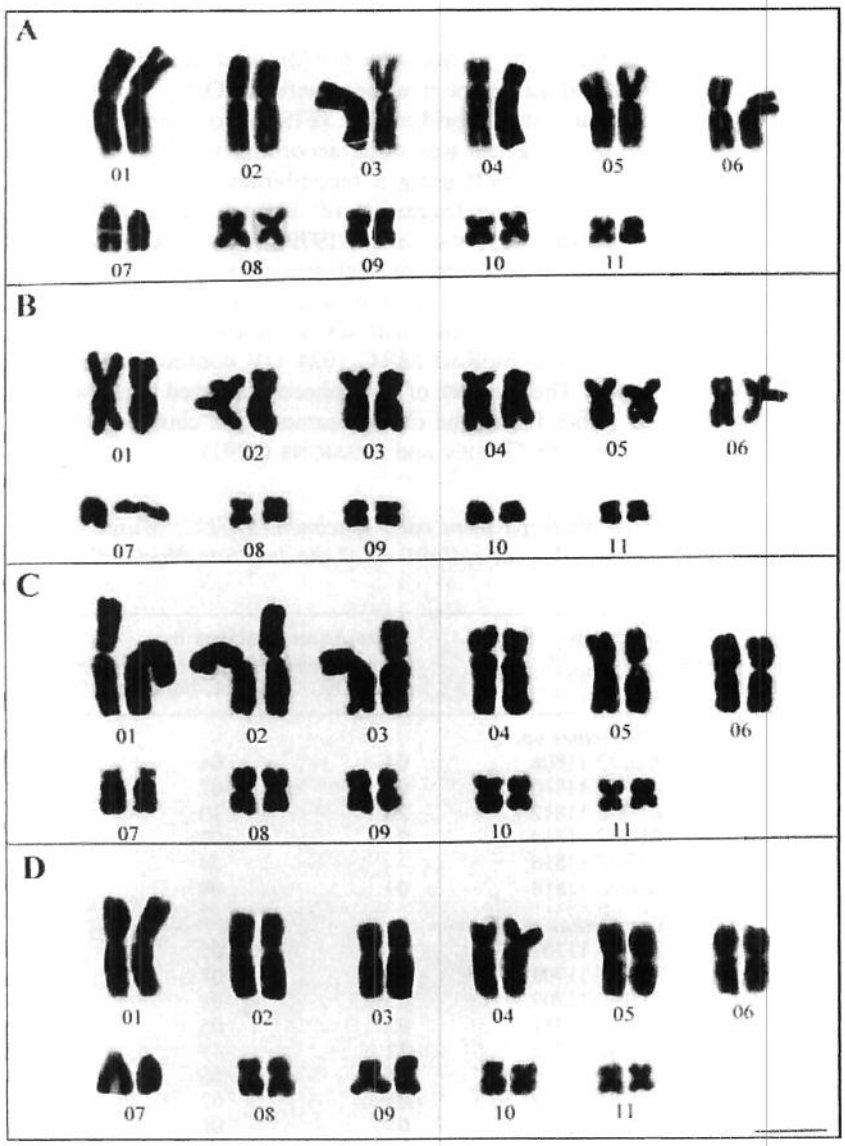

Fig. 1A–D. Giemsa-stained karyotypes of: *C. marchesianus* (**A**), *C. caeruleodactylus* (**B**), *Colostethus* sp. 1 (**C**) and *Colostethus* sp. 2 (**D**). Bar = 10 μm.

Table 2. *Morphometric analysis of the chromosomes of four Colostethus species. Centromeric classification follows that of* GREEN *and* SESSIONS *(1991). CH: chromosome, IC: centromeric index, RL: relative length (%), CC: centromeric classification, M: metacentric, SM: submetacentric, ST: subtelocentric. (*): obtained values for one of the homologs of the respective pairs that showed in heteromorphism C-banding size. n: number of measured metaphases.*

Colostethus marchesianus (n = 17)											
CH	1	2	3	4	5	6	7	8	9	10	11
RL	16.0	14.2	12.5	12.2	11.1	10.0	6.1	5.5	4.7	4.3	3.3
IC	0.439	0.428	0.374	0.252	0.400	0.426	0.145	0.456	0.447	0.468	0.465
CC	M	M	SM	SM	M	M	ST	M	M	M	M
Colostethus caeruleodactylus (n = 16)											
CH	1	2	3	4	5	6	7	8	9	10	11
RL	16.7	14.0	12.5	12.1	10.9	9.6	6.0	5.5	4.8	4.2	3.7
IC	0.414	0.382	0.354	0.260	0.410	0.411	0.132	0.462	0.450	0.460	0.458
CC	M	M	SM	SM	M	M	ST	M	M	M	M
Colostethus sp. 1 (n = 18)											
CH	1	2	3	4	5	6	7	8	9	10	11
RL	15.7	13.7	12.5	11.7	11.2	10.4	5.5	5.4	5.1	4.7	4.0
IC	0.488	0.484	0.395	0.314	0.418	0.430	0.175	0.489	0.468	0.427	0.474
CC	M	M	M	SM	M	M	ST	M	M	M	M
Colostethus sp. 2 (n = 39)											
CH	1	2	3	4	5	6	7	8	9	10	11
RL	15.3	13.2	11.7	11.5	10.7	10.1	6.3	6.2	5.7 6.8*	4.9	4.3
IC	0.436	0.429	0.419	0.324	0.435	0.442	0.162	0.461	0.456 0.357*	0.424	0.438
CC	M	M	M	SM	M	M	ST	M	M/SM*	M	M

additional NOR site was detected in the interstitial region on the long arm of pair 1, whereas in one specimen of *Colostethus* sp. 1, an additional NOR site was also detected on the short arm of pair 9. Such extra NOR sites were seen in only one of the homologs. In situ hybridization with an rDNA probe confirmed the location of all NORs detected by silver staining. However, an additional marking on the long arm of pair 5 in one specimen of *Colostethus* sp. 2 (ZUEC 11712), undetected by AgNOR, was also observed (Fig. 3).

C-banding revealed interspecific variations among the karyotypes. C-bands were detected in the centromeric region of all chromosomes in the four species. Pericentromeric, interstitial and terminal bands were also observed, although some stained faintly and were difficult to observe. In the four species, a small block of constitutive heterochromatin was present in the interstitial region on the long arm of pair 7. One specimen of *Colostethus* sp. 2 (ZUEC 11719) showed a heteromorphic C-block in the interstitial region on the long arm of pair 9 (Fig. 4 and 5). This block was detected as a secondary constriction in conventionally and AgNOR stained chromosomes. None of the C-positive blocks was coincident with the NORs in any of the species (Fig. 5).

DISCUSSION

Despite the large number of species in the genus *Colostethus*, only 13 of them have been karyotyped. The studies of RADA de MARTÍNEZ (1976) and BOGART (1991) were restricted to a description of chromosomal number and morphology using conventional staining, and chromosomal banding data were recently described by KAISER et al. (2003) and VEIGA-MENONCELLO et al. (2003) for only four *Colostethus* species. Except for *C. chalcopis* (KAISER et al. 2003) and *C. nidicola* (VEIGA-MENONCELLO et al. 2003), as well as the species studied here all, the other *Colostethus* species have 2n = 24 chromosomes.

The presence of 22 chromosomes indicates that there is karyotypic variability in *Colostethus*. Within the Dendrobatidae, a diploid number of 22 chromosomes has been found only in *Dendrobates opisthomelas* (*Minyobates opisthomelas* – BOGART 1991). Intrageneric variation in chromosome number among dendrobatids has been reported only in *Dendrobates*, with 2n = 18, 20 and 22 chromosomes (LEÓN 1970; RASOTTO et al. 1987; BOGART 1991). Thus, neither *Dendrobates* nor *Colostethus* appear to be karyotypically conserved. Further analysis with more species should improve our understanding of the relationships between these genera.

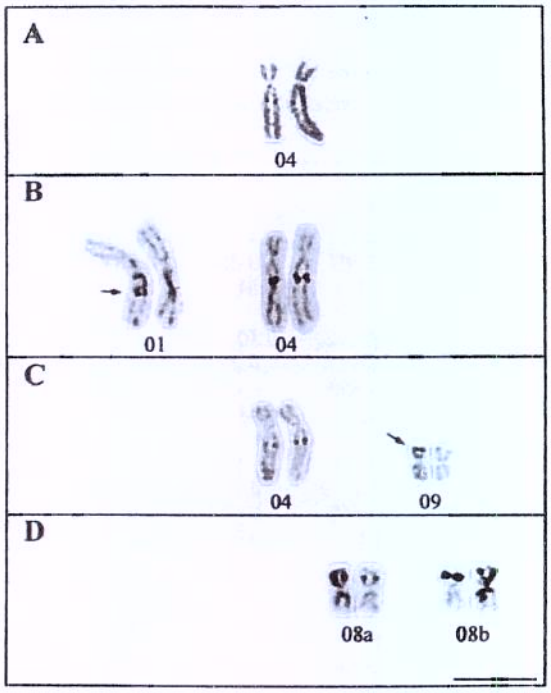

Fig. 2A–D. Silver-stained, NOR-bearing chromosome pairs of: *C. marchesianus* – pair 4 (**A**), *C. caeruleodactylus*- the arrow indicates an additional NOR site on one of the homologs of pair 1, in addition to that of pair 4 (**B**), *Colostethus* sp. 1 – the arrow indicates an additional NOR site on one of the homologs of pair 9, in addition that of pair 4 (**C**). *Colostethus* sp. 2 – pair 8 with homomorphic (a) and heteromorphic (b) NOR sites (**D**). Bar = 10 μm.

Some species belonging to different dendrobatid genera have telocentric chromosomes which suggests centric fusion and fission as possible mechanisms for changes in the chromosomal number in this family, as also found in other anuran groups (BOGART and HEDGES 1995; MIURA et al. 1995; BUSIN et al. 2001). According to BOGART (1991), other chromosomal rearrangements, such as translocations and inversions, are probably involved in the karyotypic evolution of *Colostethus*, since even within the 2n = 24 group some species have no telocentric chromosomes.

The species of *Colostethus* analyzed by BOGART (1991) showed extensive intrageneric variation in their chromosomal morphology, but this was not observed in *C. marchesianus* or in the species related to *C. marchesianus* studied here. The karyotypes of the four species shared some common characteristics with other dendrobatids, including the morphology

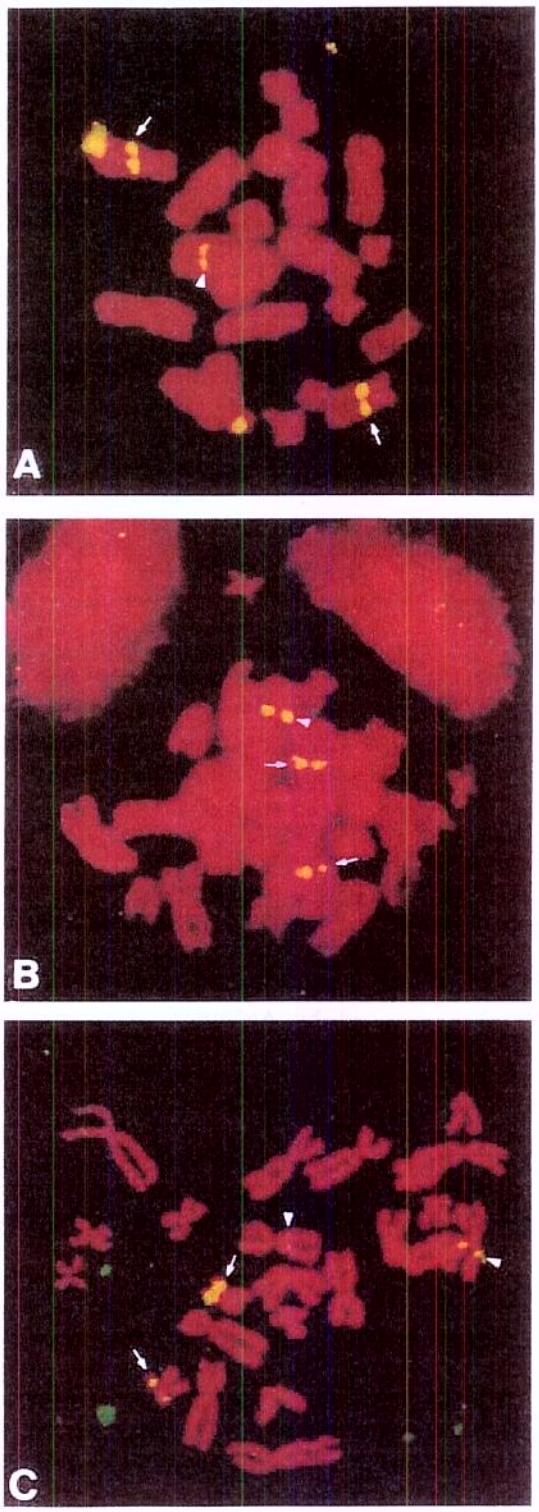

Fig. 3A–C. Mitotic metaphases following FISH with an rDNA probe. *Colostethus caeruleodactylus* – the arrows indicate the NOR sites of pair 4. The arrowheads indicate an additional site on one of the homologs of pair 1 (**A**). *Colostethus* sp.1 – note the NOR on pair 4 (arrows). An additional site can be seen on one of the homologs of pair 9 (arrowhead) (**B**). *Colostethus* sp. 2 – the arrows indicate heteromorphic sites of rDNA on pair 8 and the arrowheads indicate an additional site on pair 5 (**C**).

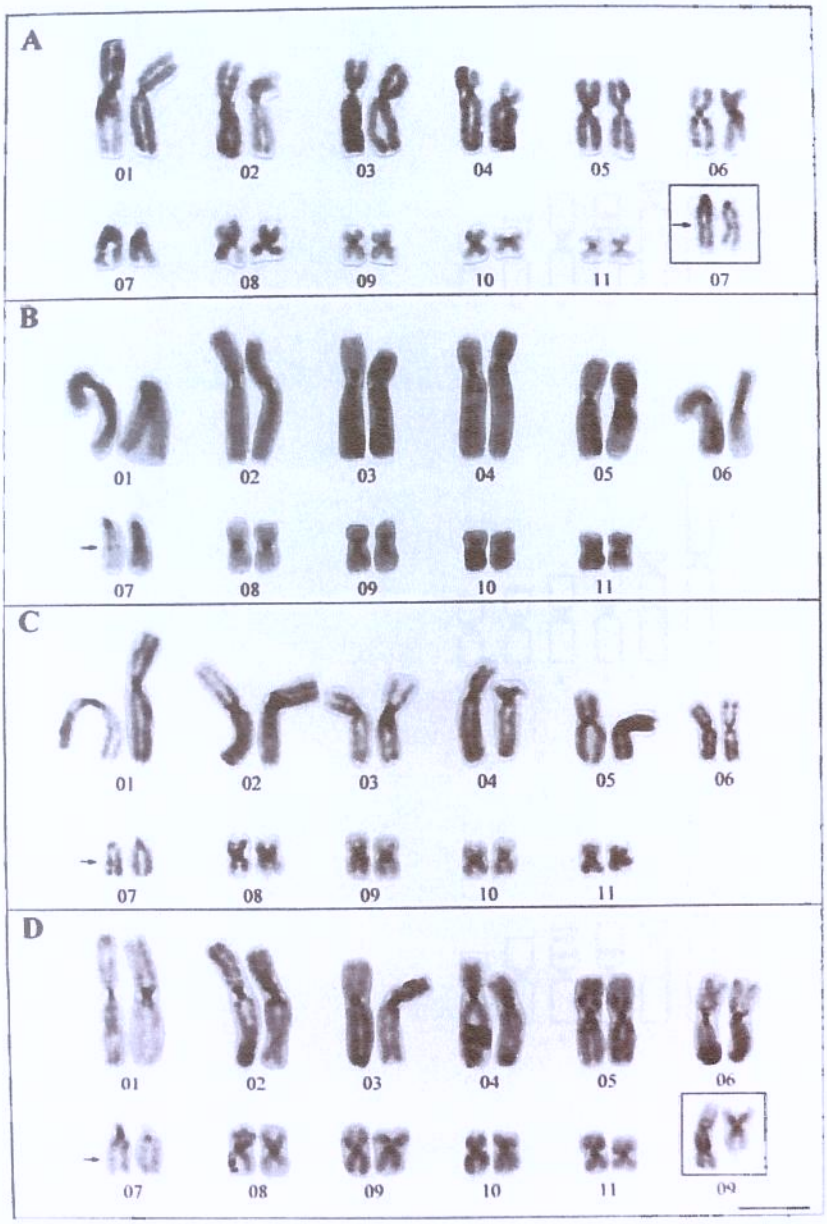

Fig. 4A–D. C-banded karyotypes of: *C. marchesianus* (**A**) – the box shows a faint band on the long arm of pair 7. *C. caeruleodactylus* (**B**), *Colostethus* sp. 1 (**C**) and *Colostethus* sp. 2 (**D**). One of the homologs of pair 9 shows an increase in the amount of heterochromatin (indicated by the square) (**D**). The arrows indicate a faint band on the long arm of pair 7 in the four species. Bar = 10 μm.

of pair 1, which was always metacentric, and a bimodal karyotype.

The karyotypes of *C. marchesianus* and *C. caeruleodactylus* differed from those of *Colostethus* sp. 1 and *Colostethus* sp. 2 only in the morphology of pair 3. Despite differences in the centromeric index, the morphology of this chromosome was similar in the two species. In conventional karyotypical analysis, the difference in the centromeric index was not sufficient to unequivocally distinguish these species.

Despite the great similarity among the four karyotypes, only *Colostethus* sp. 2 could be distinguished from the other species by the NOR location. *Colostethus marchesianus*, *C. caeruleodactylus* and *Colostethus* sp.1 had the NOR site on the long arm of pair 4, whereas *Colostethus* sp. 2 had the NOR on pair 8. According to SCHMID (1982) and SCHMID et al. (1990), variations in NOR location indicate that

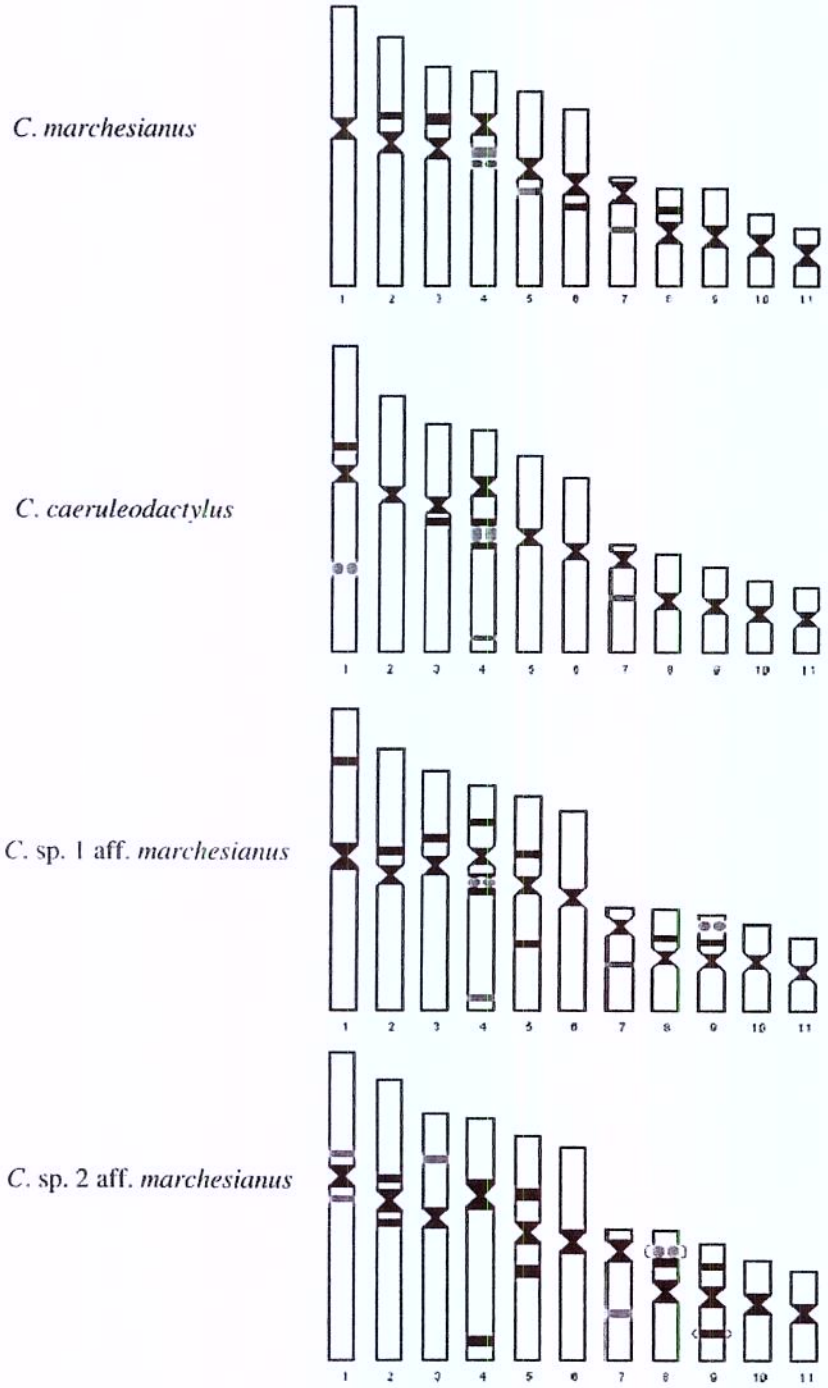

Fig. 5. Representative ideograms of the chromosomal numbers, NOR locations and C-banding patterns of four species of *Colostethus* from the Brazilian Amazon. Solid blocks: dark C-bands. Gray blocks: faint C-bands. Open regions: secondary constrictions. Gray circles: NORs. The parentheses indicate heteromorphic markings.

chromosomal rearrangements occurred during evolution since NOR sites are always located in the same chromosomal region in species of the same or related groups. Our results for the NOR location suggest that the karyotype of *Colostethus* sp. 2 is less conservative than that of other species in this parameter.

Heteromorphism in NOR size is frequent in a large number of anuran species (SCHMID 1982; LOURENÇO et al. 2000; BUSIN et al. 2001). The NOR heteromorphism in *Colostethus* sp. 2 probably resulted from the amplification of some ribosomal sequences in one of the homologs since our results did not indicate a complete duplication similar to that observed in most of the species analyzed by SCHMID (1982). Homomorphism or heteromorphism in Ag-stained NORs was seen in interphase nuclei, as also observed by SCHMID (1980, 1982).

Intraspecific polymorphism in the number and location of NORs has been described in a few anuran species, including *Hyla versicolor*, *Hyla chrysoscelis* (WILEY et al. 1989), *Bufo terrestris* (FOOTE et al. 1991), *Agalychnis callidryas* (SCHMID et al. 1995), *Physalaemus petersi* (LOURENÇO et al. 1998), *Physalaemus cuvieri* (SILVA et al. 1999), *Paratelmatobius poecilogaster* (LOURENÇO et al. 2000), *Pseudis minuta* and *Pseudis* sp. aff. *minuta* (BUSIN et al. 2001). In *C. caeruleodactylus* and *Colostethus* sp. 1, as well as in *A. callidryas* and *B. terrestris*, an additional NOR occurred in one of the homologs. According to SCHMID et al. (1995), these NORs appear to have been excised from or inserted into chromosomes without altering their morphology. FOOTE et al. (1991) suggested some probable mechanisms to explain the origin of this additional NOR site, including NORs functioning as mobile genetic elements, "orphan" rDNA copies, and reinsertion errors during ribosomal cistron amplification. However, additional evidence is needed to support such mechanisms.

Other small markers, such as that revealed by in situ hybridization on the long arm of pair 5 in one specimen of *Colostethus* sp. 2, which was undetected by AgNOR, have also been observed in *Hyla versicolor* and *Hyla chrysoscelis* (WILEY et al. 1989). According to SCHMID (1978b), small NORs cannot be detected by AgNOR because of their size. However, SCHMID et al. (1995), KING et al. (1990), FOOTE et al. (1991) and LOURENÇO et al. (1998) reported that in anurans all NORs detected by the AgNOR technique were also detected by in situ hybridization. Hence, a probable hypothesis to explain the additional marker present on pair 5 in specimen ZUEC 11712 of *Colostethus* sp. 2 is the presence of a homologous sequence of some portion of rDNA. In all specimens of *Colostethus* sp. 2, this region also had a C-band, which suggested the transposition of rDNA sequences to the heterochomatic region in this specimen.

Some of the C-band-positive it was useful for distinguishing these species. Thus, *C. marchesianus* had a pericentromeric C-block on the long arm of pair 6, which was not observed in the other species. *C. caeruleodactylus* differed from the other species by the absence of a pericentromeric C-block on pairs 2 and 5, and *Colostethus* sp. 2 had a C-block on the long arm of pair 9 that was characteristic only of this species.

Despite the variability detected in the C-banding pattern, the four species of *Colostethus* examined had a common, faintly staining band on the long arm of pair 7 which could be considered a landmark band for the 22-chromosome *Colostethus* species, as *C. chalcopis* (KAISER et al. 2003) also had this band. Since all species examined here had the same chromosomal number and a similar karyotype, but a different C-banding pattern, it is probable that the transformation of euchromatic segments to heterochromatic ones had a role in the separation of these *Colostethus* species. However, other events related to heterochromatin (and not detectable by the methods used here) may have been involved.

The heteromorphism in C-band size observed on the long arm of one of the homologs of pair 9 in a specimen of *Colostethus* sp. 2 (ZUEC 11719) probably resulted from the amplification of certain repetitive DNA sequences, and may have caused a change in chromosomal morphology. Changes in chromosomal morphology resulting from the addition of heterochromatin have also been reported by KING (1980) for species of *Litoria* (Hylidae).

Despite their similar chromosomal morphology, the species of *Colostethus* examined here were distinguished from each other by their C-banding pattern, and *Colostethus* sp. 2 could also be distinguished from the other species by it NOR location. Moreover, these species could be distinguished from *C. chalcopis* (KAISER et al. 2003) by differences in the position of the centromere of some chromosome pairs, in addition to the NOR location and C-banding pattern.

In conclusion, chromosomal rearrangements and heterochromatin-related events may have been involved in karyotypic differentiation in these species. Further analysis using molecular approaches could be useful for understanding the phylogenetic relationships of the 22-chromosome *Colostethus* species from Central Amazonia and the 22-chromosome *C. chalcopis*.

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ARTIGO II

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Chromosomal study of *Colostethus brunneus* from the type locality and two related species (Anura – Dendrobatidae)

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Key words: *Colostethus, cytogenetics, Dendrobatidae, karyotype*

Abstract

In this paper, we report a chromosomal study of three Brazilian species of *Colostethus*, *C. brunneus* from the type locality, *Colostethus* sp. (aff. *trilineatus*), and *Colostethus* sp., which is morphologically similar to *C. brunneus*. The diploid number for *C. brunneus* was 2n = 24 chromosomes, in agreement with that previously described for specimens from Peru. *Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp. showed a very similar karayotype with 22 chromosomes. The NOR was located on pair 3 in *C. brunneus*, on pair 4 in *Colostethus* sp. (aff. *trilineatus*), and on pair 2 in *Colostethus* sp. In one specimen of *Colostethus* sp., an additional NOR site was located on pair 7 in only one of the homologs. This extra Ag-NOR site was confirmed by FISH using an rDNA probe. In addition to the NOR location, the C-banding pattern was also species-specific, despite the similar chromosomal morphology of the species. These results indicate that although these species may be closely related, there is a clear dichotomy in their chromosome number.

Introduction

Colostethus is the largest genus of Dendrobatidae, with 128 nominal species (Frost, 2004), although many more undescribed species are known. This genus is widespread in Neotropical forests, from Costa Rica and the Caribbean region (Antilles and Martinique), to Bolivia and Brazil (Coloma, 1995; Frost, 2004). In general, the frogs of this genus are diagnosed by their cryptic coloration and the absence of skin alkaloids (Coloma, 1995).

Despite the large number of described species, the intrageneric relationships of this genus are poorly understood. Lynch (1982), Rivero ("1988" 1990) and Rivero & Serna ("1988" 1989) have proposed some *Colostethus* species groups based mainly on morphological data. However, some of these species groups have been criticized. According to Coloma (1995) and Grant et al. (1997), many of the hypothesized synapomorphic characters may represent symplesiomorphies or homoplasies.

The *Colostethus brunneus* group – group II (*sensu* Rivero "1988" 1990) contains various species, including *C. brunneus, C. marchesianus* and *C. trilineatus*, that resemble each other. This similarity has lead to confusion, with aspects of *C. trilineatus* ecology being reported under the name *C. marchesianus* (see Grant & Rodríguez, 2001).

Recently, Veiga-Menoncello et al. (2003b) showed that a cytogenetic analysis of four species of *Colostethus* with 22 chromosomes was able to distinguish these morphologically similar species. To obtain additional information that could be useful in assessing the systematics of *Colostethus*, we have done a chromosomal study of *Colostethus brunneus* from the type locality. Previous data for this species was restricted to the number and morphology of the chromosomes of some specimens from Peru (Bogart, 1991). We also analyzed two other species, *Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp., which is morphologically similar to *C. brunneus*.

Material and methods

Specimens

We examined 15 specimens of *Colostethus brunneus* (three females and 12 males) obtained from the type-locality (Chapada dos Guimarães, Mato Grosso State, Brazil 15°.16′.00″.S,

55°.31′.52′.W), 12 specimens of *Colostethus* sp (aff. *trilineatus*) (all males) from Rio Branco, Acre State (9°.57′.S, 67°.52′.W), and 12 specimens of *Colostethus* sp. (three females and nine males) from Santarém, Pará State (Long –54.84028, Lat –3.14912).

All specimens were collected by A. P. Lima under a permit issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (Proc. no. 02005.001367/99-58-AM). Voucher specimens of all species studied were deposited in the Museu de História Natural "Professor Adão José Cardoso" (ZUEC), Universidade Estadual de Campinas, Brazil, in the Célio F. B. Haddad collection (CFBH), Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, Brazil, and in the herpetological collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Brazil, under the following accession numbers: *Colostethus brunneus:* CFBH 05203, 05206, 05208, 05212, 05213, 05215, 05216, 05219, 05220 and 05221; INPA 10149, 10151, 10152, 10153 and 10154. *Colostethus* sp. (aff. *trilineatus*): ZUEC 12992, 12994, 12995, 12996, 12997; INPA 11975, 11976, 11979, 11982, 11984, 11985, 11987. *Colostethus* sp.: INPA 10170, 10167 and 10164; ZUEC 13007, 13005, 13006, 13008, 13009, 13010, 13013, 13018 and 13021.

Chromosome preparation and techniques

After treatment with 2% colchicine for at least 4 h, the frogs were killed and the chromosomal preparations were obtained according to King and Rofe (1976) and Schmid (1978a). Conventional staining with 10% Giemsa solution, $AgNO_3$ labeling (Howell & Black, 1980) and C banding (Sumner, 1972), with modifications in the duration of treatment with HCl and $Ba(OH)_2$, were done for the three species. Fluorescence *in situ* hybridization (FISH) (Viegas-Péquignot, 1992) using an rDNA probe consisting of a recombinant plasmid, HM 123, containing a fragment of *Xenopus laevis* rDNA (Meunier-Rotival et al., 1979) was done only in *Colostethus* sp.

Results

All specimens of *Colostethus brunneus* had 24 chromosomes, whereas the specimens of *Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp. had 22 chromosomes (Figures 1 and 5 and Table 1).

The karyotype of *C. brunneus* consisted of eight pairs of metacentric chromosomes (1, 2, 5-10), three pairs of submetacentric chromosomes (3, 4 and 11) and one telocentric pair (12) (Figure 1A and Table 1). The karyotypes of the *Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp. were very similar to each other. *Colostethus* sp. (aff. *trilineatus*) showed eight pairs of metacentric chromosomes (1, 2, 5, 6, 8-11), two submetacentric pairs (3 and 4) and one subtelocentric pair (7), whereas the karyotype of *Colostethus* sp. differed from that of *Colostethus* sp. (aff. *trilineatus*) only in the morphology of pair 3, which was classified as metacentric in this species. In conventional karyotypical analysis, the difference in the centromeric index was not sufficient to distinguish these species (Figure 1B and 1C and Table 1).

In some Giemsa-stained metaphases a discreet secondary constriction was observed and was always coincident with the nucleolus organizer region (NOR) detected by silver staining. In *C. brunneus,* the NORs were located in the interstitial region on the long arms of pair 3, whereas in *Colostethus* sp. (aff. *trilineatus*) the NORs were located on the short arms of pair 4, which showed the same morphology as pair 3 of *C. brunneus* (Figures 2A and 2B and 5A and B). In one specimen of *Colostethus* sp., in addition to the main NOR located in the interstitial region on the short arm of pair 2, further Ag-NOR staining was detected on the long arm of pair 7 in only one of the homologs. This extra NOR were confirmed by FISH with an rDNA probe (Figures 2C and 3A and B and 5C).

In addition to the NOR location, the pattern of heterochromatin distribution was also species-specific. C-bands were detected in the centromeric region of all chromosomes of the three species. However, only a few interstitial bands were shared by the species, such as the C-positive blocks located on the short arms of pairs 4 and 8 in *C. brunneus* and *Colostethus* sp. (aff. *trilineatus*) and the faint band present in the interstitial region on the long arms of pair 7 in both of the 22-chromosome *Colostethus* species (Figures 4A-C and

5A-C). C-positive blocks were detected adjacent to the NOR sites in the three species (Figure 5).

Discussion

The diploid complement number of 24 chromosomes seen in all specimens of *C. brunneus* was the same as reported that by Bogart (1991) for specimens from Peru identified as *C. brunneus*. However, when the karyotypes were compared, conspicuous differences were detected in the morphology of some chromosomal pairs. The karyotype of *C. brunneus* from the type locality had one telocentric pair, whereas that reported by Bogart (1991) had two pairs of telocentric chromosomes (pairs 9 and 10). Moreover, in *C. brunneus* from the type locality the arm ratio obtained for chromosome pairs 4, 6 and 12 indicated that they corresponded to submetacentric, metacentric and telocentric chromosomes respectively, in contrast to Bogart (1991), who classified them as subtelocentric, submetacentric and submetacentric, respectively. The divergence between the karyotype observed here and that described by Bogart (1991) may be indicate interpopulational variation since the chromosomal classification (see Green & Sessions, 1991) used in both studies was the same. However, interpopulational variation in anuran karyotypes is not very common (Lourenço et al., 2003). Unfortunately, Bogart (1991) did not report any chromosomal banding that could be useful for indicating chromosomal homeologies between these populations. Moreover, since we analyzed the type locality specimens of *C. brunneus,* we cannot exclude the possibility that the specimens analyzed by Bogart (1991) had been misidentified, especially since many *Colostethus* species are morphologically similar and have a drab coloration.

Although the external morphology of *Colostethus* sp. is very similar to that of *C. brunneus* (A.P. Lima, personal observation), the chromosomal data obtained here revealed two distinct taxonomic units of *Colostethus*. The distinction between these species was strongly supported by the difference in chromosome number, in addition to the divergent patterns of heterochromatin distribution and the NOR location (on the long arm of pair 3 in *C. brunneus* and on the short arm of pair 2 in *Colostethus* sp.).

The diploid number of 22 chromosomes in *Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp. was the same as in *C. nidicola, C. marchesianus, C. caeruleodactylus, Colostethus* sp. 1 (aff. *marchesianus*) and *Colostethus* sp. 2 (aff. *marchesianus*) (Veiga-Menoncello et al., 2003a,b) and *C. chalcopis* (Kaiser et al., 2003). The chromosomal morphology of *Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp. was similar to the species mentioned above.

Despite the difference in chromosomal number between *C. brunneus* and *Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp., the morphology of the six largest chromosomes is very similar and diverged only in the arrangement of pairs 3 and 4 in *C. brunneus*. The difference in the arrangement of these chromosomal pairs may be related to the extent of the secondary constriction (AgNOR - positive) present on the long arm of pair 3.

The chromosomal C-banding and NOR staining patterns were very useful for distinguishing *Colostethus* sp. (aff. *trilineatus*) from *Colostethus* sp. since these species had the same chromosomal number and similar karyotypes. The NOR location on the short arm of pair 4 in *Colostethus* sp. (aff. *trilineatus*) also distinguished this species from the other 22-chromosome *Colostethus* species previously analyzed. *Colostethus marchesiansus, C. caeruleodactylus* and *Colostethus* sp. (aff. *marchesianus*) (Veiga-Menoncello et al., 2003b) had the NOR on the same pair as *Colostethus* sp. (aff. *trilineatus*), but on long arm of this chromosomal pair. Our results indicate that structural rearrangements involving the rDNA region may have had a role in the separation of this species. According to Silva et al. (2000), who analyzed *Leptodactylus* species, a shift in the NOR position in homeologous chromosomes at the interspecific level may be useful for characterizing distinct species with similar karyotypes.

An additional NOR site, such as that seen in one specimen of *Colostethus* sp., has been described in a few anuran species, including *Colostethus caeruleodactylus* and *Colostethus* sp. 1 (aff. *marchesianus*) (Veiga-Menoncello et al., 2003b) and *Agalychnis callidryas* (Schmid et al., 1995), in which an additional NOR also occurred in only one of the homologs. In agreement with Schmid et al. (1995), the additional NOR seen here also appeared to have been excised from or inserted into the chromosome without altering its

morphology. According to Foote et al. (1991), probable mechanisms to explain the origin of this additional NOR site include transposition by mobile genetic elements, "orphan" rDNA copies, and reinsertional errors during ribosomal cistron amplification.

Despite the interspecific variability seen in the C-banding pattern, some chromosomal homeologies were observed. Both of the 22-chromosome *Colostethus* species examined had a common, faintly stained band on the long arm of pair 7. This has also been observed in other 22-chromosome species (Veiga-Menoncello et al., 2003a,b), and appears to be a characteristic of Brazilian 22-chromosome *Colostethus* species.

To date, various *Colostethus* species have been used in molecular studies in attempts to elucidate the relationships within the Dendrobatidae (e.g. Vences et al., 2000; La Marca et al., 2002; Vences et al., 2003). The molecular phylogenetic analyses that show a close relationship between *C. marchesianus* and *C. trilineatus* (Vences et al., 2003) and among *C. trilineatus, C. humilis* and *C. talamancae* (La Marca et al., 2002) generally agree with the phenetic data based on morphology used by Rivero ("1988" 1990) to propose the "brunneus" group. Based on the results obtained here and on previous work (Veiga-Menoncello et al., 2003b), we conclude that although these species may be closely affiliated, there is a clear dichotomy in chromosome number in the "brunneus" group (*sensu* Rivero "1988" 1990), since *C. talamancae* (Bogart, 1991) *C. brunneus* have 2n = 24 chromosomes and *C. marchesianus* (Veiga-Menoncello et al., 2003b) and *Colostethus* sp. (aff. *trilineatus*) have 2n = 22 chromosomes. Additional molecular analyses involving *C. brunneus* and other species of this group are needed to corroborate this dichotomy.

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Legends

Figure 1. Karyotypes of *C. brunneus* (A), *Colostethus* sp. (aff. *trilineatus*) (B) and *Colostethus* sp. (C) after conventional staining with Giemsa. Bar = 10 μm.

Fugure 2. Silver-stained karyotypes. The arrows show the NOR sites in *C. brunneus* (A), *Colostethus* sp. (aff. *trilineatus*) (B) and *Colostethus* sp. (C). Bar = 10 μm.

Figure 3. The NOR-bearing chromosomal pairs of *Colostethus* sp. The arrow shows an additional NOR site on the long arm of pair 7 in only one of the homologs (A). Metaphase from one specimen of *Colostethus* sp. submitted to FISH using an rDNA probe (B). The arrowhead shows an extra NOR site on chromosome 7 in addition to the fixed NOR-bearing on pair 2.

Figure 4. Karyotypes of *C. brunneus* (A), *Colostethus* sp. (aff. *trilineatus*) (B) and *Colostethus* sp. (C) after C-banding. The arrows show a faintly stained band on the long arm of pair 7 in *Colostehus* sp. (aff. *trilineatus*) (B) and *Colostethus* sp. (C). Bar = 10 μm.

Figure 5. Ideograms of the karyotypes of *C. brunneus* (A), *Colostethus* sp. (aff. *trilineatus*) (B) and *Colostethus* sp. (C). Solid blocks: dark C-bands. Gray blocks: faint C-bands. Open regions: secondary constrictions. Gray circles: NORs. The parentheses indicate the region of an extra NOR site.

Table 1. Morphometric data for the *Colostethus brunneus, Colostethus* sp. (aff. *trilineatus*) and *Colostethus* sp. karyotypes. Chromosome nomenclature is based on Green & Sessions (1991).

	Chromosomes											
	1	2	3	4	5	6	7	8	9	10	11	12
Colostethus brunneus												
RL	15.9	14.0	13.4	11.8	10.1	9.4	5.6	4.8	4.5	4.0	3.6	2.5
AR	1.52	1.64	2.88	2.11	1.52	1.43	1.10	1.04	1.13	1.09	2.06	7.34
CC	M	M	SM	SM	M	M	M	M	M	M	SM	T
Colostethus sp. (aff. *trilineatus*)												
RL	16.0	12.8	11.8	11.5	11.1	10.0	6.20	6.13	5.48	4.74	4.14	
AR	1.33	1.52	1.73	2.09	1.45	1.25	5.86	1.16	1.09	1.30	1.15	
CC	M	M	SM	SM	M	M	ST	M	M	M	M	
Colostethus sp.												
RL	17.0	14.7	12.3	11.6	10.6	9.8	5.7	5.4	4.8	4.1	3.4	
AR	1.14	1.17	1.63	2.35	1.32	1.30	5.50	1.24	1.15	1.23	1.10	
CC	M	M	M	SM	M	M	ST	M	M	M	M	

RL: relative length; AR: ratio arm; CC: centromeric classification; M: metacentric; SM: submetacentric; ST: subtelocentric; T: telocentric.

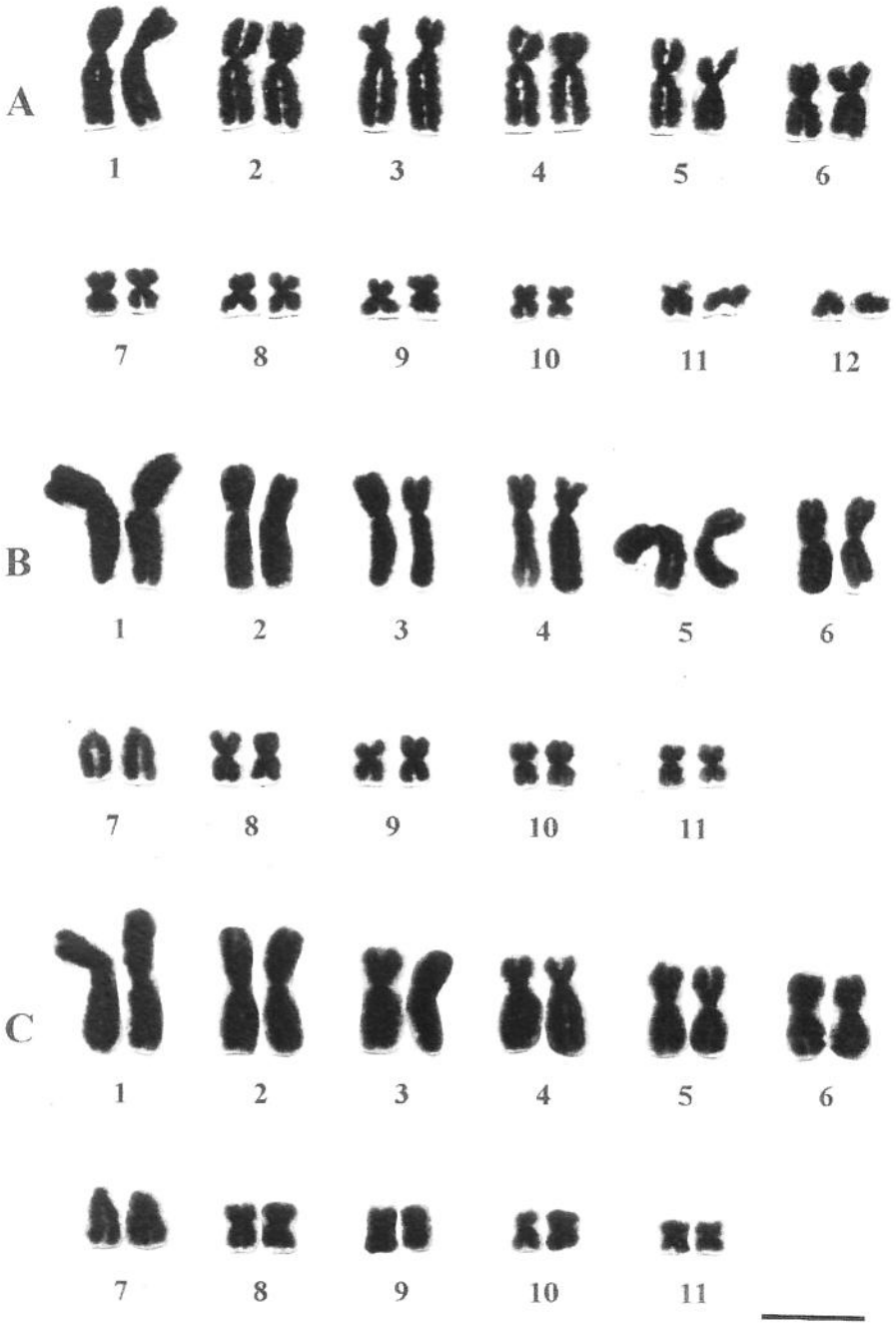


Figure 1

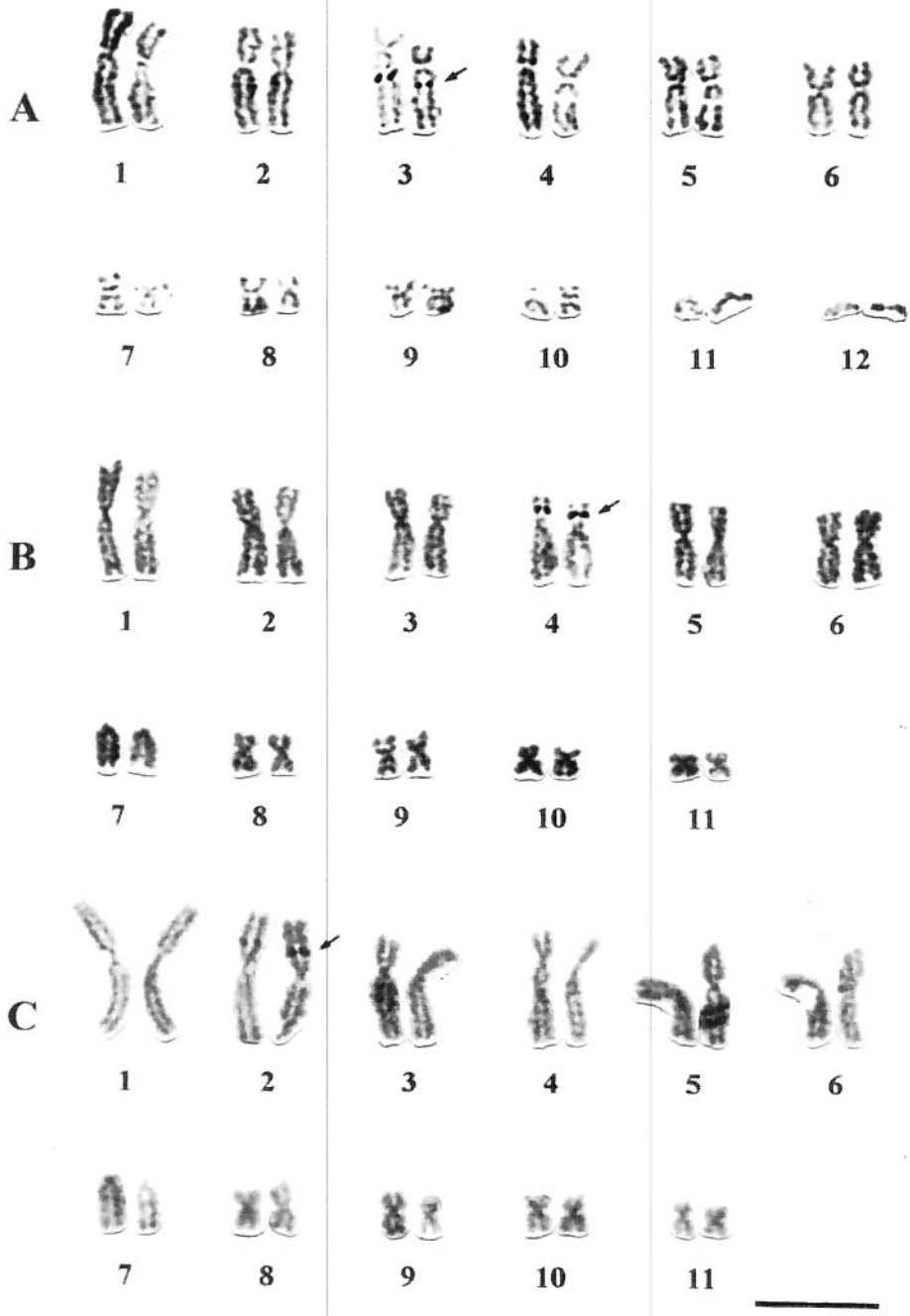
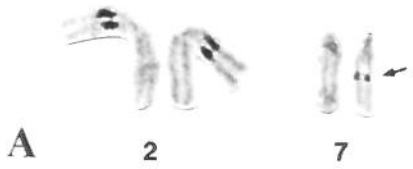


Figure 2



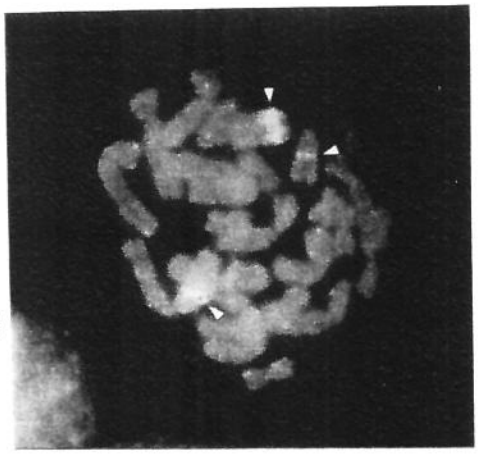


Figure 3

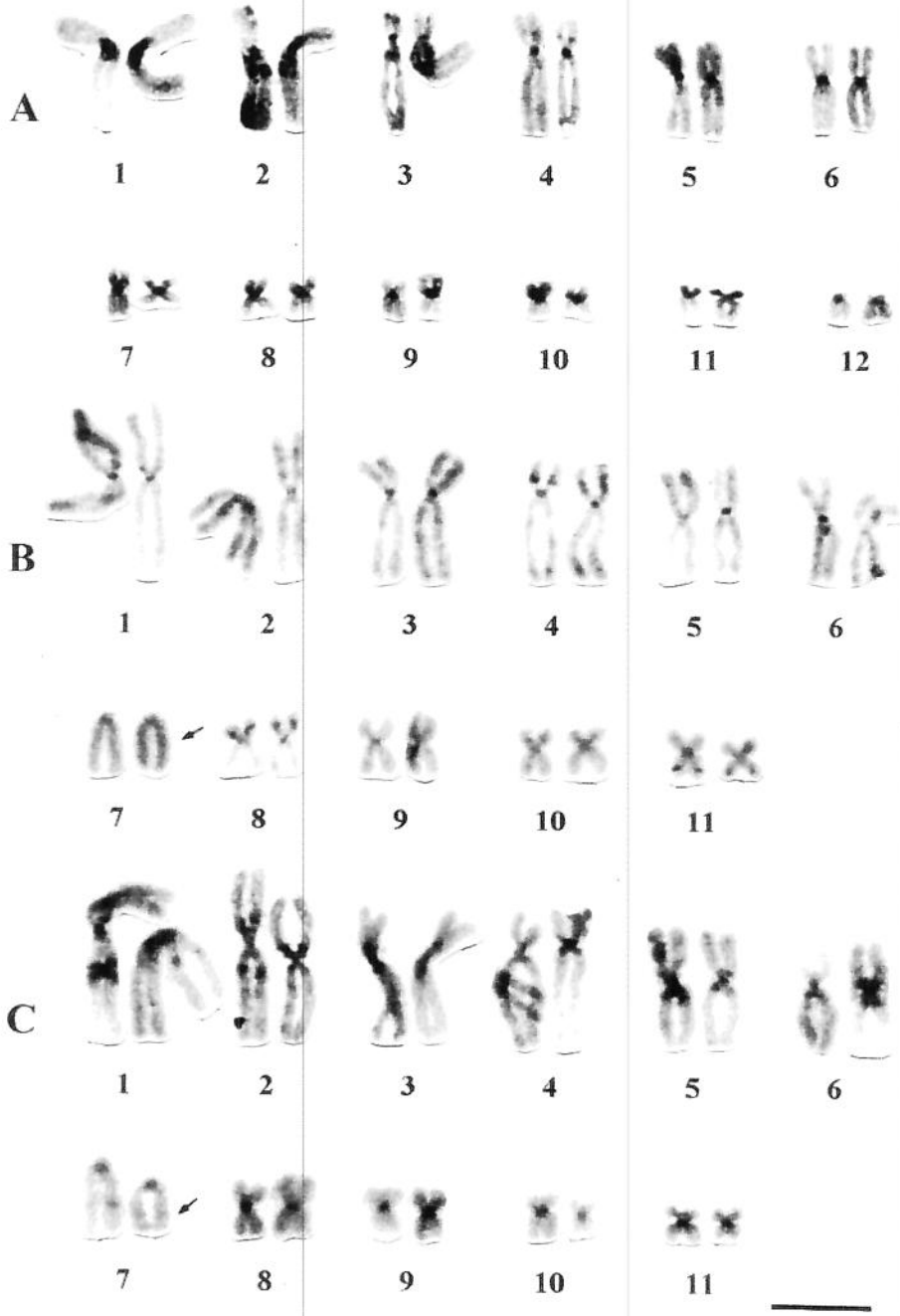


Figure 4

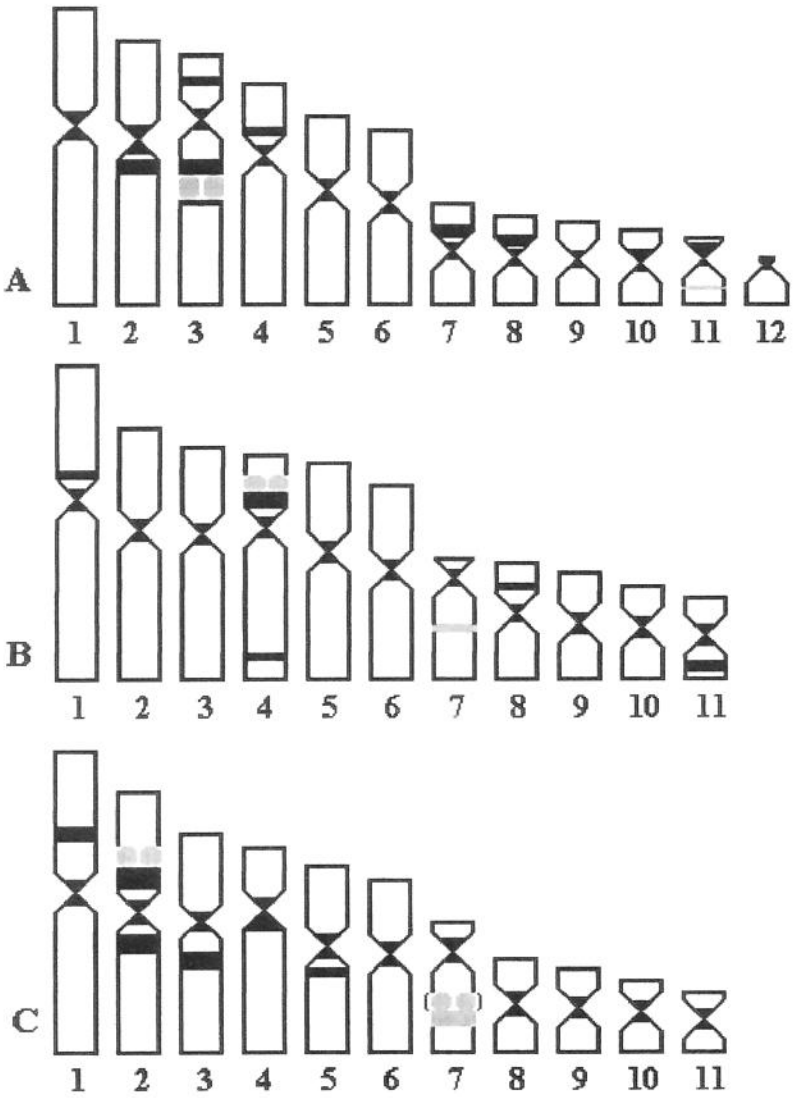


Figure 5

3. ANÁLISE DA ULTRA-ESTRUTURA DO ESPERMATOZÓIDE

ARTIGO III

Scanning and transmission electron microscopy analysis of the biflagellate spermatozoa of *Colostethus marchesianus* (Anura, Dendrobatidae) from the type locality and a related species

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Short title: Spermatozoa of *Colostethus* species

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Key word: sperm ultrastructure, Anura, Dendrobatidae, *Colostethus*.

ABSTRACT

Scanning and transmission electron microscopy and fluorochrome staining with DAPI were used to study the spermatozoa of *Colostethus marchesianus* from the type locality and *Colostethus* sp. (aff. *marchesianus*). The sperm cell of these species resembles those of most neobatrachian species, except for the presence of two complete flagella, a characteristic previously described only for four anuran species belonging to three families. Although very similar the spermatozoa described here showed slight differences in the nuclear length, extension of the subacrosomal cone above the nucleus and in the curvature of the axial fiber. The absence of a juxtaxonemal fiber and the presence of a comma-shaped axial fiber seem to be common characteristics of dendrobatid spermatozoa. The differences found, allied to previous suggestions from the literature, may indicate that the taxa here studied are distinct species. The evolutionary significance of biflagellarity is still unclear, although the presence of this trait in unrelated groups suggests independent origins.

INTRODUCTION

The ultrastructural characteristics of spermatozoa have been used in an increasing number of studies in a variety of animal groups such as platyhelminths (Justine 1991), fishes (Mattei, 1991; Jamieson, 1991), and reptiles (Jamieson, 1995; Teixeira, Coli & Báo, 1999a,b).

In anurans, the sperm ultrastructure revealed by transmission electron microscopy has been helpful in clarifying some taxonomic relationships. The main sperm structures used have been the acrosome, perforatorium and tail, all of which have a variable morphology and may provide new characters for phylogenetic inferences (Lee & Jamieson, 1992, 1993; Jamieson, Lee & Long, 1993; Kwon & Lee, 1995; Meyer, Jamieson & Scheltinga, 1997). However, so far, the descriptions of spermatozoon morphology and ultrastructure have been limited to a few species in only 20 of 33 extant anuran families (Scheltinga & Jamieson, 2003; Frost, 2004).

There is considerable uncertainty about the relationships of the Dendrobatidae with another anuran families, and many of the hypotheses remain controversial (Myers, Paolillo & Daly, 1991; Ford, 1993; Ford & Cannatella, 1993; Clough & Summers, 2000; Vences *et al.*, 2000). *Colostethus* is the largest dendrobatid genus, with about 128 recognized species (Frost, 2004), and is considered to be a basal group within the dendrobatids (Lynch, 1982). Several attempts have been made to understand the systematics of this genus. Based on morphological data, putative monophyletic species groups within *Colostethus* have been proposed by Rivero & Serna ("1988" 1989), Rivero ("1988" 1990), and Lynch (1982). However, according to Coloma (1995), the monophyly of the groups given by Rivero & Serna ("1988" 1989) and Rivero ("1988" 1990) is questionable and many of the hypothesized synapomorphic characters may represent symplesiomorphies or homoplasies.

Colostethus marchesianus, included in the "brunneus" group (Rivero, "1988", 1990), is a widespread species occurring in the Amazon basin and on lower slopes of the eastern Andes. Some populations currently assigned to this species show little morphological variation. However, differences in calls suggest that more than one species is present in this nominal taxon (Coloma, 1995). In this work, we used light microscopy

and transmission electron microscopy to describe and compare the spermatozoa of *C. marchesianus* to a putative related species, *Colostethus* sp. (aff. *marchesianus*). To ensure an appropriate comparison we used specimens of *C. marchesianus* from its type locality. Our objective was to contribute with additional data which may be useful to clarify the systematics of the genus *Colostethus*.

As the spermatozoa of these species are biflagellate, a condition very uncommon in anurans, we have also provided for the first time a structural analysis through the scanning electron microscopy.

MATERIAL AND METHODS

Specimens

Two specimens of *C. marchesianus* from the type locality at Missão de Taracuá (00°07'56''N, 68°33'03''W) in the state of Amazonas, Brazil, and 2 specimens from São Gabriel da Cachoeira, located 150 km east of the type locality, in Amazonas, and 4 specimens of *Colostethus* sp. (aff. *marchesianus*) from Reserva Florestal Adolfo Ducke (RFAD), Manaus, Amazonas (03°,08' S, 60°,04' W) were analyzed by light, transmission and scanning microscopy. The specimens were collected by A. P. Lima in February 2000 under a permit issued by the Instituto Brasileiro de Meio Ambiente e Recursos Naturais Renováveis (IBAMA) (Proc. no. 02005.001367/99-58-AM). We deposited the animals in the herpetological collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, or in the Museu de História Natural "Professor Adão José Cardoso" (ZUEC), Universidade Estadual de Campinas (UNICAMP), Brazil, under the following accession numbers: *Colostethus marhesianus* - INPA 10200, 10202, 8007 and 8003; *Colostethus* sp. (aff. *marchesianus*) – ZUEC 11811, 11818, 12969 and 12970.

Techniques

The specimens were killed by narcosis with ether. The testes were removed and, for light microscopy (LM), a suspension of spermatozoa was prepared in 0.1 M cacodylate buffer,

pH 7.2, then spotted on glass slide, and fixed in this buffer containing 2% paraformaldehyde-glutaraldehyde, 5 mM $CaCl_2$ and 3% sucrose. The slides were stained with 0.2 µg of 4,6-diamino-2-phenylindole (DAPI)/ml for 15 min, rinsed in water, and placed in 0.1 M McIlvane buffer for 5 min. The slides were examined with an Olympus BX 60 microscope.

For scanning electron microscopy (SEM), an aliquot of the suspension was spotted on cover slips and fixed in the same fixative solution as above. The samples were dehydrated in an ascending ethanol series, and after critical-point drying and sputter coating with gold, the material was examined with a JMS 5800 LV scanning microscope.

For transmission electron microscopy (TEM), fragments of the testes were fixed overnight at 4° C in the fixative solution described above, post-fixed for 1 h at 4° C in 0.1 M sodium cacodylate buffer, pH 7.2, containing 1% osmium tetroxide and 1.6% potassium ferricyanide, and left for 2 h in a solution of 0.5% uranyl acetate for "en block" contrasting. The samples were then rinsed in the same buffer, dehydrated in an increasing acetone series and embedded in Epon 812 resin. Sections were placed on copper grids and stained with uranyl acetate and lead citrate prior to examination with a Leo 906 transmission electron microscope.

RESULTS

In both species, the spermatozoa were filiform, with two complete flagella inserted into the midpiece. The nucleus was elongated and cylindrical, and differed in length between them, with 28.7 µm in *Colostethus marchesianus* and 31.6 µm in *Colostethus* sp. (aff. *marchesianus*) (Figs. 1a and 1e, 2a and 3b).

In SEM, the spermatozoa of *C. marchesianus* and *Colostethus* sp. (aff. *marchesianus*) showed two axial rods, two undulating membranes and two axonemes (Fig. 1a and 1e). In *Colostethus marchesianus* the beginning of the acrosomal complex over the nucleus was seen as a pronounced concavity (Fig. 1b). At the level of the midpiece, the nucleus was sometimes separated from the flagella, and revealed a connection between the

two structures (Fig. 1c). The axonemes were arranged in waves connected to the rods via an undulating membrane (Fig. 1d and f).

In TEM, a conic acrosomal vesicle containing homogenous material of moderate electron density covered approximately one-third of the nucleus in both species (Figs. 2b and 3b). In *C. marchesianus*, this material was concentrated in the anteriormost portion of the vesicle and was not observed on the acrosome extension (Figs. 2b-d), whereas in *Colostethus* sp. (aff. *marchesianus*), the vesicle was entirely filled (Figs. 3b and c). The acrosome was not seen in cross-section of the middle and basal portions of the nucleus in both species (Figs. 2e–f, 3d and e). A subacrosomal cone occurred below the acrosomal vesicle and extended beyond the limits of the vesicle. This cone was separated from the nucleus by a very discrete subacrosomal space (Figs. 2b–e, 3b-d) and presented a larger extension above the nucleus in *C. marchesianus* when compared to *Colostethus* sp. (aff. *marchesianus*) (Figs. 2b and 3b). The subacrosomal cone progressively narrowed and was not observed in transversal sections of the nuclear base (Fig. 2f and 3e).

The nucleus had a strongly condensed, electron-dense chromatin (Figs. 2b, e and f, 3b, d and e). A pronounced nuclear fossa was observed in the distal portion of the nucleus where the two centrioles were inserted. The centrioles were arranged parallel to each other and gave rise to two independent axonemes (Figs. 2g and h, and 3f). Also in this region, a cytoplasmic expansion containing mitochondria (mitochondrial collar) surrounded the initial portion of the flagella (Fig. 2i, 3f and g). The flagella were formed by two axonemes with a typical 9+2 pattern, an undulating membrane, and a comma-shaped axial fiber, which was connected to the axonemes through the axial sheath (Figs. 2i and j, 3g and h). In *Colostethus* sp. (aff. *marchesianus*), the axial fiber presented a less marked curvature (Figs. 3g and h).

In the final portion of the flagella, the undulating membrane was shortened and the axial fiber was close to the axoneme (Fig. 2k and 3i). In cross-sections of posterior portions, only the axoneme was observed (Fig. 2 l and 3j).

A diagrammatic representation of the spermatozoon of *C. marchesianus* is shown in Figure 4.

DISCUSSION

Although the family Dendrobatidae contains approximately 228 species distributed in nine genera (Frost, 2004), in only three species (one in the genus *Colostethus* and one in the genus *Allobates* and three in the genus *Epipedobates*) has the sperm ultrastructure been described (Garda *et al.*, 2002; Aguiar-Jr. *et al.*, 2003, 2004). Biflagellarity, the most striking characteristic found in *Colostethus*, is shared with one species of *Allobates* (*A. femoralis*, formerly named *Epipedobates femoralis*) described by Aguiar-Jr. *et al.* (2003). Whereas biflagellarity is found in the species of *Colostethus* and *Allobates* that have been investigated, this condition is exceptional in *Epipedobates*, in which monoflagelatte spermatozoa have been observed (Garda *et al.*, 2002; Aguiar-Jr. *et al.*, 2004). Phylogenetic relationships among the species of *Allobates* and some species of *Colostethus* (including *C. marchesianus*) have been suggested by La Marca, Vences & Lötters (2002) and Vences *et al.* (2003).

The head structures of *C. marchesianus* and *Colostethus* sp. (aff. *marchesianus*) spermatozoa resembled those of most neobatrachian species (Báo, Dalton & Oliveira, 1991; Lee & Jamieson, 1993; Amaral *et al.*, 2000). The conical acrosomal vesicle and the subacrosomal cone are shared with most anuran species. According to Scheltinga *et al.* (2001), a subacrosomal cone covering the tip of the nucleus is a synapomorphy of lisamphibians and amniotes. In agreement with Garda *et al.* (2002), we consider the subacrosomal cone to be homologous with the conical perforatorium seen in bufonoid species.

The spermatozoa of *C. marchesianus* and *Colostethus* sp. (aff. *marchesianus*) was very similar to that of *Colostethus* sp. (Aguiar-Jr. *et al.*, 2003), although slight differences were noted. In *Colostethus* sp. described by Aguiar-Jr *et al.* (2003), the acrosomal vesicle was shorter and entirely filled with a material of moderate electron-density, as in *Colostethus* sp. (aff. *marchesianus*) described here, whereas in *C. marchesianus* the acrosomal vesicle was filled only in the anteriormost portion. In addition, the extension of the subacrosomal space was very long in *Colostethus* sp., but restricted in *C. marchesianus* and *Colostethus* sp. (aff. *marchesianus*). Although the overall structure of spermatozoa of

Colostethus species study so far is very uniform, the differences found in the present work may corroborate the hypothesis that the taxa here analyzed are distinct species as suggested by Caldwell, Lima & Biavati (2002) based on larval characteristics.

A mitochondrial collar, such as that seen in *C. marchesianus* and *Colostethus* sp. (aff. *marchesianus*) and other dendrobatid species, was considered by Lee & Jamieson (1992) to be a synapomorphic characteristic of most Eubufonoidea. However, Scheltinga *et al.* (2001) considered that no extant species have an arrangement of mitochondria which could confidently be regarded as plesiomorphic for the Anura. Based on their findings in *Leiopelma hochstetteri*, these authors argued that this structure appeared to be a plesiomorphy in anurans since a well-developed mitochondrial collar like that of eubufonoids resembled a condition seen in many acanthopterygian fishes.

The recurrent presence of a comma-shaped axial fiber and absence of a juxtaxonemal fiber (at least as separate entity) in the dendrobatid species analyzed may indicate that both characteristics are common features of this family, as suggested by Aguiar-Jr. *et al.* (2003). These pattern also occur in monoflagelate species (see Garda *et al.*, 2002; Aguir-Jr *et al.*, 2004).

In Anura, biflagellate spermatozoa have been described for *Telmatobufo australis* (Leptodactylidae) (Pugin-Rios & Garrido, 1981), *Chiromantis xerampelina* (Rhacophoridae) (Wilson, Van der Horst & Channing, 1991; Jamieson, 1999) and *Allobates femoralis* and *Colostethus* sp. (Dendrobatidae) (see Aguiar-Jr *et al.*, 2003). The presence of this trait in unrelated groups suggests an independent origin. Thus, we agree with Aguiar-Jr *et al.* (2003) that there is no evidence that biflagellarity has phylogenetic significance in the Anura. However, a general trend towards the simplification of anuran sperm cells, including the lack of some structures such as accessory fibers and an undulating membrane, was noted by Lee and Jamieson (1993) and Jamieson *et al.* (1993). This simplification, according to those authors, culminates in the presence of biflagellate spermatozoa (with only the axonemes). The biflagellarity seen here and in *Colostethus* sp. and *Allobates femoralis* (Aguiar-Jr *et al.*, 2003). is quite different since the two flagella are complete (with accessory fibers and undulating membrane) and thus this pattern do not appear to be the result of simplification.

According to J. P. Caldwell (personal communication), although the *Colostethus* sp. analyzed by Aguiar-Jr *et al.* (2003) has not yet been described, it is probably a member of the *C. brunneus* group, as is *C. marchesianus* (sensu Rivero, "1988" 1990) and *Colostethus* sp. (aff. *marchesianus*). However, considering the low number of species examined in this genus, it is unclear whether biflagellarity is common feature to this species group (*brunneus* group) within *Colostethus*. With the analysis of a greater number of species, this characteristic could be useful in elucidating the relationships within and among the currently proposed species groups, particularly since these groups are defined by combinations of character states variously present in other anurans groups, rather than by unambiguous synapormophies (Grant, Humphrey & Myers, 1997).

In conclusion, when a greater number of species is analyzed, the ultrastructural characteristics of sperm cells, together with morphological data and vocalization patterns, may be useful in characterizing species currently referred to *C. marchesianus* as well as the remaining species of the *C. brunneus* group.

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Figure legends

Fig. 1. Scanning electron micrograph.

A. and E. Panoramic view showing the nucleus and two complete flagella of a spermatozoon from *Colostethus marchesianus* and *Colostethus* sp. (aff. *marchesianus*) respectively. **B.** Detail of the head, with emphasis on the acrosomal concavity (asterisk) in *C. marchesianus*. **C.** Detail of the midpiece showing the connection between the head and flagella in *C. marchesianus*. **D and F.** Detail of the tail showing two axial rods, two undulating membranes, and two axonemes in *Colostethus marchesianus* and *Colostethus* sp. (aff. *marchesianus*) respectively.

Fig. 2 Spermatozoon of *Colostethus marchesianus* under light and transmission electron microscopy. **A.** DAPI staining. **B.** Longitudinal section of the head. The acrosomal vesicle is a cap-like structure covering the first portion of the nucleus, with a subacrosomal cone below this vesicle. The subacrosomal cone is separated from the nucleus by a subacrosomal space. **C-F.** Transversal sections of the acrosomal complex and nucleus showing a narrowing of the subacrosomal cone. **G and H.** Transversal sections of the midpiece showing two centrioles arranged in parallel which give rise to two independent axonemes. **I.** The initial portion of the flagella is surrounded by a mitochondrial sheath. **J and K.** Transversal sections of the flagella showing progressive narrowing of the axial sheath, undulating membrane and axial fiber. **L.** In the final portion of the flagella, the axial fiber is close to the axoneme and some sections only the axonemes are seen.

Fig. 3 Spermatozoon of *Colostethus* sp. (aff. *marchesianus*) under light and transmission electron microscopy. **A.** DAPI staining. **B.** Longitudinal section of the head. The acrosomal vesicle is a cap-like structure covering the first portion of the nucleus, filled by an homogenous material of moderate electron density. Note a subacrosomal cone below this vesicle. This cone is separated from the nucleus by a discreet subacrosomal space. **C-E.** Transversal sections of the acrosomal complex and nucleus showing a narrowing of the subacrosomal cone. **F.** Longitudinal section showing the nuclear fossa and mitochondrial

sheath, also observed in surrounding the flagella in transversal section **(G)**. **H and I.** Transversal sections of the flagella showing progressive narrowing of the axial sheath, undulating membrane and axial fiber. **J.** In the final portion of the flagella, only the axonemes are seen.

Abbreviations: a = acrosome; af = axial fiber; as = axial sheath; av = acrosomal vesicle; ax = axoneme; cc = cytoplasmic canal; f = flagellum; m = mitochondria; mc = mitochondrial collar; N = nucleus; nf = nuclear fossa; ns = nuclear space; pr = paraxonemal rod; sc = subacrosomal cone; ss = subacrosomal space; um = undulating membrane.

Fig. 4 A diagrammatic representation of spermatozoon ultrastructure of *C. marchesianus* as seen in longitudinal and transverse sections by transmission electron microscopy. The differences when compared to *Colostethus* sp. (aff. *marchesianus*) are described on the text.

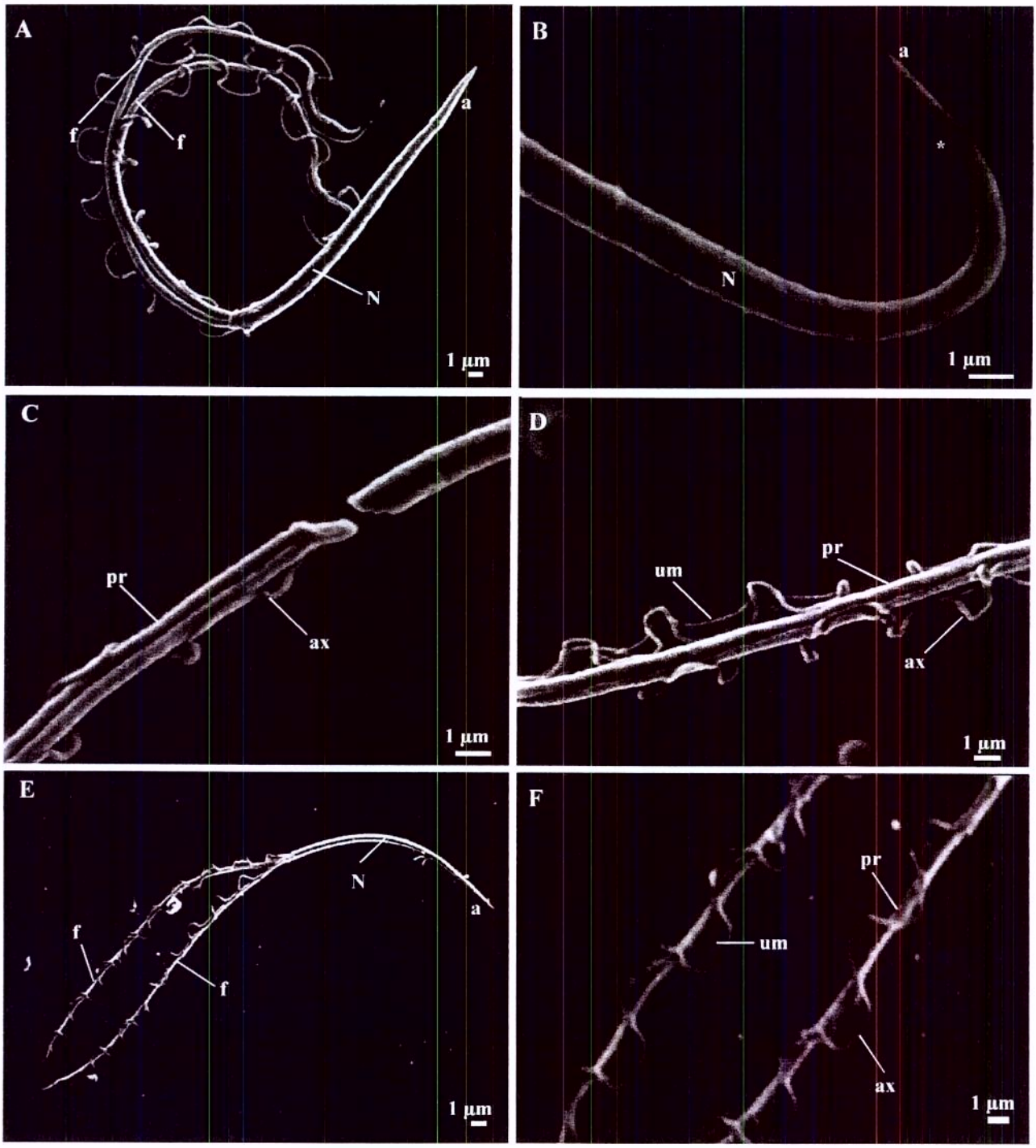
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Figure 1

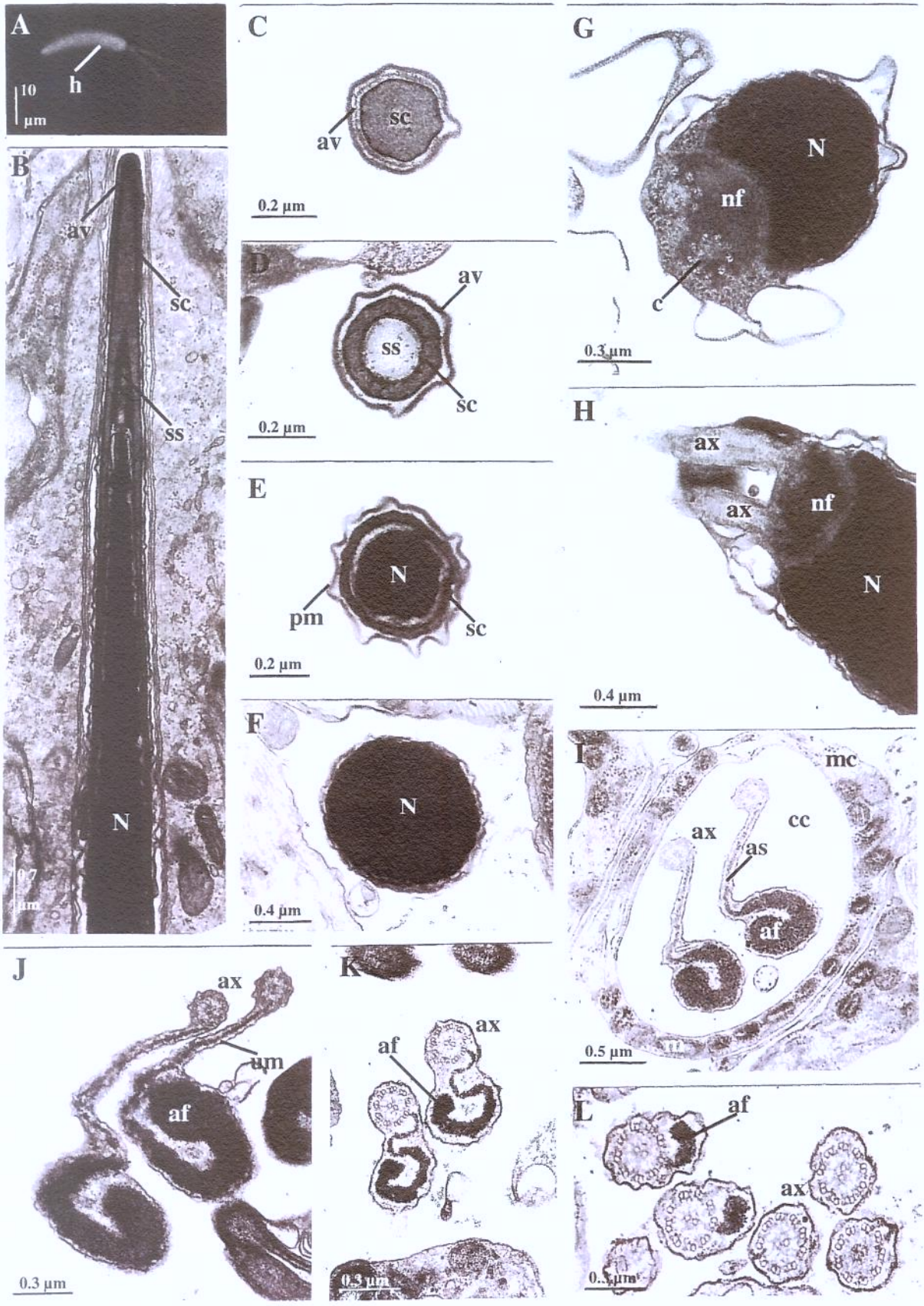
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Figure 2

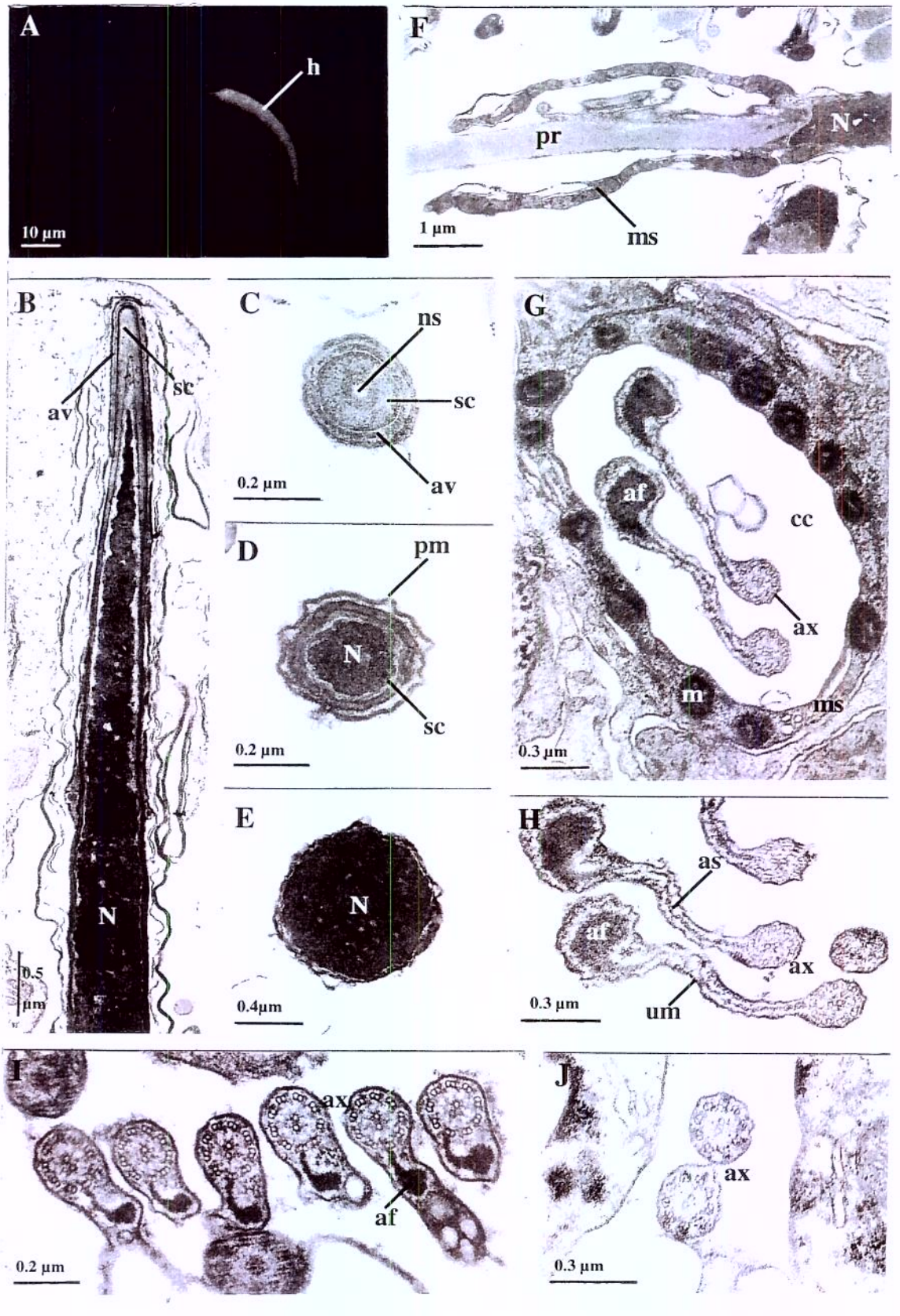
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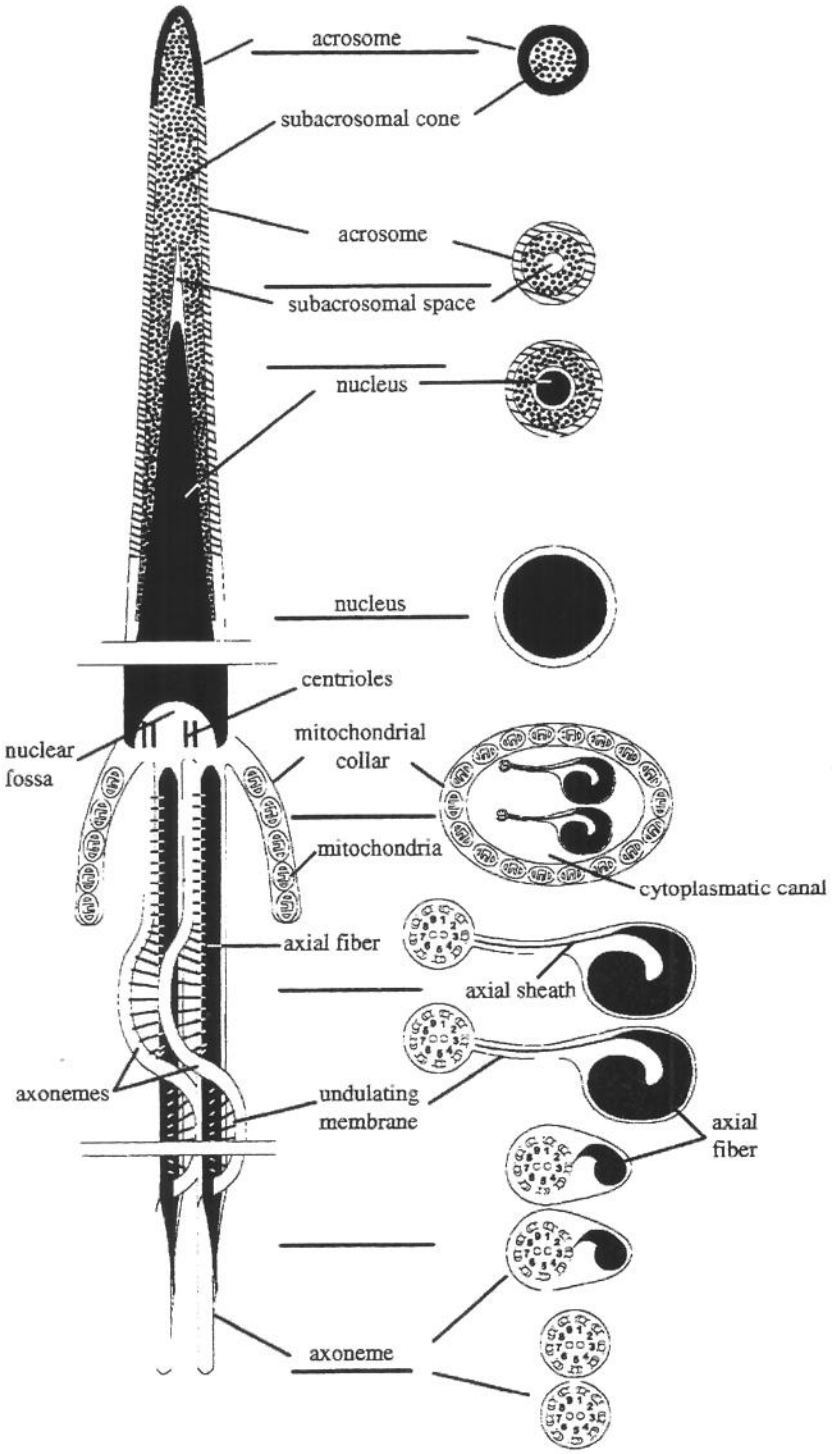
Figure 3


acrosome
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acrosome
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centrioles
nuclear fossa
mitochondrial collar
mitochondria
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axial fiber
axial sheath
axonemes
undulating membrane
axial fiber
axoneme

Figure 4

ARTIGO IV

Sperm morphology of five species of *Colostethus* (Anura, Dendrobatidae), with phylogenetic comments

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Keywords: Anura, Dendrobatidae, *Colostethus,* spermatozoon, ultrastructure

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Abstract

In this report, we describe the ultrastructure of sperm from *Colostethus brunneus, Colostethus* sp. (aff. *trilineatus*), *Colostethus nidicola, Colostethus* sp. and *Colostethus stepheni.* The general structures of the spermatozoa (acrosomal complex and flagellar apparatus) were shared with species of the Bufonoidea lineage. The main difference among the spermatozoa was the presence of a single flagellum in *Colostethus stepheni*, a characteristic previously unknown for this genus. In addition, the spermatozoa of this species showed some mitochondria within the undulating membrane, which was very thick. These characteristics appear to be common to dendrobatid species that have a single flagellum. In all spermatozoa except for those of *C. stepheni*, the slight differences in the nuclear length and in the extent of the acrosomal vesicle, as well as the occurrence of the nuclear space, corroborated the chromosomal data, and were useful for distinguishing these morphologically similar species. The results obtained here, together with molecular data, do not support the proposed regrouping of the "brunneus" and "alagoanus" groups in a monophyletic "trilineatus" group.

Introduction

The 128 species of nonpoisonous, cryptically colored dendrobatid frogs of the genus *Colostethus* have a widespread central Costa Rica to northern Peru and the Guianas and through the Amazon Basin to southeastern Brazil, in addition to the Caribbean island of Martinique (Frost 2004).

Although *Colostethus* is the largest genus of the family Dendrobatidae, the intrageneric relationships of this genus remain unclear. Some *Colostethus* species groups have been proposed by Lynch (1982), Rivero ("1988" 1990) and Rivero & Serna ("1988" 1989) based mainly on morphological data. However, according to Coloma (1995) and Grant *et al.* (1997), many of the hypothesized synapomorphic characters used to assemble the *Colostethus* species into different groups may represent symplesiomorphies or homoplasies. In addition, the groupings within the *Colostethus* genus have undergone some changes. Morphological studies by La Marca (1992, 1994) removed most of the species from two species groups (VII and VIII) proposed by Rivero ("1988" 1990), and elevated them to generic status (*Mannophryne* and *Nephelobates,* respectively). Recently, Morales (2000) reported a systematic review of the "brunneus" and "alagoanus" groups (groups II and III, respectively - *sensu* Rivero,"1988" 1990) and proposed unite them a new monophyletic group named "trilineatus". This new group contains various species, including *C. brunnues, C. marchesianus, C. trilineatus, C. stepheni, C. alagoanus, C. capixaba* and *C. carioca* (Morales, 2000).

Despite the large number of *Colostethus* species described so far, few species of this genus have been used in molecular studies of the Dendrobatidae. In general, *Colostethus* species have been represented as outgroups in phylogenetic reconstructions since this genus is considered a basal group (Summers *et al.* 1997, 1999; Clough & Summers, 2000). However, Vences *et al.* (2003) used a large number of *Colostethus* species in a phylogenetic analysis based on mitochondrial DNA sequences and showed that several well-defined groups of this contained aposematic species.

Controversy still surrounds the intrageneric relationships of the *Colostethus*, since morphological have shown that species from groups II and III (*sensu* Rivero,"1988" 1990)

compose the group "trilineatus" (Morales, 2000), whereas molecular data have shown that *Colostethus stepheni* (group III) is not closely related to *Colostethus* species from group II (Vences *et al.* 2003).

The ultrastructural analysis of sperm has been used as an additional source of data in taxonomic investigations of many anuran groups (Lee & Jamieson, 1993; Jamieson *et al.*, 1993; Kwon & Lee, 1995; Meyer *et al.*, 1997). The few studies of this type that have investigated the Dendrobatidae have been useful for clarifying inter- and intrageneric relationships (Aguiar-Jr *et al.* 2002, 2003; Garda *et al.* 2002).

In this work, we describe the sperm ultrastructure of *C. brunneus* from the type locality, of *Colostethus* sp. (aff. *trilineatus*) and *C. stepheni*, which represent the groups "brunneus" and "alagoanus", respectively, and of *Colostethus nidicola* and *Colostethus* sp. which have not been included in any species group. The data obtained in this study may be useful for understanding the intrageneric relationships of these *Colostethus* species.

Material and Methods

Five Brazilian *Colostethus* species were analyzed by light and transmission electron microscopy. Three specimens of *C. stepheni* were collected in Reserva Florestal Adolfo Ducke (RFAD), Manaus, in the state of Amazonas, (03°,08' S, 60°,04' W), five specimens of *C. brunneus* were obtained from the type locality, Chapada dos Guimarães, in the state of Mato Grosso (15°,16',00"S, 55°,31',52"W), four specimens of *C. nidicola* were from the municipality of Careiro, at km 12 on the road to Autazes, state of Amazonas (03°,37',10.4"S, 59°,86',78.4" W), four specimens of *Colostethus* sp. (aff. *trilineatus*) were from Rio Branco, in the state of Acre (09°,57' S, 67°,52' W) and three specimens of *Colostethus* sp. were from Santarém, in the state of Pará (Long – 54.84028, Lat – 3.14912).

All of the specimens were colleted by A. P. Lima under a permit issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (Proc. no. 02005.001367/99-58-AM). Voucher specimens were deposited in the Museu de História Natural "Professor Adão José Cardoso" (ZUEC), Universidade Estadual de

Campinas, Brazil, in the Célio F. B. Haddad collection (CFBH), Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, Brazil, and in the herpetological collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Brazil, under the following accession numbers: ZUEC 11625, 5638 and 5639 (*C. stepheni*); CFBH 05215; INPA 10122, 10135, 10147 and 10155 (*Colostethus brunneus*); ZUEC 13067, 12999, 13000 and 13002 (*Colostethus nidicola*); ZUEC 12990, 12992; INPA 11981 and 11982 (*Colostethus* sp. (aff. *trilineatus*)) and INPA 10170, 10163 and 10169. (*Colostethus* sp.).

Techniques

The testes of the specimens were removed and cut into small pieces. For light microscopy (LM), a suspension of spermatozoa was prepared in 0.1 M cacodylate buffer, pH 7.2, then spotted on to a glass slide and fixed in the same buffer containing 2% paraformaldehyde-glutaraldehyde, 5 mM $CaCl_2$ and 3% sucrose. The slides were stained in 0.2 µg of 4,6-diamino-2-phenylindole (DAPI)/ml for 15 min, rinsed in water, and placed in 0.1 M McIlvane buffer for 5 min. The slides were examined with an Olympus BX60 microscope and the measurement of head length was done using Image Pro-Plus software, version 3 (Media Cybernetics).

For transmission electron microscopy (TEM), fragments of the testes were fixed overnight at 4 °C in the fixative solution described above. After rising in 0.1 M sodium cacodylate buffer, pH 7.2, the samples were post-fixed for 1 h at 4 °C in 1% osmium tetroxide and 1.6% potassium ferricyanide in the same buffer, and left for 2 h in a solution of 0.5% uranyl acetate for "en block" contrast staining. The samples were then rinsed in distilled water, dehydrated in an increasing acetone series (50-100%) and embedded in Epon 812 resin. The ultra-thin sections were placed on copper grids and stained with uranyl acetate and lead citrate prior to examination with a Leo 906 transmission electron microscope.

Results

Light microscopy

The spermatozoa of the five species studied were filiform, with two complete flagella, except for *Colostehus stepheni,* which a single flagellum. The general structure of the testicular spermatozoa is shown in Figure 1A-B.

The average nuclear length of the biflagellate spermatozoa was similar among four species: *C. brunneus* (19.2 ± 2.3 μm, n = 10), *Colostethus* sp. (aff. *trilineatus*) (24.8 ± 1.5μm, n = 10), *C. nidicola* (23.8 ± 1.8 μm, n = 10) and *Colostethus* sp. (25.3 ± 1.8μm, n = 10), with *C. stepheni* having significantly longer nuclei (33 ± 2.5 μm, n = 10).

Electron microscopy

Ultrastructurally, the spermatozoa of *C. brunneus, Colostethus* sp. (aff. *trilineatus*), *C. nidicola* and *Colostethus* sp. were very similar so that only one description is provided, with the differences being emphasized when necessary. The spermatozoa of *Colostehus stepheni* are described separately.

Colostethus brunneus, Colostethus sp. (aff. *trilineatus*), *C. nidicola* and *Colostethus* sp.

In these species, the nucleus was conical in longitudinal sections (Figs. 2A,B, 3A, 4A,B and 5A), and circular in transversal sections (Figs. 2D-F, 3D-F, 4D-F and 5C, D). The acrosomal complex (acrosomal vesicle and subacrosomal cone) covered the anterior portion of the nucleus. The acrosome is a conical, membrane-bound vesicle filled with electron-dense material (Figs. 2B-E, 3A-C, 4A-C and 5B,C). This structure was not seen in cross-sections of the middle and basal portions of the nucleus.

Below the acrosomal vesicle, a subacrosomal cone composed of diffuse electron-dense material was observed. This cone extended beyond the limits of the acrosomal vesicle and narrowed progressively (Figs. 2C-E, 3B-E, 4C-E and 5B,C), but was not seen in transversal sections of the nuclear base. In *C. nidicola*, the subacrosomal cone was not separated from the nucleus by a nuclear space as in other biflagellate spermatozoa (Fig. 4A,C). Nuclear shoulders were not seen in the spermatozoa of the species analyzed. The

chromatin was not totally compacted in most spermatozoa and electron-lucent nuclear lacunae were occasionally seen (Fig. 4A,D).

The midpiece consisted of a nuclear fossa, one pair of centrioles, axonemes, mitochondria and paraxonemal rods (Figs. 2G, 3G, 4G and 5E). In spermatids of *C. nidicola*, the nuclear fossa was observed as a pronounced concavity that differed from the other species (Fig. 4G). In the midpiece region, a mitochondrial collar surrounded the anterior portion of the flagella (Figs. 2H, 3H, 4H and 5F).

In transversal sections, both of the flagella consisted of a 9 + 2 axoneme, an undulating membrane and an axial fiber. The axial fiber was curved or U-shaped, and was connected directly to the axoneme through the axial sheath (Figs. 2G-I, 3G-I, 4H,I and 5F,G). In the posterior portions of the tail, the undulating membrane was shortened and the axial fiber became closely associated with the axoneme (Figs. 2J, 3J,K, 4J and 5H). At the end of flagella, only the axonemes were seen (Figs. 2K, 3L, 4K and 5H).

Colostethus stepheni.

In general, the testicular spermatozoa of *C. stepheni* were similar to those of the *Colostethus* species described above, but differed in the structure of the tail. In longitudinal sections, the nucleus was elongate and curved, and in transversal sections it was circular (Fig. 6A,E). The acrosomal vesicle was filled with moderately electron-dense material and extended further down the anterior portion of the nucleus (Fig. 6A-D). Below the acrosome, a discreet, subacrosomal cone was observed, which also narrowed progressively and was not present in transversal sections of the nuclear base (Fig. 6B-D). There was no evident nuclear space. The high electron-density of the condensed chromatin indicated that the nucleus extended to the tip of the head (Fig. 6B).

Numerous electron-lucent lacunae were present (Fig.6A,B) and distinct nuclear shoulders were absent. Transversal sections showed that the nucleus increased in diameter throughout its length (Fig. 6C-E).

In the region of midpiece, the final portion of the nucleus had a pronounced concavity, or nuclear fossa in which lay the centrioles (Fig. 6F). The paraxonemal rod

(*sensu* Jamieson *et al.*, 1993) extended anteriorly into the neck region to the level of the distal centriole (Fig. 6F).

A mitochondrial collar surrounded the flagellum in the anterior portion of the tail of the spermatids (Fig. 6G). Some mitochondria were observed within the undulating membrane in the anterior portion of the tail (Fig. 6H), but no mitochondria were seen further down (Fig. 6H,I). The tail complex consisted of a 9 + 2 axoneme, an axial fiber and an undulating membrane, in which the axial sheath (*sensu* Garda *et al.*, 2002) connected the axoneme to the axial fiber. The axial fiber was not U-shaped and the undulating membrane and the axial sheath were thick (Fig. 6H-J).

The undulating membrane and the axial sheath shortened progressively, leaving the axial fiber and the axoneme close together (Fig. 6K,L). At the and of the flagellum, only the axoneme was observed (Fig. 6M).

Discussion

The biflagellate spermatozoa of *C. brunneus, Colostethus* sp. (aff. *trilineatus*), *C. nidicola* and *Colostethus* sp. were very similar to those of *Allobates femorales* and *Colostethus* sp. described by Aguiar-Jr *et al.* (2003). The spermatozoa of *Cololostehus stepheni* resembled those of *Epipedobates flavopictus, E. hahneli* and *E. trivittatus* (Garda *et al.*, 2002; Aguiar-Jr *et al.*, 2004) since some mitochondria were observed within the undulating membrane in the initial portion of the single flagellum in these species. The general structure of the spermatozoa of the five species analyzed here, was similar to that of other dendrobatids described by Garda *et al.* (2002) and Aguiar-Jr *et al.* (2003, 2004), and supported the suggested grouping of the Dendrobatidae family with in the Bufonoidea lineage, based mainly on the similarity of the flagellar apparatus and the structure of the acrosomal complex (Garda *et al.* 2002; Aguiar-Jr *et al.* 2004).

Recent molecular studies have suggested that the Dendrobatidae family have phylogentic relationships with the Bufonoidea rather than with the Ranoidea (Hegdes and Maxon, 1993; Hay *et al.* 1995; Ruvinsky and Maxon 1996; Vences *et al* 2000). The

presence of a conical acrosomal vesicle and subacrosmal cone (homologous with the subacrosomal cone of *Ascaphus truei* and the conical perforatorium of bufonoids - see Garda *et al.* 2002), as well as the flagellar apparatus, composed of an axoneme and accessory fibers within the undulating membrane, were considered a plesiomorphic condition (Kwon and Lee 1995; Jamieson 1999; Scheltinga and Jamieson 2003). Hence, these spermatological characteristics shared among dendrobatids (*Allobates, Colostethus* and *Epipedobates* species studied so far) and bufonoids not allow any phylogenetic relationships to be established

The presence of a mitochondrial collar is another similarity shared with bufonoid species, and could indicate close relationships among the dendrobatid and bufonoids since this structure was considered a synapomorphy by Lee and Jamieson (1993). Nevertheless, Scheltinga *et al.* (2001) described the presence of a poorly developed cytoplasmic collar and a rudimentary cytoplasmic canal in the spermatozoa of *Leiopelma hochstetteri* (Archeobatrachia), which was similar to that seen in the lungfish *Neoceratodus forsteri* (Jespersen 1971 *appud* Scheltinga and Jamieson 2003). Until the spermatozoa of *Leiopelma* were studied, no other species was known to have a mitochondria arrangement that could be regarded as plesiomoephic for the Anura. On the other hand, the well-developed mitochondrial collar, which may be lost at maturity (Garrido *et al.* 1989; Lee and Jamieson 1992; Pugin-Rios 1980; Pugin and Garrido 1981; Meyer *et al.* 1997), resembled a condition seen in many acanthopterygian fishes, and for this reason, appears to be plesiomorphic. However, a reversal to the pre-lissamphibian condition was also considered by Scheltinga *et al.* (2001).

In *C. stepheni,* some mitochondria were seen scattered within the undulating membrane in the anterior portion of the flagellum. This mitochondrial arrangement has already been observed in the dendrobatids *Epipedobates flavopictus* (Garda *et al.* 2002) and *E. hahneli* and *E. trivitatus* (Aguiar-Jr *et al.* 2004). Because of the uncertainties about the phylogenetic significance of the mitochondrial arrangement in anuran spermatozoa, we considered the presence of mitochondria within the undulating membrane in dendrobatids to be a similarity shared among species with a single flagellum. Moreover, mitochondria within the undulating membrane have been described in two other unrelated species,

Nimbaphrynoides occidentalis – a neobatrachian (Pugin-Rios 1980) and *Ascaphus truei* – an archeobatrachian (Jamieson *et al.* 1993). So far, the absence of mitochondria within the undulating membrane appears to be a common trait of the biflagellate spermatozoa of *Allobates femoralis* and *Colostethus* sp. (Aguiar-Jr *et al* 2003) and of the *Colostethus* species studied here.

In addition to the complete single flagellum, the spermatozoa of *C. stepheni* had a thick undulating membrane and axial sheath, that differed from the pattern described for the other *Colostethus* species analyzed here and for those studied by Aguiar-Jr *et al* (2003) and Veiga-Menoncello *et al.* (2001).

According to Scheltinga and Jamieson (2003), the widly separated axial and justaxonemal fibers that occur in many anurans are considered homologous with the paraxonemal rod observed in *Ascaphus*, as suggested by Jamieson *et al.* (2003), or with the simple unmodified axial fiber of other anurans. The axial fiber can be simple (circular in cross-section) or modified to be elongated and thick, or be divided into a juxtaxonemal fiber that may be reduced or absent, and joined by an axial sheath to the axial fiber. Modification of the axial fiber is considered by Scheltinga and Jamieson (2003) to be an apomorphic condition since in primitive urodeles and gymnophionans a simple axial fiber was observed in transversal sections of the sperm tail. In all dendrobatids studied so far, the undulating membrane is shorter and the juxtaxonemal fiber is absent, at least as a separate entity. Nevertheless, in *C. stepheni*, *Epipedobates falvopictus* (Garda *et al.* 2002), *E. hahneli* and *E. trivitatus*, the undulating membrane is very thick. This arrangement of the undulating membrane appears to be common in the species with a single flagellum.

The slight differences in nuclear length, in the extent of the acrosomal vesicle, and in the occurrence of the subacrosomal space in biflagellate spermatozoa of *C. brunneus, Colostethus* sp. (aff. *trilineatus*), *C. nidicola* and *Colostethus* sp., corroborated the chromosomal data, and were useful to for distinguishing these species, mainly based on differences in chromosomal number and chromosomal banding pattern (Veiga-Menoncello *et al.* 2003a; Veiga-Menoncello *et al.* – in prep.).

The spermatological results described here, and those reported by Garda *et al.* (2002) and Aguiar-Jr *et al.* (2003, 2004) were not very informative about the placement of

the Dendrobatidae within the Bufonoidea lineage originally proposed by Hay *et al.* (1995) and Vences *et al* (2000). Nevertheless, the structure of the acrosomal complex and the "bufonoid-like" flagellar apparatus suggest the exclusion of the dendrobatids from the ranoid lineage.

Our results did not support the proposed unification of the "brunneus" and "alagoanus" groups (*sensu* Rivero "1988" 1990) into a monophyletic "trilineatus" group proposed by Morales (2000). The distinctive presence of biflagellate spermatozoa in *A. femoralis* (Aguiar-Jr. *et al.* 2003) and in *C. brunneus* and *Colostethus* sp. (aff. *trilineatus*) appears to be phylogenetically informative. In a recent molecular study involving a large number of dendrobatid species (Vences *et al.* 2003), *A. femoralis* was considered a sister group of the clade formed by "brunneus" group species (*sensu* Rivero "1988" 1990), whereas *C. stepheni* was related to *Mannophryne* + *Nephelobates* species. If biflagellarity is considered a relationship character, in dendrobatids, then the "brunneus" group (*sensu* Rivero "1988" 1990) may be valid and the other biflagellate *Colostethus* species should be included in this group.

Acknowledgements

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Legends

Figure 1. Light microscopy. A. Representative biflagellate spermatozoa of *Colostethus brunneus*. B. Spermatozoon of *Colostethus stepheni* with a single flagellum. Bar = 10μm.

Figure 2. TEM of *Colostethus brunneus.* A and B. Longitudinal sections of the head. The acrosomal vesicle caps the anterior portion of the nucleus. Below this vesicle, a subacrosomal cone is separated from the nucleus by a subacrosomal space. C-F. Transversal sections of the acrosomal complex and nucleus showing a progressive narrowing of the subacrosomal cone. G. Longitudinal section through the midpiece showing the mitochondrial collar and the implantation of both axonemes in the nuclear fossa. H. The initial portion of the flagella is surrounded by the mitochondrial collar. I and J. Transversal sections of the tail showing narrowing of the axial sheath and the undulating membrane. K. Final portion of the flagella where only the axonemes are seen. Bars: A = 1.5 μm; B = 1.0 μm; G, H and I = 0.4 μm; C - F, J and K = 0.2 μm.

Figure 3. TEM of *Colostethus* sp. (aff. *trilineatus*). A. Longitudinal section of the head. B and C. Transversal sections of the initial portion of the head showing the acrosomal complex. D-F. Cross-section of the nucleus. Note the narrowing of the subacrosomal cone and the increase in nuclear diameter. G. Longitudinal section through the midpiece showing the axonemes. H. Transversal section of the proximal region of the midpiece showing the mitochondrial collar surrounding the flagella. I-K. Transversal sections through the flagella, showing the axial sheath, axial fiber and undulating membrane, all of which become narrower in the posterior portion. L. Final portion of the flagella in which only the axonemes are seen. Bars: A, E and F = 0.4 μm; C and D = 0.5 μm; B, G - L = 0.2 μm.

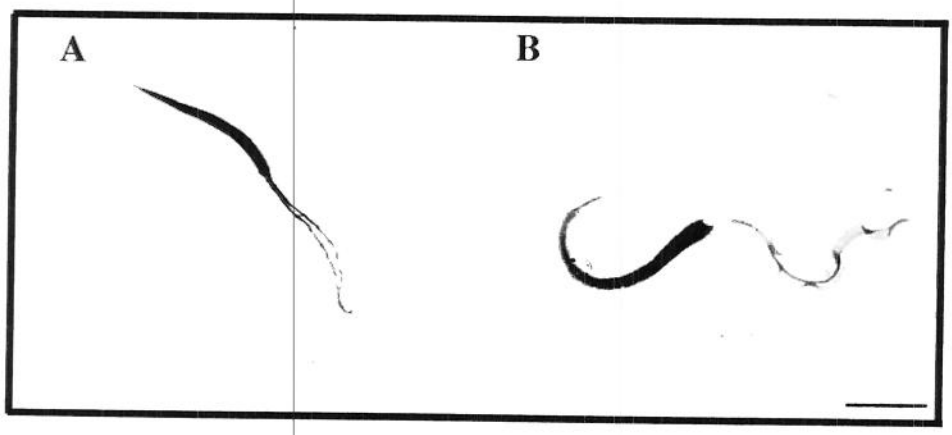
Figure 4. TEM of *Colostethus nidicola.* A and B. Longitudinal sections of the head. Note the absence of the nuclear space and the presence of some electron-lucent inclusions in the chromatin. C-F. Serial sections of the head showing progressive enlargement of the nucleus and reduction of the subacrosomal cone. G. Transversal section of a spermatid showing the

midpiece. Note the pronounced nuclear fossa where two centrioles are inserted and the presence of a mitochondrial collar. H. Transversal section of the proximal region of the midpiece, showing the mitochondrial collar surrounding the flagella. I. Transversal section of the tail showing the axial axoneme, accessory fibers and undulating membrane. J-K. Final portion of the tail showing narrowing of the undulating membrane and the axial sheath. Further down, only the axonemes are seen. Bars: G = 1.0 μm; A and B = 0.8 μm; E, F and H = 0.5 μm; C, D and I = 0.3 μm; J and K = 0.1 μm.

Figure 5. TEM of *Colostethus* sp. A. Longitudinal section of the head. B-D. Transversal sections of the acrosomal complex and the nucleus. Note the presence of a nuclear space in the anterior portion of the head, and the progressive narrowing of the subacrosomal cone, which is not seen in the posterior portion of the nucleus. E. Oblique section of the midpiece. Note the presence of parallel centrioles surrounded by a pericentriolar material. F. Transversal section of the initial portion of the tail. The mitochondrial collar surrounds the flagella, but is separated from it by the cytoplasmic canal. G and H. Distal and final portions of the tail. Bars: A = 1.5 μm; B - H = 0.3 μm.

Figure 6. TEM of *Colostethus stepheni*. A and B. Longitudinal sections of the head showing the acrosomal complex and the nucleus. Note the presence of a discreet subacrosomal cone. Some electron-lucent lacunae are seen in the chromatin. C-E. Transversal sections of the nucleus. Note the absence of the nuclear space and a progressive increase in the diameter of the nucleus. F. Longitudinal section through the midpiece showing the proximal centriole which is inserted deep into the nuclear fossa. G. Transversal section of a spermatid showing the presence of a mitochondrial collar surronding the flagellum. H-J. Transversal sections of the tail. In the anterior portion, some mitochondria are present within the undulating membrane, but were not seen in the posterior portion. Note the thickened axial sheath and undulating membrane. K-M. Final portion of the tail showing the narrowing of the axial sheath and undulating membrane, at the tip of the flagellum, only the axoneme is seen. Bars: A = 1.0 μm; B – E, G and H = 0.4 μm; F = 0.7 μm; I – M = 0.2 μm.

Abbreviations: **af** = axial fiber; **as** = axial sheath; **av** = acrosomal vesicle; **ax** = axoneme; **c** = centriole; **cc** = cytoplasmic canal; **f** = flagellum; **l** = lacuna; **m** = mitochondria; **mc** = mitochondrial collar; **N** = nucleus; **nf** = nuclear fossa; **ns** = nuclear space; **pm** = pericentriolar material; **pr** = paraxonemal rod; **sc** = subacrosomal cone; **um** = undulating membrane; **v** = vesicle.



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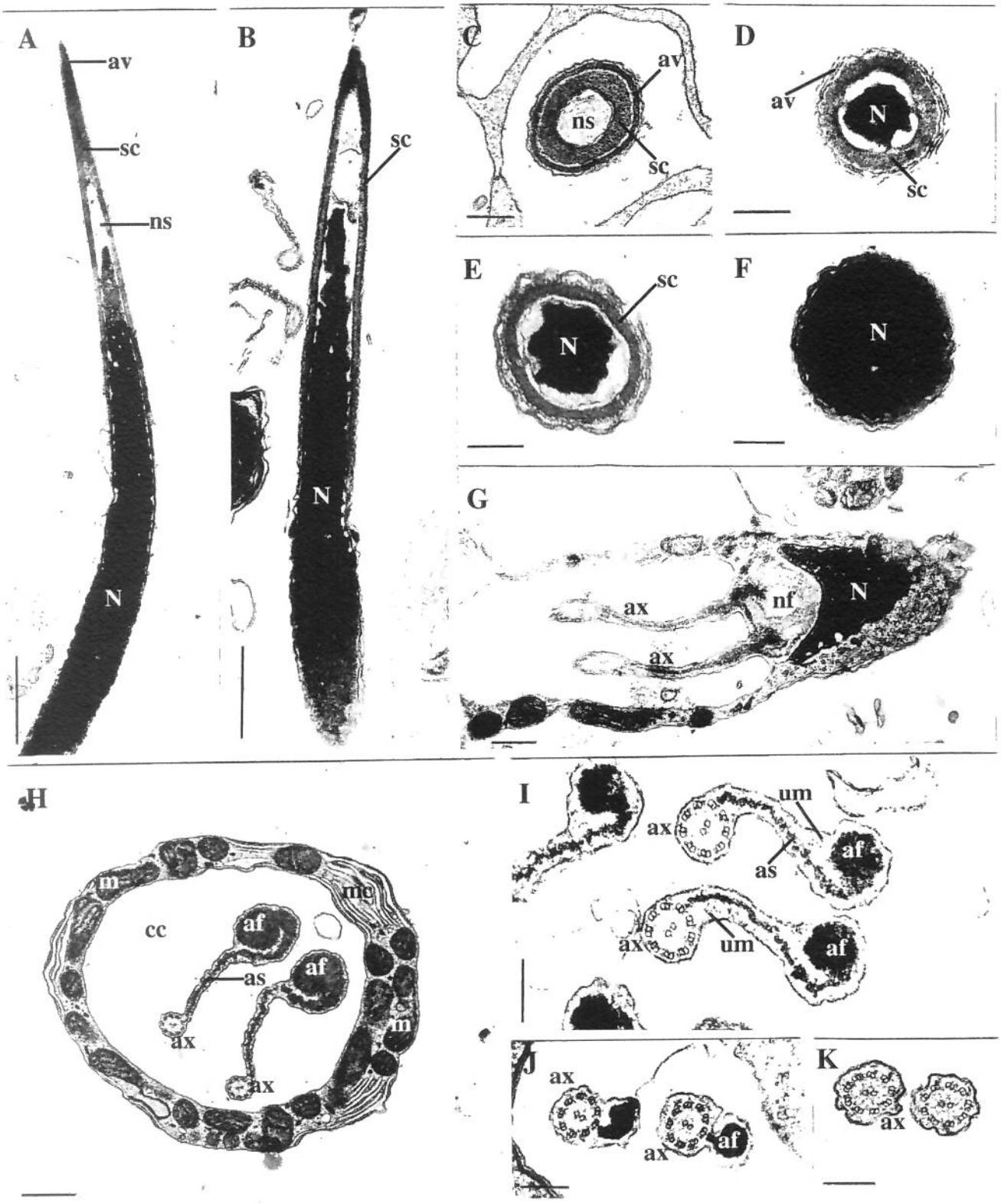
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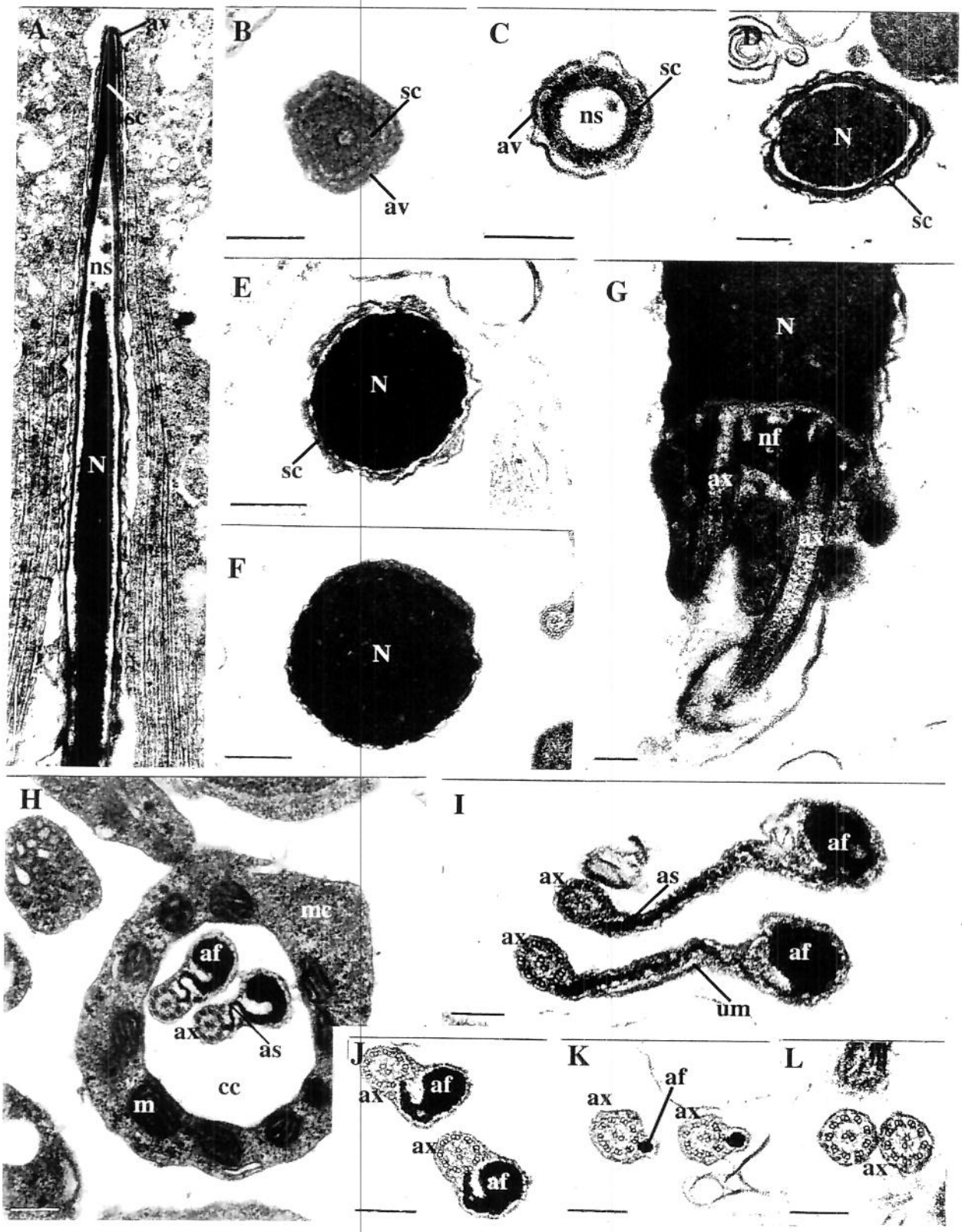
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Figure 3

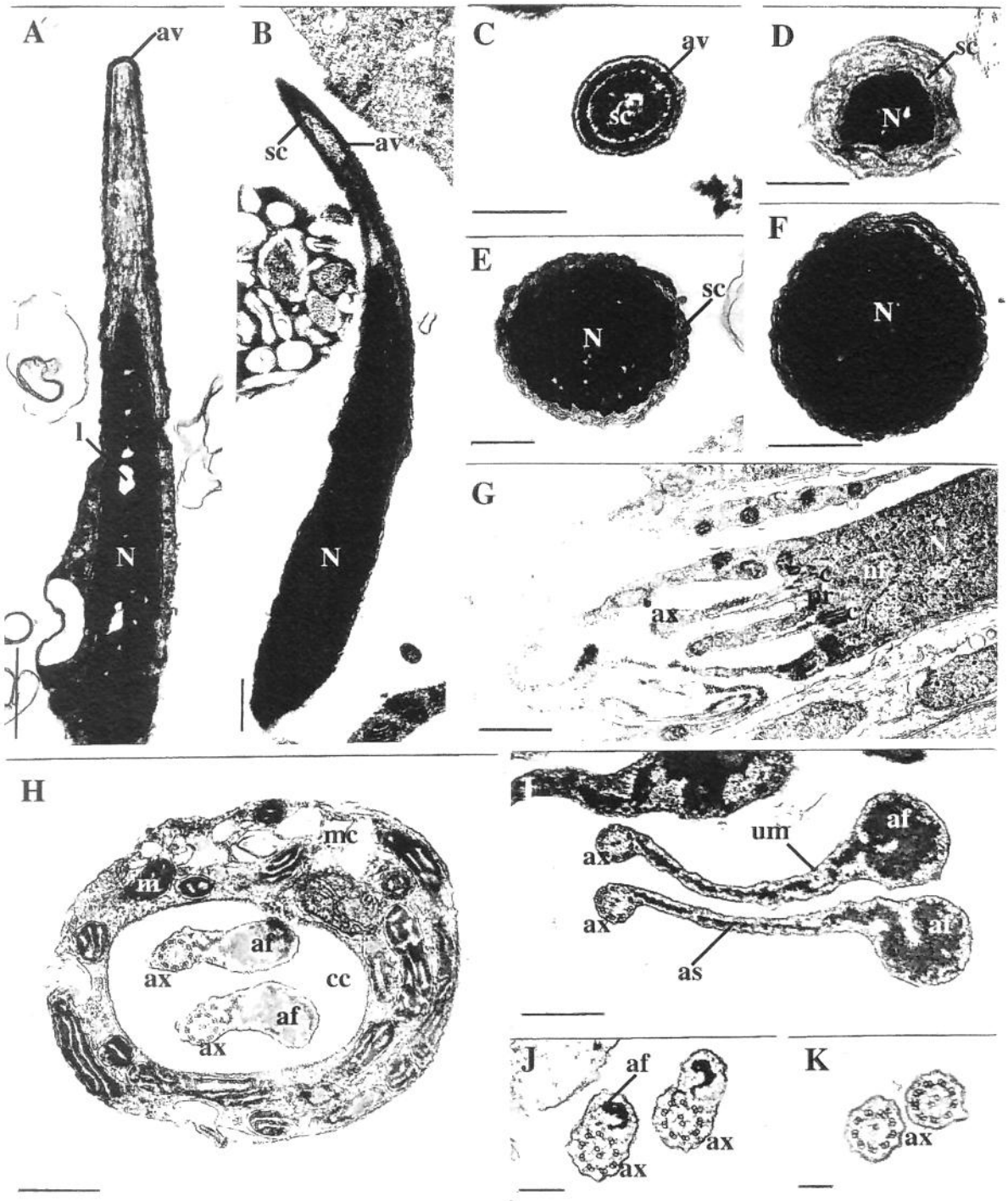
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Figure 4

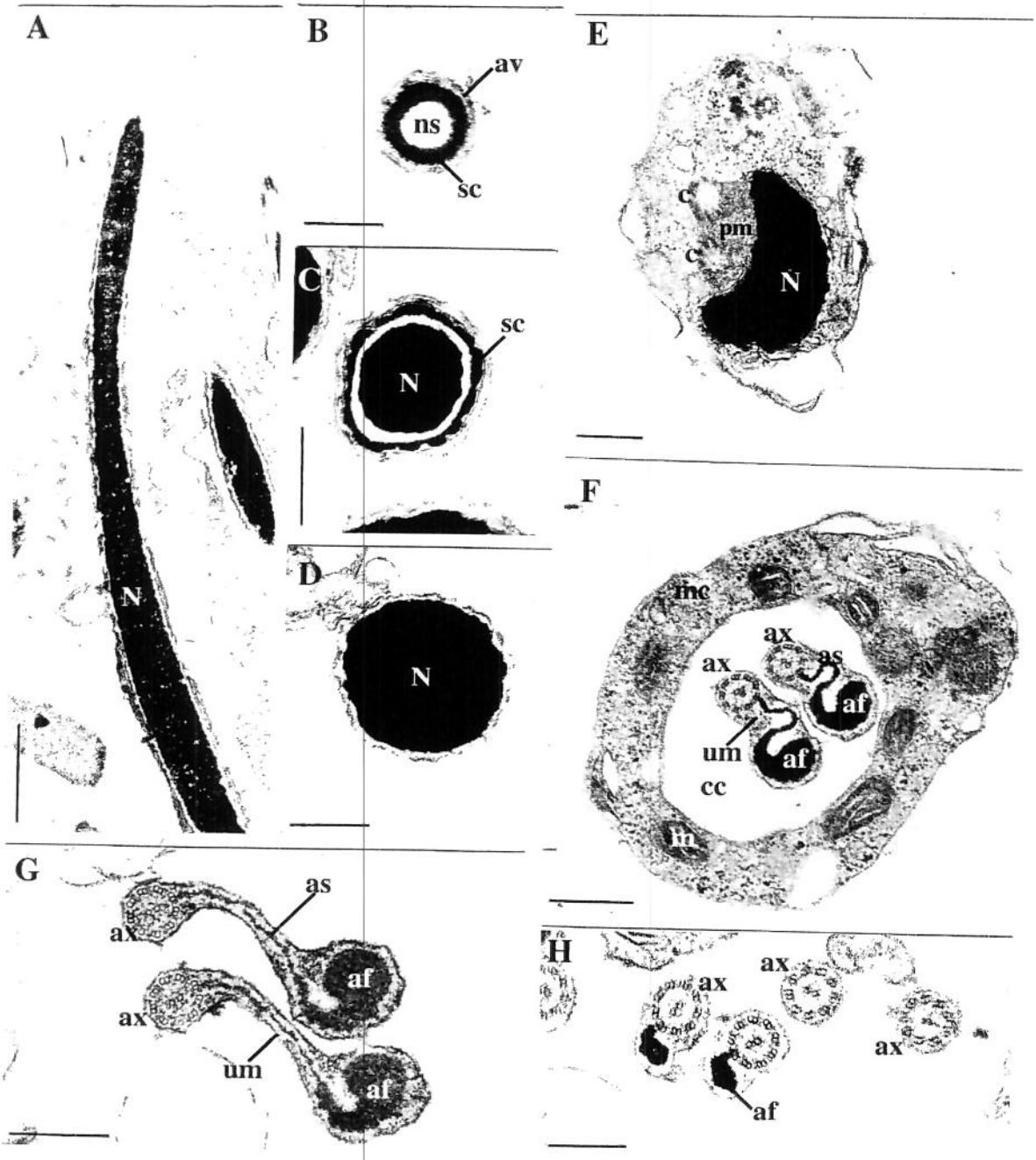
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Figure 5

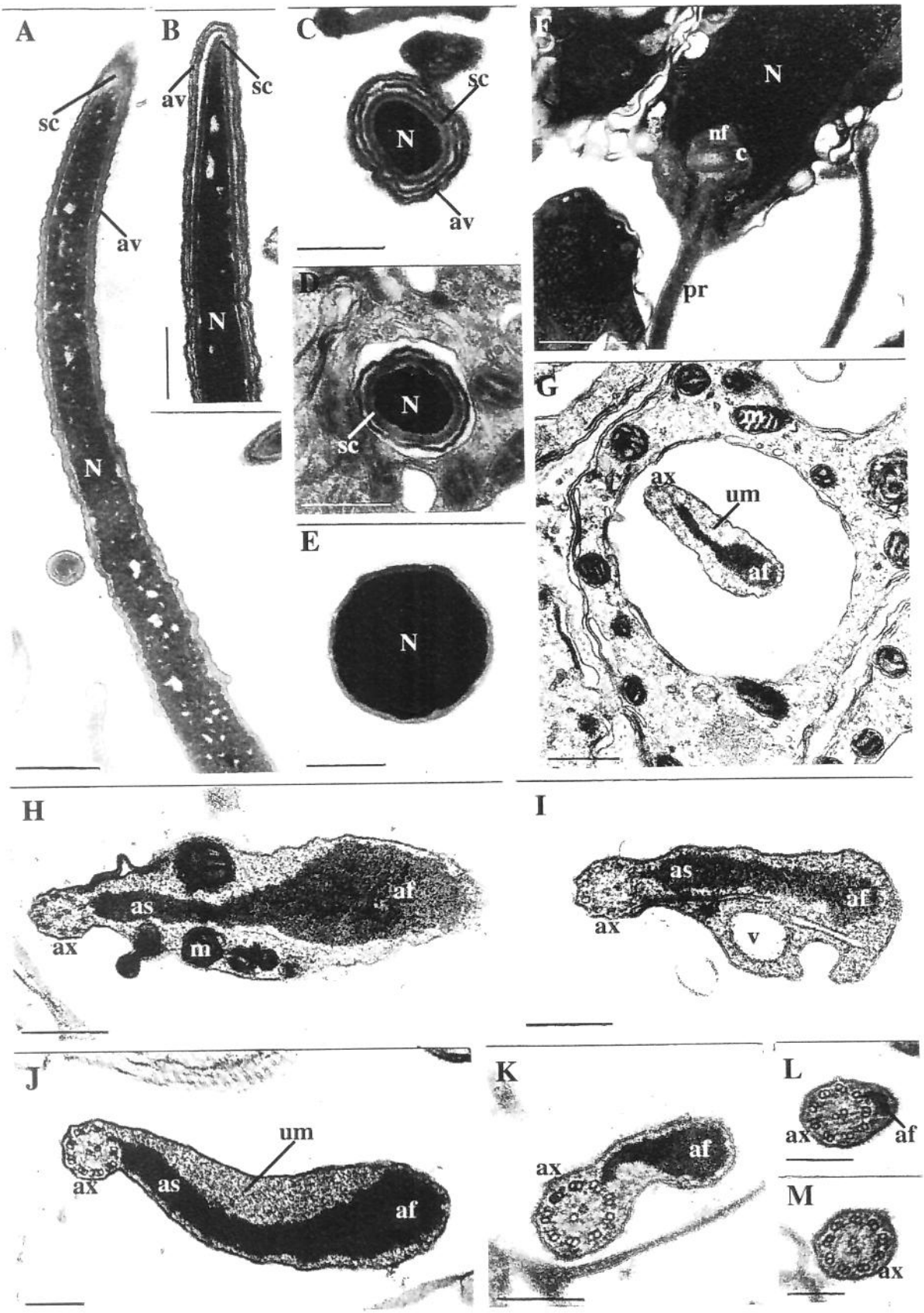
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Figure 6

4. CONCLUSÕES GERAIS

4. Conclusões Gerais

Os resultados obtidos para as espécies de *Colostethus* a partir da análise citogenética e da ultra-estrutura do espermatozóide contribuem para um melhor entendimento das relações de parentesco intragenéricas, através de dados referentes ao padrão de distribuição de heterocromatina e localização da NOR, assim como espermatológicos. Com os dados citogenéticos, foi possível detectar uma dicotomia em relação ao número de cromossomos para as espécies de *Colostethus* pertencentes ao grupo "brunneus" com 2n = 22 e 2n =24 (artigos I e II desta tese).

A variabilidade no número de cromossomos em *Colostethus*, embora pequena, e restrita a poucas espécies analisadas, contradiz a hipótese de Bogart (1991). Segundo o autor, na maioria dos gêneros de anuros que apresentavam características reprodutivas como desova com baixo número de ovos, cuidado parental mais elaborado, podendo envolver ambos os parentais e desenvolvimento terrestre e/ou direto, uma maior variabilidade cromossômica seria observada. Além disso, estas características reprodutivas estariam presentes em espécies com tendência a formar pequenos demes, o que facilitaria a fixação de mudanças cromossômicas. Nos dendrobatídeos analisados por Bogart (1991), variação no número de cromossomos foi encontrada apenas em espécies do gênero *Dendrobates* (*D. quinquivittatus, D. pumilio, D. histrionicus* com 2n = 20 e *D. truncatus* com 2n = 18 cromossomos). Este gênero foi tido pelo autor como o mais especializado, especialização esta correlacionada com a diminuição do número de cromossomos e a estratégia de vida. Por outro lado, os dados, embora empíricos, apontavam *Colostethus* como um gênero menos especializado, em função das espécies produzirem mais ovos, apresentarem uma ampla distribuição geográfica com grande tamanho populacional, menor cuidado parental, além de terem um número cromossômico mais elevado, e portanto, seria plausível que as espécies pertencentes a este gênero apresentassem menor variabilidade cariotípica. Desta forma, parece provável pelo menos no gênero *Colostehus,* que a evolução cariotípica não está diretamente associada a fatores relacionados à estratégia de vida.

Entre as espécies de *Colostethus* com 2n = 22 cromossomos do presente estudo, a morfologia cariotípica foi bastante semelhante, o que tornou difícil a separação entre elas

baseada apenas na coloração convencional de seus cromossomos. Porém, o emprego das técnicas de bandamento, além de ter possibilitado uma distinção mais segura, permitiu o reconhecimento de prováveis homeologias cromossômicas. A presença de uma banda heterocromática fracamente corada na região intersticial do braço longo do par 7, sugere uma possível proximidade entre elas. Além disso, a região organizadora do nucléolo (NOR) localizada no par 4 nas espécies *Colostethus marchesianus, Colostethus* sp.1 (aff. *marchesianus*), *Colostethus caeruleodactylus* e *Colostethus* aff. *trilineatus*, também pode ser considerada uma homeologia cromossômica. Embora a NOR em *Colostethus brunneus* tenha sido localizada no par 3, este é morfologicamente semelhante ao par 4 das outras espécies. O posicionamento dos pares 3 e 4 supostamente invertidos em *C. brunneus*, não invalida a hipótese de uma relação mais próxima entre estas espécies baseada na localização da NOR.

Com relação as outras espécies brasileiras de *Colostethus* com 22 cromossomos, (*Colostethus nidicola* – Veiga-Menoncello *et al,*. 2003; *Colostethus* sp. 2 aff. *marchesianus* e *Colostethus* sp. – artigos I e II desta tese), estas apesar de compartilharem a banda C na região intersticial do par 7, diferiram quanto a posição e localização da NOR, indicando a ocorrência de rearranjos cromossômicos envolvendo esta região, os quais possivelmente contribuíram para a diferenciação destas espécies. *Colostethus nidicola* difere ainda, quanto à morfologia do par 7 o que reforça a hipótese levantada acima.

Os dados citogenéticos obtidos para *Colostethus marchesianus* e *C. brunneus* provenientes de suas localidades-tipo foram muito importantes para definir cromossomicamente estas espécies. Além disso, possibilitou a distinção das unidades taxonômicas, *Colostethus* sp. 1 (aff. *marchesianus*), *Colostethus* sp. 2 (aff. *marchesianus*) (artigo I desta tese) e *Colostethus* sp (artigo II desta tese), as quais por apresentarem grande semelhança morfológica, são informalmente referidas como *C. marchesianus* e *C. brunneus.*

No que diz respeito às relações intragenéricas de *Colostethus,* os dados citogenéticos foram pouco informativos em função da variabilidade no número de cromossomos encontrado nas espécies estudadas do grupo "brunneus". *Colostethus stepheni* (Veiga-Menoncello *et al.,* 2003) apresentou 2n = 24 cromossomos, assim como *C.*

brunneus. Porém, de acordo com os dados obtidos através da análise filogenética molecular (Vences *et al.*, 2003), *C. stepheni* seria mais aparentado com as espécies do gênero *Mannophryne* ou *Nephelobates*, do que com os membros do grupo "brunneus". Da mesma forma, *Colostethus chalcopis* apresentou 2n = 22 cromossomos (Kaiser *et al.*, 2003), assim como as espécies *C. marchesianus, C. caeruleodactylus, Colostethus* sp. 1 (aff. *marchesianus*), *Colostethus* sp. 2 (aff. *marchesianus*), *Colostethus* sp. (aff. *trilineatus*), *Colostethus* sp. e *C. nidicola* (artigos I e II desta tese; Veiga-Menoncello *et al.*, 2003). No entanto, de acordo com similaridades morfológicas observadas, Kaiser *et al.* (1994, 2003) sugerem que *C. chalcopis* provavelmente seja relacionada aos dendrobatídeos do gênero *Mannophryne*. Sendo assim, faz-se necessária uma análise mais refinada, a fim de tentar obter um maior esclarecimento das relações de parentesco entre estas espécies e entre os gêneros de dendrobatídeos.

Por outro lado, os resultados obtidos a partir da análise da ultra-estrutura do espermatozóide podem ser considerados, pelo menos até o momento, mais informativos para questões intragenéricas.

Os espermatozóides das espécies *Colostethus marchesianus, Colostethus* sp. (aff. *marchesianus*) (artigo III desta tese), *Colostethus brunneus, Colostethus nidicola, Colostethus* sp. (aff. *trilineatus*) e *Colostethus* sp. (artigo IV desta tese) apresentaram dois flagelos completos, enquanto *C. stepheni* (artigo IV desta tese) apresentou um único flagelo.

Com relação às espécies biflageladas, as pequenas diferenças encontradas na ultra-estrutura dos espermatozóides, bem como nas dimensões do núcleo, corroboraram os dados citogenéticos, na distinção entre as espécies. Portanto, as variações espermatológicas podem também ser consideradas como auxiliares na identificação dos táxons, principalmente no contexto de determinar se as diferentes populações podem representar espécies distintas.

Até o momento ainda não se conhece o significado filogenético da biflagelaridade. No entanto, a presença de espermatozóide biflagelar parece ser mais freqüente na família Dendrobatidae. Como mencionado anteriormente, os estudos filogenéticos moleculares (Vences *et al,*. 2003; La Marca *et al.*, 2002) mostram que algumas espécies de *Colostethus*,

entre elas *C. marchesinaus* e *C. trilineatus,* são mais relacionadas com o gênero *Allobates*, enquanto que *C. stepheni* provavelmente é mais relacionado às espécies do gênero *Mannophryne* ou *Nephelobates*. Desta forma, a presença de espermatozóides com dois flagelos em *Allobates femoralis* e *Colostethus* sp. (Aguiar-Jr *et al.,* 2003) e em *Colostethus marchesianus, Colostethus* sp. (aff. *marchesianus*) (artigo III desta tese), *Colostethus brunneus, Colostethus nidicola, Colostethus* sp. (aff. *trilineatus*) e *Colostethus* sp. (artigo IV desta tese), parece até o momento corroborar os relacionamentos obtidos através de estudos moleculares.

Sendo assim, os resultados da ultra-estrutura do espermatozóide não estão de acordo com a fusão dos grupos "brunneus" e "alagoanus" (grupo "trilineatus") como propôs Morales em 2000, e sugerem então, que o grupo "brunneus" seja mantido. Porém, um estudo adicional da ultra-estrutura do espermatozóide de espécies dos gêneros *Mannophryne* e *Nephelobates*, bem como de *Colostethus chalcopis* poderá contribuir para o esclarecimento das relações entre estes táxons, revelando se a presença de dois flagelos é uma condição comum somente às espécies pertencentes ao clado formado por membros do grupo "brunneus", cujo provável grupo irmão são as espécies do gênero *Allobates* (veja Vences *et al.,* 2003). Além disso, uma análise molecular também poderá propor um posicionamento para *C. chalcopis*, visto que esta espécie possui características cromossômicas similares aos *Colostethus* com 2n = 22, e morfologia externa semelhante aos *Manophryne*.

Os resultados obtidos nesta tese indicam a necessidade de uma revisão do gênero *Colostethus,* uma vez que os dados citogenéticos sugerem um relacionamento entre as espécies analisadas, porém algumas delas não estão alocadas formalmente em nenhum grupo. Da mesma maneira, os dados de ultra-estrutura do espermatozóide revelaram uma variabilidade intragenérica em relação ao número de flagelos. Até o momento, o número de flagelos presentes nos espermatozóides das espécies de Dendrobatidae estudadas está de acordo a proposta das relações de parentesco intra- e intergenérica obtida através da análise filogenética molecular, a qual sugere a possibilidade de futuras divisões dentro do gênero *Colostethus*, visto que muitas de suas espécies estão mais aparentadas com membros de outros gêneros da família.

4.1. Referências Bibliográficas

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