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"ANÁLISE DE ESPÉCIES BRASILEIRAS DE TERRARANA (AMPHIBIA: ANURA) UTILIZANDO ESTUDOS CROMOSSÔMICOS E DA ULTRA-ESTRUTURA DO ESPERMATOZÓIDE"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) SÉRGIO SIQUEIRA JÚNIOR

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Sumário

I. Resumo

Terrarana é atualmente o maior táxon da ordem Anura, possuindo cerca de 900 espécies. Recentemente, esse grupo de anuros foi alvo de uma série de modificações taxonômicas. Primeiramente essas espécies foram removidas da família Leptodactylidae e colocadas na sinonímia de Brachycephalidae, e mais tarde alocadas em um novo táxon chamado Terrarana. Atualmente as espécies deste grupo estão distribuídas nas famílias, Eleutherodactylidae, Craugastoridae, Strabomantidae e Brachycephalidae. Embora tenha sido bastante estudado, alguns dos relacionamentos inter- e intragenéricos permanecem não esclarecidos. Devido ao baixo número de espécies amostradas nos estudos filogenéticos, os clados da América do Sul, Strabomantidae e Brachycephalidae, são os grupos que têm as relações filogenéticas menos desvendadas. Neste trabalho foram realizados estudos citogenéticos e da ultra-estrutura dos espermatozóides de algumas espécies de Terrarana dos gêneros, *Pristimantis*, *Ischnocnema*, *Barycholos*, *Haddadus* e *Brachycephalus*. As metáfases foram obtidas por suspensão do epitélio intestinal e coradas com Giemsa ou submetidas a técnicas de impregnação por prata (Ag-NOR) e hibridação *in situ* (FISH) para a detecção das regiões organizadoras do nucléolo e bndamento-C, para a localização da heterocromatina. Os dados da ultra-estrutura dos espermatozóides foram analisados ao microscópio eletrônico de transmissão. As espécies estudadas foram *Pristimantis fenestratus* (Borba e Manaus, Reserva Florestal Adolfo Ducke, Amazonas e Rio Branco, Acre), *P. dundeei* (Chapada dos Guimarães, Rondonópolis e Aripuanã, Mato Grosso), *P. crepitans* (Chapada dos Guimarães e São Vicente, Cuiabá, Mato Grosso), *Ischnocnema paulodutrai* (Ilhéus, Bahia); *I. juipoca* (Atibaia e Campos do Jordão, São Paulo)*, I. guentheri* e *I. parva* (Mogi das Cruzes, São Paulo), *Brachycephalus ephippium* (Mogi das Cruzes e Atibaia, São Paulo, *Barycholos ternetzi* (Uberlândia, Minas Gerais e Porto Nacional, Tocantins), e *Haddadus binotatus* (Mogi das Cruzes e Ilhabela, São Paulo). Os dados citogenéticos foram importantes na caracterização cromossômica das espécies *P. fenestratus* 2n=34 cromossomos, *P. crepitans* 2n=22, *P. dundeei* 2n=28 e *I. paulodutrai* 2n=30 e ainda *B. ternetzi* e *I. juipoca* 2n=22. Os dados citogenéticos revelaram diferenças no bandamento-C e Ag-NOR, sugerindo a existência de espécies ainda não descritas de *Pristimantis* nas localidades de Manaus, Rio Branco, e Aripuanã, e de *Barycholos* (Tocantins) identificadas incorretamente como *P. fenestratus, P. dundeei* e *B. ternetzi.* A análise citogenética indicou ainda que o baixo número cromossômico em *P. crepitans* é único dentro do gênero e sugere que esta a mesma não seja proximamente relacionada a

espécies congenéricas. As similaridades cariotípicas entre *I. paulodutrai* e *P. dundeei* indicam que estas espécies podem ser proximamente relacionadas. Além disso, foi encontrado um caso incomun de variação intra-individual cromossômica no número fundamental de células somáticas de *P. fenestratus*, que pode estar relacionada com a segregação anômala de cromatides irmãs durante a mitose. A ultra-estrutura dos espermatozóides das espécies brasileiras de *P. fenestratus, P. dundeei, P. crepitans, B. ternetzi*, *B. ephippiun, I. guentheri, I. parva, I. juipoca* e *H. binotatus* revealaram diferenças na estrutura básica do acrossomo, peça intermediária e flagelo, apresentando características distintas dentro da família Brachycephalidae e subfamília Strabomantinae, assim como dentro do gênero *Pristimantis.* Como característica marcante da ultra estrutura dos espermatozóides, observou-se a presença de um bastão paraxonemal em *H. binotatus, B. ephippium, B. ternetzi* e *P. crepitans* e a ausência desta estrutura em *P. fenestratus, P. dundeei* e todos os *Ischnocnema* amostrados. A divergência ultra-estrutural no bastão paraxonemal é coerente com dados recentes de filogenia molecular, que suportam transferência de *H. binotatus* do gênero *Ischnocnema* para um novo táxon e também agrupa as espécies *I. guentheri, I. parva* e *I. juipoca* em um mesmo clado. Além disso, a ultra-estructura dos espermatozóides indicou que *B. ephippium* não é proximamente relacionado as espécies de *Ischnocnema*, e que a taxonomia das espécie de Brachycephalidae deve ser reexaminada. As análises da ultra-estructura dos espermatozóides também corroboram os dados cromossômicos que indicam que *P. crepitans* estão erroneamente alocados no gênero *Pristimantis* e que as espécies *I. paulodutrai* e *P. dundeei* são proximamente relacionadas. Apesar do pequeno número de espécies amostradas, os dados citogenéticos e da ultra-estrutura dos espermatozóides de espécies das três famílias dos Terrarana do Brazil, apresentaram características inter- e intragenéricas não conservadas, levantamento de novos questionamentos e fortes evitências da existência de novas espécies.

II. Abstract

The Terrarana is the largest taxon of the Anura order, comprising 900 species. Recently this anuran group has undergone a series of taxonomical rearrangements. At first, these species were removed from the Leptodactylidae family and transferred to the synonymy of Brachycephalidae, and lately they were placed into a new taxon named Terrarana. Currently, the Terrarana species are distributed in the families, Eleutherodactylidae, Craugastoridae, Strabomantidae and Brachycephalidae. Despite being intensively studied, many aspects of their inter- and intrageneric relationships remain unclear. The South American Strabomantidae and Brachycephalidae are the least known clades regarding molecular phylogeny. In the present work, Brazilian Terrarana species of *Pristimantis*, *Ischnocnema*, *Barycholos*, *Haddadus* and *Brachycephalus* were studied by means of cytogenetical and sperm ultrastructural characteristics. Metaphases obtained from suspensions of intestinal epithelium were stained with Giemsa or submitted to silver staining (Ag-NOR) and fluorescence *in situ* hybridization (FISH) techniques, in order to detect the nucleolus organizing region, and to C-banding for heterochromatin localization. Sperm ultrastructure was analyzed by transmission electron microscopy. The studied species were *P. fenestratus* (Borba and Manaus at Reserva Florestal Adolfo Ducke, Amazonas, and Rio Branco, Acre), *P. dundeei* (Chapada dos Guimarães, Rondonópolis, and Aripuanã, Mato Grosso), *P. crepitans* (Chapada dos Guimarães and São Vicente, Cuiabá, Mato Grosso), *Ischnocnema paulodutrai* (Ilhéus, Bahia), *I. juipoca* (Atibaia and Campos do Jordão, São Paulo), *I. guentheri* and *I. parva* (Mogi das Cruzes, São Paulo), *B. ephippium* (Mogi das Cruzes and Atibaia, São Paulo), *Barycholos ternetzi* (Uberlândia, Minas Gerais, and Porto Nacional, Tocantins), and *Haddadus binotatus* (Mogi das Cruzes and Ilhabela, São Paulo). The cytogenetical analyses allowed chromosomal characterization of the species *P. fenestratus* 2n=34 chromosomes, *P. crepitans* 2n=22, *P. dundeei* 2n=28 and *I. paulodutrai* 2n=30, which have not been previously analyzed in phylogenetical studies, in addition to *B. ternetzi* and *I. juipoca* 2n=22. The data revealed differences in C-banding and Ag-NOR, which indicated the existence of not yet described *Pristimantis* species in the Manaus, Rio Branco, and Aripuanã localities, and of *Barycholos* (from Tocantins) uncorrectly identified as *P. fenestratus, P. dundeei* and *B. ternetzi.* The cytogenetical analysis also indicates that the low chromosome number in *P. crepitans* is unique within this genus and suggests that this species is not closely related to its congeneric species. The karyotypical similarities between *I. paulodutrai* and *P. dundeei* indicate that these species could

be close relatives. Moreover, an unusual case of intra-individual chromosomal variation in the fundamental number of several somatic cells was found in the *P. fenestratus* species, which may be a consequence of anomalous segregation of sister chromatids during mitosis. The sperm ultrastructure of the Brazilian species *P. fenestratus, P. dundeei, P. crepitans, B. ternetzi*, *B. ephippiun, I. guentheri, I. parva, I. juipoca* and *H. binotatus* revealed differences in the basic structures of the acrosome, midpiece and flagellum, showing distinct characteristics within the family Brachycephalidae and subfamily Strabomantinae, as well as within the genus *Pristimantis.* The most remarkable sperm characteristic was an axonemal fiber in *H. binotatus, B. ephippium, B. ternetzi* and *P. crepitans,* which has not been observed in *P. fenestratus, P. dundeei* and in all of the analyzed *Ischnocnema* specimens. The ultrastructural divergence in the axonemal fiber is in accordance with recent molecular data, which supported the transfer of *H. binotatus* from the *Ischnocnema* genus to a new taxon and also grouped the species *I. guentheri, I. parva* and *I. juipoca* in a same clade. In addition, the sperm ultrastructure showed that *B. ephippium* is not closely related to the *Ischnocnema* species, and that the taxonomy of the Brachycephalidae species should be reexamined. Furthermore, the sperm ultrastructure analysis corroborated chromosomal data indicating that *P. crepitans* is misallocated in the *Pristimantis* genus and that the *I. paulodutrai* and *P. dundeei* species are close relatives. Despite the small number of studied species, the cytogenetical and ultrastructural data on sperm ultrastructure of the three Brazilian Terrarana families revealed inter- and intrageneric non-conservative characteristics that generate new taxonomic questions within this anuran group and provide strong evidence of the existence of undescribed species in this taxon.

III. Introdução

1. Classe Amphibia e Ordem Anura

O número das espécies descritas de anfíbios cresceu enormemente nos últimos anos, com um aumento de cerca de 50% desde 1985, refletindo a facilidade crescente de coletas em lugares remotos e um crescimento significativo de comunidades científicas ativas em vários países (Frost *et al*. 2006). Infelizmente, o aumento rápido no conhecimento da diversidade de anfíbios coincide com o declínio maciço e global de suas populações (Alford & Richards 1999; Stuart *et al*. 2004; Young *et al*. 2001) devido a uma diversidade de fatores que incluem a fragmentação e a perda de habitat (Alford *et al*. 2007; Smith & Green 2005), além das mudanças ambientais globais (Blaustein & Kiesecker 2002; Donnelly 1998) e doenças infecciosas emergentes (Collins & Storfer 2003; Toledo *et al*. 2007).

Atualmente, na classe Amphibia existem cerca de 6200 espécies distribuídas em três ordens: Caudata, que reúne as salamandras com aproximadamente 560 espécies, Gymnophiona que representa as cobras-cegas, com cerca de 170 espécies, e a ordem Anura, representada pelas rãs, sapos e pererecas, constituída atualmente por cerca de 5600 espécies descritas (Frost 2008). Os Anura são ainda subdivididos em três subordens: Archaeobatrachia, que inclui quatro famílias de rãs consideradas mais primitivas; Mesobatrachia, que inclui seis famílias de rãs em um nível evolutivo intermediário, e Neobatrachia, o maior grupo, englobando cerca de 95% do total de espécies de anuros (Ford & Cannatella 1993). Esta classificação é baseada em características morfologicas como o número de vértebras, estrutura do cinturão peitoral, e morfologia dos girinos (Frost *et al*. 2006).

De acordo com Duellman & Trueb (1986) e Frost (2008), em contraste com o número limitado de espécies e distribuição geográfica restrita das salamandras e cobras-cegas, os anuros ocorrem em todos os continentes, exceto na Antártida e algumas ilhas oceânicas, apresentando 77% de suas famílias com distribuição tropical e 14% em áreas subtropicais do hemisfério Norte.

Apesar do grande número de espécies viventes e da diversidade de habitats, os anuros variam pouco morfologicamente, o que torna as investigações filogenéticas baseadas exclusivamente nestes caracteres extremamente difíceis (Hillis & Dixon 1991). A compreensão geral sobre a filogenia de anfíbios anuros progrediu substancialmente desde os primeiros trabalhos dos anos 60 (exemplos, Inger 1967; Kluge & Farris 1969), e os principais avanços na

taxonomia de anuros, ainda baseados na classificação fenotípica, aconteceram entre os anos 80 e 90. Entretanto, até o fim da década de 90, apenas as relações entre as famílias basais estavam bem estabelecidas (ver referênncias em Frost *et al*. 2006).

A partir do ano 2000, estudos baseados em análises principalmente moleculares com acréscimo de quantidades significativas de caracteres, tiveram grande contribuição no entendimento das relações filogenéticas, principalmente entre os neobatráquios (exemplo, Biju & Bossuyt 2003; Darst & Cannatella 2004; Faivovich 2002; Faivovich *et al*. 2004). Uma das mais importantes contribuições foi realizada por Frost *et al*. (2006) e Grant *et al.* (2006) que fizeram extensos estudos englobando todas ordens de anfíbios e praticamente todas as famílias de anuros, fornecendo um mapeamento geral dos relacionamentos filogenéticos e embasamentos para estudos subsequentes de grupos de anfíbios não amostrados anteriormente por dados moleculares, como é o caso dos trabalhos de Heinicke *et al*. (2007) e Hedges *et al*. (2008).

2. Os "eleutherodactyline" e o táxon Terrarana

A partir do fim da década de 50 até o ano de 2006, os "eleutherodactyline" (sensu Frost *et al.* 2006) foram alvo de uma série de inferências taxonômicas, sofrendo algumas reestruturações baseadas apenas em caracteres morfológicos, sem que houvesse algum trabalho que esclarecesse completamente a sistemática deste grupo que, inicialmente, pertencia a subfamília Eleutherodactylinae (família Leptodactylidae) (Lynch 1971). Entretanto, a diversidade e heterogeneidade dos *Eleutherodactylus* indicavam que a manutenção deste grupo de anuros em um único gênero é reflexo de uma pobre compreensão dos seus relacionamentos (Heinicke *et al*. 2007). As relações desse táxon mudaram a partir de análises de dados de filogenia molecular no decorrer dos últimos anos, indicando que família Leptodactylidae não é monofilética e que os "eleutherodactyline" e o gênero *Brachycephalus* pertencem a uma linhagem separada de anuros neobatráquios (Ruvinsky & Maxson, 1996; Darst & Cannatella, 2004, Frost *et al*. 2006).

Para garantir uma taxonomia coerente com os princípios da filogenia, Frost *et al*. (2006), alocaram Eleutherodactylinae Lutz, 1954, em sinonímia com Brachycephalidae Gunther, 1858, corroborando o trabalho de Darst & Cannatella (2004), e dividiu *Eleutherodactylus* em cinco gêneros, *Euhyas*, *Craugastor, Pelorius*, *Syrrhopus* e *Eleutherodactylus*, concordando com agrupamentos anteriores baseados em caracteres morfológicos feitos por Lynch & Duellman (1997). Posteriormente, Heinicke *et al*. (2007) analisando 277 espécies, reorganizam este grupo

de anuros novamente, formando dois gêneros adicionais, *Pristimantis* e *Lymnophis,* revalidando o gênero *Ischnocnema*, e alocarando *Euhyas, Pelorius* e *Syhrophus* novamente em *Eleutherodacylus.* A árvore filogenética resultante deste trabalho forneceu resolução para quatro grupos: um clado do Caribe composto por 185 espécies de *Eleutherodactylus* (140 espécies analisadas), um clado com espécies da América Central com 111 espécies de *Craugastor* (14 espécies amostradas), um clado com espécies do norte da América do Sul com 397 espécies de *Pristimantis* (87 espécies analisadas) e um clado restrito ao Sudeste do Brasil, com de 5 espécies amostradas de *Ischnocnema* (representando 29 espécies). As espécies que estavam alocadas anteriormente no gênero *Ischnocnema* foram alocadas na sinonímia de *Oreobates*.

Hedges *et al*. (2008), no mais recente dos trabalhos, analisando 344 espécies, alocou todo o grupo de Brachycephalidae (sensu Frost *et al.* 2006) em um novo táxon, denominado Terrarana, listando as 882 espécies descritas, e sugerindo uma nova taxonomia, composta por quatro famílias (Brachycephalidae, Craugastoridae, Eleutherodactylidae e Strabomantidae), quatro subfamílias, 24 gêneros, 11 subgêneros, 33 séries de espécies, 56 grupos de espécies e 11 subgrupos de espécies. Ainda segundo estes autores, cada um dos quatro clados reconhecidos como novas famílias apresentam também alguma sustentação de dados de morfologia e distribuição geográfica. Apenas os gêneros *Brachycephalus* e *Ischnocnema* permaneceram em Brachycephalidae (sensu Frost *et al*. 2006), que é um clado relativamente pequeno, com menos de 40 espécies, restrito à mata Atlântica que vai do Sul ao Nordeste do Brasil.

A atual família Eleutherodactylidae está distribuída principalmente na região do Caribe e inclui as subfamílias Eleutherodactylinae e Phyzelaphryninae, com quatro gêneros (*Diasporus, Adelophryne, Phyzelaphryne* e *Eleutherodactylus*). Os Craugastoridae estão distribuídos principalmente na América Central e contêm dois gêneros (*Haddadus* e *Craugastor*), sendo duas de suas espécies (*H. binotatus* e *H. plicifer*) encontradas na mata Atlântica do Brasil. A família Strabomantidae é um clado Sul Americano que inclui as subfamílias Holoadeninae, com seis gêneros (*Barycholos, Bryophryne, Euparkerella, Holoaden, Noblella* e *Psychrophrynellae*) e Strabomantinae, com dez gêneros (*Atopophrynus, Dischidodactylus, Geobatrachus, Isodactylus, Lynchius, Niceforonia, Oreobates, Phrynopus, Strabomantis* e *Pristimantis*) e tem a mais ampla distribuição, abrangendo os Andes e toda a América do Sul

Segundo Hedges *et al*. (2008), novas espécies continuam a ser descritas para o táxon Terrarana em uma taxa crescente, taxa esta que era de aproximadamente de 1 a 2 espécies por o

ano durante o século 19, elevando-se para 20 a 15 por o ano durante a última metade do século passado.

Os anuros deste grande táxon, Terrarana, ocupam uma ampla variedade de habitats, desde semi-aquáticos até arbóreos, estão amplamente distribuídos nos trópicos e subtrópicos, desde o Texas no sul dos EUA, por todo o México, América Central, América do Sul tropical e subtropical até o norte da Argentina. De acordo com Schmid *et al*. (2002), esses anuros ainda estão em fase de extrema radiação e especiação no norte da América do Sul e na América Central.

Exceto por *Eleutherodactylus jasperi,* que era ovovivíparo (Drewry & Jones 1976), todos anfíbios de Terrarana têm desenvolvimento direto terrestre (Elinson 1994; Hanken *et al*. 1997), que faz com que o sucesso reprodutivo seja muito maior do que em outras espécies (Wake 1978). Seu tamanho pode variar de 10-11 milímetros em *Brachycephalus didactylus* e *Eleutherodactylus* (*Euhyas*) *iberia* a 110 milímetros em *Craugastor pelorus*.

3. Os Terrarana do Brasil

De acordo com dados de Frost (2008) e Hedges *et al*. (2008), no Brasil existem cerca de 90 espécies deste novo táxon (Terrarana), divididas entre as famílias: Strabomantidae, com os gêneros *Strabomantis* (2 espécie), *Euparkerella* (4 espécies), *Holoaden* (2 espécie), *Noblella* (1 espécie), *Barycholos* (1 espécie), *Oreobates* (2 espécie) e *Pristimantis* (29 espécies); Craugastoridae, *Haddadus* (2 espécies); Eleutherodactylidae, que compreende, *Adelophryne* (5 espécies) e *Phyzelaphryne* (1 espécie) e, Brachycephalidae, com os gêneros *Brachycephalus* (11 espécies) e *Ischnocnema* (30 espécies). Contrapondo a este pequeno número de espécies brasileiras de Terrarana descritas até o momento, Frost (2008) lista mais de 300 espécies adjacentes às fronteiras do Brasil, principalmente em países próximos aos trópicos incluídos na Amazônia Legal, indicando um número de espécies amplamente subestimado para o território brasileiro e a necessidade de se ampliar os estudos nas regiões da Amazônica brasileira, para este grande grupo de anuros.

Segundo Hedges *et al*. (2008), de um ponto de vista evolutivo e taxonômico, os clados da América do Sul, Strabomantidae e Brachycephalidae, são os grupos menos conhecidos de vertebrados. Ainda segundo estes autores, embora novas espécies estejam sendo descritas constantemente, os taxonomistas têm sido incapazes de concordar, na maioria das vezes, com a

organização das espécies em gêneros, subgêneros e em grupos de espécie (exemplo, Lynch 1976; Savage 1987; Hedges 1989; Lynch & Duellman 1997). Dentro da família Brachycephalidae, Hedges *et al*. (2008) analisaram menos de 15% das espécies: *Brachycephalus* (apenas uma espécie, *B. ephippium*) e *Ischnocnema* (cinco espécies, *I. guentheri, I. hoehnei, I. parva, I. juipoca* e *I. holti*), o que deixa ainda uma necessidade de ampliar esses dados para a melhor compreensão dos seus relacionamentos. Além disso, baseando-se apenas na morfologia externa, o gênero *Ischnocnema* e *Brachycephalus* apresentam problemas intra- e intergenéricos, e ainda de relacionamentos com espécies de outras famílias como Craugastoridae e Strabomantidae.

De acordo com Frost (2008) e Hedges *et al*. (2008), na família Strabomantidae, cerca de 30 espécies de seis grupos de *Pristimantis* são encontradas no Brasil, desde o centroeste até o extremo norte do país e, dentre estas, 13 espécies (*Pristimantis altamazonicus*, *P. conspicillatus*, *P. croceoinguinis*, *P. diadematus*, *P. fenestratus*, *P. lanthanites*, *P. malkini*, *P. marmoratus*, *P. ockendeni*, *P. peruvianus*, *P. skydmainos*, *P. toftae* e *P. zeuctotylus*), já foram amostradas em estudos moleculares, mas nenhuma delas foi coletada em território brasileiro.

Seis gêneros da subfamília Holoadeninae (*Barycholos, Bryophryne*, *Euparkerella, Holoaden*, *Noblella* e *Psychrophrynella*) estão confinados à América do Sul e dois destes, *Euparkerella* e *Holoaden*, são endêmicos da floresta Atlântica brasileira. Apesar do grande número de espécies de Strabomantidae estudadas (104) no trabalho de Hedges *et al*. (2008), esse número representa menos de um terço das espécies desta família. Ainda, outros quatro gêneros desta subfamília (*Atopophrynus*, *Dischidodactylus*, *Geobatrachus* e *Niceforonia*) e um de Holoadeninae (*Euparkerella*) não foram incluídos em análises moleculares.

4. Caracterização cromossômica em Anura

Ao contrário do observado em nível morfológico, os estudos citogenéticos têm mostrado grande variabilidade dentro de muitos grupos de Anura que apresentam morfologia conservada, incluindo os Terrarana, com isso, contribuído bastante em investigações de ordem sistemática, além de permitir formulação de hipóteses sobre a relação filogenética entre diferentes grupos (ver referências em Lourenço 2001).

As investigações filogenéticas em Anura baseadas apenas em dados morfológicos são de difícil interpretação, uma vez que, apesar do grande número de espécies e da diversidade de habitats, os Anura são muito conservados anatomicamente e segundo Hillis (1991) essa pequena

divergência anatômica entre os Neobatrachia pode ser, em parte, explicada pelo fato de a maioria das famílias desse grupo ter derivado do ancestral há pouco tempo na escala evolutiva. No entanto, isso não impede que caracteres citogenéticos e moleculares tenham sofrido maior divergência do que os anatômicos, visto que a taxa evolutiva entre diferentes caracteres pode variar (Futuyma 1992).

Até a década de 80 muitos estudos citogenéticos em Anura foram desenvolvidos com base na análise de cariótipos corados convencionalmente com Giemsa onde a maior preocupação dessas investigações era a caracterização do número e morfologia cromossômica. Essas primeiras descrições cariotípicas consistiam na determinação do número cromossômico, identificação de constrições secundárias, posições centroméricas e tamanho relativo dos cromossomos (para referências, ver lista de Kuramoto 1990). Esses estudos iniciais permitiram detectar a ocorrência de certa variabilidade entre os anuros, que se mostrou grande em determinados grupos, como nos atuais Terrarana (*sensu* Hedges *et al.* 2008) e pequena em outros, como em Bufonidae e ainda, indicaram a possível existência de rearranjos, como fusão e fissão que poderiam contribuir para alterações no número cromossômico em Anura (Bogart 1973).

Uma melhor compreensão dos rearranjos cromossômicos envolvidos na diferenciação cariotípica desses animais passou a ocorrer após o uso de técnicas citogenéticas mais avançadas a partir da década de 80, com uso do bandamento-C, o método de Ag-NOR, e a partir da década de 90 com as colorações com fluorocromos e hibridação *in situ*, que possibilitaram a melhor caracterização de segmentos cromossômicos e indicaram também eventos como, translocações, inversões, adições, deleções e amplificações, como rearranjos que também podem estar envolvidos na evolução cariotípica dos anuros (exemplos, Bogart 1981; King 1990; Miura 1995; Lourenço *et al.* 1999; Medeiros *et al.* 2003; Siqueira et al. 2004).

Apesar dessas técnicas adicionarem evidenciarem vários tipos de rearranjos cromossômicos, estes foram pouco descritos para poucas espécies até agora e evidenciaram rearranjos envolvendo principalmente as NORs (exemplos, Lourenço *et al*. 1998, 2003a; Medeiros *et al*. 2003; Siqueira *et al*. 2004). Em células meióticas foram observados anéis multivalentes possivelmente resultantes da presença de translocações cromossômicas múltiplas e inversões pericêntricas e paracêntricas (exemplos, Beçak & Beçak 1974; Schmid *et al*. 1995; Lourenço *et al*. 2000, 2003a, 2003b; Siqueira *et al*. 2004).

Cromossomos sexuais heteromórficos são raros em anuros e foram observados em pouco

mais de 20 espécies já analisadas citogeneticamente, e apresentaram os sistemas XY/XX, ZZ/ZW, XAA^{Y}/XXAA, 00/W0 (ver, entre outros, Schmid *et al*. 1991, 1992, 1993; Nishioka *et al*. 1993; Miura 1994; Lourenço *et al*., 1998; Schmid & Steilein 2001; Schmid *et al*. 2003; Ananias *et al*. 2007; Busin *et al*. 2008).

Apesar de se mostrar como uma ferramenta valiosa no estudo dos anuros, até o momento, aproximadamente 1000 espécies foram estudadas citogeneticamente sendo que, menos de um terço destas foram analisadas com técnicas de coloração diferencial como, bandamento-C, Ag-NOR e hibridação *in situ* fluorescente (ver referências, King 1990; Lourenço *et al.* 2003a; Schmid *et al.* 2003; Siqueira *et al.* 2004; Silva *et al.* 2006)

4.1. Região organizadora do nucléolo (NOR)

As NORs são sítios cromossômicos ricos em seqüências GC, formados por numerosas cópias de genes ribossomais que codificam os RNAs ribossomais (RNAr) 18S, 5,8S e 28S, arranjadas *in tandem* e separadas por seqüências espaçadoras. Esses RNAr tornam-se parte integrante do ribossomo (Galls & Pardue 1969; Sumner 1990). Também compõem as NORs, um grupo peculiar das proteínas ácidas altamente argirofílicas, permitindo assim que elas sejam muito clara e rapidamente visualizadas por procedimentos de impregnação por nitrato de prata (Goodpasture & Bloom 1975). Geralmente são regiões de constrições secundárias, inicialmente observadas por Henderson *et al*. (1972). No entanto, nem todas as NORs aparecem como constrições secundárias e em alguns casos contêm heterocromatina (Goessens 1984; King 1980; Schmid, 1982). Em alguns casos ainda, a NOR localiza-se adjacente a regiões de heterocromatina (Schmid 1978a; 1982; King 1980).

A detecção das NORs para a análise do número e da localização cromossômica dessas regiões pode ser feita através de hibridação *in situ* com sonda de DNAr ou por impregnação pelo íon prata. Dentre esses dois métodos, a hibridação *in situ* com sondas de DNA ribossômico é o mais específico e evidencia todas as NORs, independente da atividade transcricional destas (exemplos, Schmid *et al.* 1986; Medeiros *et al.* 2003; Siqueira *et al.* 2004).

As NORs evidenciadas por impregnação por prata são conhecidas como Ag-NORs, e as proteínas argirofílicas como proteínas Ag-NOR (Derenzini 2000). Uma série de investigações ultra-estruturais demonstrou que durante a intérfase as NORs se situam nos componentes fibrilares do nucléolo (Hernandez-Verdun 1983, 1986). Há evidências de que os centros fibrilares

mais o componente fibrilar denso em torno delas são os únicos componentes nucleolares que são corados e visualizados pelos métodos de Ag-NOR, assim como as NORs em cromossomos metafásicos (Derenzini 2000). A NOR interfásica é uma unidade estrutural/funcional para a síntese e transcrição dos genes de RNAr em uma estrutura não-nucleossômica estendida (em configuração pronta para a transcrição), a RNA polimerase I, o fator de ligação da RNA polimerase I e a topoisomerase I (Derenzini *et al*. 1990).

A impregnação por prata só é possível de ser observada em NORs que estão ou estiveram engajadas à síntese de RNA (Miller *et al*. 1976a, b), sendo que a especificidade pela prata se dá em razão da reação desta com as proteínas acídicas, associadas ao RNAr recém-transcrito (Schwarzacher & Wachtler 1993). Portanto, apenas as NORs ativas na intérfase anterior serão evidenciadas pelo método em cromossomos metafásicos. Algumas proteínas Ag-NOR específicas identificadas foram a RNA polimerase I (Scheer & Rose 1984), a nucleolina (Ochs *et al*. 1983), a DNA topoisomerase I (Guldner *et al*. 1986), p135 (Pfeifle *et al*. 1986) e o UBF (Chan *et al*. 1991; Hernandez-Verdun *et al*. 1993). Outras análises citoquímicas também mostraram que a impregnação por prata consiste de proteínas não histônicas ricas em grupos sulfidril e dissulfeto com rápida associação com o RNAr recém transcrito (Buys & Osinga 1980). Resíduos destas proteínas que permanacem associadas com as NORs nas metáfases podem ser detectados com a impregnação por prata, embora este estágio das NORs seja transcricionalmente inativo (Fan & Penman 1970). Suja *et al*. (1993) propuseram ainda que a afinidade pela prata seja promovida pelo domínio acido amino-terminal das proteínas Ag-NOR.

A análise das regiões organizadoras de nucléolos (NORs) representa uma importante ferramenta para o estudo de cariótipos em anfíbios (exemplos, Schmid 1978a, 1978b, Wiley *et al*. 1989, Kaiser *et al*. 1996, Lourenço *et al*. 1998, Silva *et al*. 1999, Silva *et al*. 2006, Medeiros *et al*. 2003, Siqueira et al. 2004). Normalmente em um grupo de espécies consideradas próximas, a NOR se encontra no mesmo par e região cromossômica, sendo que seu tamanho vai estar relacionado ao tamanho da contrição secundária (Schmid 1982). A localização das NORs pode variar entre as famílias, gêneros e até mesmo dentro de espécies (Schmid 1978). Além disso, os heteromorfismos no tamanho e presença de NORs adicionais, ou ainda, NORs associadas à heterocromatina, são características que podem ser usadas nas comparações citogenéticas (Lourenço 2001).

O tamanho das diferentes NORs (mesmo que sejam homólogas) também pode variar entre

diferentes indivíduos da mesma espécie, devido ao diferente número de cópias do gene ribossomal presente em cada NOR (Mellink *et al*. 1994). Em anuros, esse fenômeno é bastante comum e amplamente descrito (Schmid 1978a, 1978b, 1980; 1982; Silva *et al*. 1999; Formas & Cuevas 2000; Siqueira *et al*. 2004). Segundo Schmid (1982), NORs duplicadas ou triplicadas não ocorrem em homozigose em populações selvagens, estando sempre associadas a NORs de tamanho "normal".

Na maioria dos anuros estudados, as regiões evidenciadas pelo método Ag-NOR e por hibridação *in situ* com sondas de DNAr são coincidentes (Schmid et at. 1986; King *et al*. 1990; Foote *et al*. 1991; Lourenço *et al*. 1998). Até hoje, em apenas cinco espécies de anuros, *Hyla chrysoscelis, Hyla versicolor* (Wiley *et al*. 1989), *Dendropsophus nanus* (Medeiros *et al.* 2003), *Colostethus* sp. (Veiga-Menoncello *et al.* 2003) e *Haddadus binotatus* (Siqueira *et al*. 2004) o método de hibridação *in situ* revelou sítios adicionais não detectados por impregnação por prata, além das NORs evidenciadas por ambos os métodos. Nesses casos, tais sítios de homologia com DNAr nunca foram evidenciados como NORs ativas, o que, segundo Lourenço (2001) torna a regulação diferencial na expressão dessa região uma hipótese pouco provável para a explicação do fenômeno observado.

A inativação de genes ribossomais foi levantada para explicar a não-detecção de NORs em todos os cromossomos homólogos no portador de NOR na espécie tetraplóide de *Odontophrynus americanus* (Ruiz *et al*. 1981; Cortadas & Ruiz 1988; Ruiz & Brison 1989), embora nesse caso a técnica de hibridação *in situ* não tenha sido utilizada para verificar a existência desses sítios possivelmente inativos. Já em híbridos interespecíficos de *Xenopus laevis* e *Xenopus mulleri,* a regulação da atividade de transcrição de DNAr foi descrita (Honjo & Reeder 1973). Nesse estudo os autores mostraram a transcrição preferencial do DNAr de *X. laevis* e a supressão da atividade do DNAr de *X. mulleri.* Estudos também relacionam polimorfismo de bandas heterocromáticas com variação no padrão de NOR, e reforçam estudos que indicam que a associação de NORs a regiões de heterocromatina está intimamente relacionada com mecanismos de expressão e também silenciamento gênico (ver revisão de Grewal & Jia 2007).

Na maioria dos casos de heteromorfismos de tamanho descritos para anuros, nenhuma variação intra-individual no padrão de NORs foi detectada (Lourenço 2001). No entanto, King *et al*. (1990) relataram variações de NORs entre diferentes células de um mesmo indivíduo em espécies de *Litoria* e em *Cyclorana novaehollandiae* (Anura, Hylidae) e, recentemente, Quindere

et al. (2007) encontrou variação intra-individual de tamanho de NOR em uma população de *Physalaemus cuvieri.* Em ambos os casos essas variações foram detectadas tanto pela técnica de Ag-NOR quanto pela hibridação *in situ*.

O número e a localização das NORs tendem a ser característicos de cada população ou espécie (para exemplos, Schmid 1978a, 1978b), entretanto variações já foram relatadas nos anuros *Hyla chrysoscelis* e *Hyla versicolor* (Wiley *et al*. 1989), *Anaxyrus terrestris* (Foote *et al*. 1991), *Agalychnis callidryas* (Schmid *et al*. 1995), *Dendropsophus ebraccatus* (Kaiser *et al*. 1996), *Engystomops petersi* (Lourenço *et al*. 1998), *Physalaemus cuvieri* (Silva *et al*. 1999), *Leptodactylus mystacinus* (Silva *et al*. 2006) e *Dendropsophus nanus* (Medeiros *et al*. 2003). No caso de *Dendropsophus ebraccatus,* a variação encontrada foi interpopulacional e se referia apenas a localização da NOR. Nas demais espécies citadas, puderam ser observados exemplos de variação intrapopulacional na ocorrência de NORs.

A maioria dos anuros apresenta um par de NORs por genoma diplóide, condição observada tanto em famílias primitivas como derivadas (para referências ver Kuramoto 1990; King 1990). Tal observação levou King *et al*. (1990) à sugestão de que, em Anura, a presença de apenas um par de NORs por genoma diplóide seria uma condição primitiva em relação à ocorrência de NORs múltiplas. Alguns mecanismos de dispersão de NORs, possivelmente envolvidos no surgimento de NORs múltiplas, têm sido considerados por diversos autores (Wiley *et al*. 1989; King *et al*. 1990; Foote *et al*. 1991; Schmid *et al*. 1995; Kaiser *et al*. 1996; Lourenço *et al*. 1998; Silva *et al*. 1999; Ocalewicz *et al*. 2007). Estes autores sugerem que essas variações possam ser um resultado de diferenças estruturais das NORs, causadas por rearranjos como transposição e amplificação de genes do DNAr, e erros de reinserção durante a amplificação extra-cromossômica de genes ribossomais na oogênese.

A análise cuidadosa do número e da localização cromossômica das NORs pode permitir a identificação de homeologias entre diferentes populações e espécies, possibilitando, em alguns casos, o reconhecimento de alguns rearranjos cromossômicos que possivelmente diferenciaram cariotipicamente diversos grupos, podendo auxiliar também em estudos taxonômicos.

4.2. Heterocromatina e bandamento C

Em células eucarióticas, o DNA genômico é empacotado com proteínas histônicas e não-histônicas. Cada unidade da cromatina, ou nucleossomo, contém 146 pb do DNA, que envolve

um octâmero de histonas (Luger *et al*. 1997). As enzimas que modificam as caudas das histonas, o complexo de remodelamento de cromatina e a metilação de DNA são considerados componentes de mecanismos epigenéticos intricados que ajudam comprimir e organizar genomas dentro de domínios cromatínicos discretos (Jenuwein & Allis 2001; Goll & Bestor 2005). Esta organização governa também muitos aspectos do comportamento cromossômico, tais como a transcrição, a recombinação e o reparo de DNA (Kosak & Groudine 2004).

Heitz, em 1928, foi o primeiro a distingüir a heterocromatina da eucromatina, com base na compactação diferencial na intérfase. A eucromatina é menos condensada, mais acessível e geralmente transcreve mais facilmente, ao passo que a heterocromatina é altamente condensada, inacessível e possui arranjos nucleossômicos altamente organizados (Huisinga *et al*. 2006). Diversos tipos de informação são integrados na formação e manutenção da heterocromatina, incluindo a posição cromossômica, localização nuclear e a presença e densidade de elementos de DNA repetitivo (Weiler & Wakimoto 1995; Birchle *et al*. 2000; Hall & Grewal 2003; Grewal & Jia 2007). As regiões cromossômicas que contêm alta densidade de elementos repetitivos do DNA tais como seqüências satélites e elementos transponíveis, que são encontrados nos centrômeros e telômeros são os alvos principais da formação de heterocromatina (ver revisão de Grewal & Jia 2007).

Dependendo do tipo de empacotamento ou condensação, é possível distinguir três classes de cromatina: eucromatina, heterocromatina facultativa e heterocromatina constitutiva. No caso da condensação da eucromatina conhecida como heterocromatina facultativa, a inativação é reversível e ocorre em determinada geração celular. As regiões de heterocromatina constitutiva permanecem condensadas durante todo o ciclo celular em locais específicos do genoma, enquanto a heterocromatina facultativa é encontrada também nos loci de regulação, onde o estado da cromatina pode mudar em resposta aos sinais celulares e às atividades genéticas (Grewal & Jia 2007).

A heterocromatina constitutiva é predominantemente constituída por seqüência de DNA altamente repetitivo e não codificador (Sumner 1994), desempenhando um importante papel ao proteger os sítios eucromáticos adjacentes de modificações estruturais por recombinação, uma vez que diminui a formação de quiasmas (King 1991). Uma característica chave dessa heterocromatina é sua habilidade de influenciar a expressão gênica em uma região específica (revisão de Grewal & Jia 2007), geralmente causando repressão epigenética de seqüências

próximas dos domínios em que ela se encontra, em um processo conhecido como silenciamento (revisão de Grewal & Jia 2007). A inativação de genes localizados dentro ou próximos a heterocromatina foi um dos primeiros efeitos atribuídos a ela, desde os estudos de variegação por efeito de posição, estudados em *Drosophila* (ver Spofford 1976; Weiler & Wakimoto 1995).

Embora heterocromatinização seja quase sinônimo de silenciamento epigénetico, é possível observar que muitas das mesmas modificações histônicas e recrutamento de proteínas para o silenciamento da estrutura da cromatina são, em outras circunstâncias, essenciais para a ativação de genes (revisão de Grewal & Jia 2007).

A heterocromatina tem ainda um perfil característico na modificação das histonas a ela associadas, que é distinguido pela hipo-acetilação e pela metilação da histona H3 na lisina 9 (H3K9). Já a eucromatina é caracterizada pela acetilação da histona H4 e pela metilação da histona H3 na lisina 4 (H3K4) (Nakayama *et al*. 2001; Litt *et al*. 2001). As histonas e suas modificações têm papéis cruciais na formação de heterocromatina (Jenuwein & Allis 2001), sendo que a metilação da histona serve como "âncora molecular", recrutando as proteínas que modificam diretamente a cromatina ou recrutando outras que fazem este processo (Martin & Zhang 2005). Esta estratégia de metilação de histonas que funciona como âncora para recrutamento de efetores não é restrita a heterocromatina, sendo que mecanismos análogos são usados também nos loci eucromáticos, onde H3K4me e H3K36me recrutam proteínas para o remodelamento da cromatina (Pray-Grant *et al*. 2005; Grewal & Jia 2007).

Outra importante proteína relacionada com a inativação gênica em vários organismos é a HP1 *(heterochromatin protein I)* (Eissenberg *et al*. 1990). Sabe-se até agora que cada proteína HP1 interage com os fatores diversos que são envolvidos em aspectos diferentes da estrutura e da função da heterocromatina (ver revisão de Grewal & Jia 2007).

Cabrero *et al*. (1986), em estudos com gafanhoto, relacionaram a existência de segmentos heterocromáticos a um aumento na atividade de NORs secundárias, sugerindo que esses blocos de heterocromatina poderiam inativar genes supressores dessas NORs, encontrados adjacentes a essa heterocromatina. Também estudando gafanhotos, Lopez-Leon *et al*. (1995) evidenciaram a ocorrência de vários NORs associadas a presença de heterocromatina supernumerária.

A heterocromatina constitutiva é geralmente detectada citogeneticamente através da técnica de bandamento C (Sumner 1990). O bandamento C foi descoberto por Pardue & Gall (1970), que sugeriram que as regiões evidenciadas por esse método eram constituídas quase

exclusivamente de DNA altamente repetitivo. O mecanismo bioquímico para explicar o bandamento C foi proposto por Holmquist (1979) e apresenta, basicamente, três etapas: um tratamento ácido, que promove a remoção de purinas da molécula de DNA; um tratamento alcalino, que causa a quebra dos sítios apurínicos e a desnaturação irreversível do DNA com a eliminação da ribose do nucleotídeo que sofreu depurinação, e um tratamento salino que promove a β-elininação desses sítios depurinados. Acredita-se ainda que a extração do DNA através desse método ocorra mais lentamente nas regiões de heterocromatina constitutiva.

A técnica de banda C mais empregada é a de Sumner (1972), apresentando três etapas básicas: tratamento com ácido com HCl seguido de um tratamento alcalino com solução aquosa de hidróxido de bário [Ba_2 (OH)] saturado, e depois com solução salina de cloreto de sódio e citrato de sódio (2x SSC).

O tratamento com hidróxido de bário resulta na extração de até 50% do DNA dos cromossomos. Esse DNA é mais facilmente extraído das regiões banda C negativas, resultando em uma fraca coloração quando comparada com regiões de heterocromatina constitutiva (banda C positivas) (Schmid 1990).

O bandamento C vem sendo empregado no estudo de um grande número de espécies de anuros, com diversas finalidades, dentre as quais: identificação de espécies parentais com a identificação de padrões em determinadas espécies ou populações, procura de possíveis polimorfismos e homeologias, além da caracterização de gêneros e famílias e identificação de pares heteromórficos entre machos e fêmeas (King 1980; Schmid 1978a; Schmid 1980; Sumner 1990; Siqueira *et al.* 2008).

King (1991) sugeriu três processos gerais para indicar a evolução de heterocromatina em anfíbios, analisando várias espécies de Archaeobatrachia e Neobatracia, que são: adição de heterocromatina a sítios cromossômicos específicos, transformação de regiões cromossômicas eucromáticas em regiões heterocromáticas e evolução combinada de sítios heterocromáticos múltiplos.

Dentre as possíveis funções dessa heterocromatina, já mencionadas anteriormente, seu envolvimento na redução de recombinação entre cromossomos sexuais em processo de diferenciação parece ser comprovada em Amphibia (ver referências, King 1991).

A análise do número, da localização e do tamanho das bandas C, assim como das marcações das NORs, permite a identificação de cromossomos homeólogos entre espécies. Em

anuros foram descritas tanto variações interespecíficas como intra específicas de bandamento C, com polimorfismos em relação ao tamanho, ao número e a localização dessas regiões de bandas heterocromáticas (Schmid, 1978a, 1978b; Schmid, 1980; King, 1980, 1991; Miura *et al*. 1995; Lourenço *et al*. 1999; Silva *et al*. 1999; Formas & Cuevas 2000)

Para estudos evolutivos, o bandamento possibilita também a observação das transformações que ocorreram em grupos de espécies próximas que apresentam cariótipos muito semelhantes (Schmid 1980; Guerra 1988). Essas características da heterocromatina banda-C positiva mencionadas acima evidenciam que o seu estudo pode fornecer importantes informações evolutivas, sendo, portanto, um importante caráter citogenético a ser analisado.

5. Citogenética de Terrarana

As investigações citogenéticas em mais de 100 espécies Terrarana revelaram uma acentuada variação no número cromossômico diplóide (2n=18 a 36) e na morfologia dos cromossomos (Bogart 1973, 1991; Bogart & Hedges 1995), além da presença de sistemas sexuais cromossômicos complexos como, ZZ/ZW, XAA^{Y}/XXAA (Schmid & Steilein 2001; Schmid 2002; Schmid *et al*. 2003).

Bogart (1970, 1973) propôs que a evolução cromossômica nos "eleutherodactylines (sensu Frost *et al.* 2006) estaria relacionada com uma contínua fusão e fissão cêntrica, tendo como extremos 2n=18 (com cromossomos metacêntricos e submetacêntricos) e 2n=36 com cromossomos telocêntricos apenas. Bogart (1981) e King (1990) sugerem que translocações recíprocas e inversões podem ter ocorrido durante a filogênese recente deste gênero e segundo Schmid *et al.* (1992), rearranjos cromossômicos do tipo fusão ou fissão cêntrica também podem estar envolvidos na evolução do cariótipo deste grupo o que explicaria o número de cromossomos telocêntricos ser maior em espécies que possuem cariótipo com grande número cromossômico.

Visto que a maioria dos gêneros de anuros possui cariótipo relativamente conservado, Bogart (1973), Moreschalchi (1973) e Bogart & Hedges (1995) consideraram os "eleutherodactylines" um exemplo que contradiz a afirmação de que os anuros têm baixa taxa de evolução cromossômica. Entretanto, esses autores não levaram em consideração o alto grau de variação cariotípica como um indicativo de que este grupo de anfíbios não fosse monofilético. Lynch & Duellman (1997), apenas com estudos de morfologia, indicaram o polifiletismo deste

grupo de anuros formando alguns subgêneros, sendo a maioria destes confirmados por Frost *et al*. (2006), Heinicke *et al*. (2007) e Hedges *et al*. (2008).

Segundo Hedges *et al*. (2008), a grande variação descrita acerca do número cromossômico por si só não foi suficiente para elucidar a classificação dos "eleutherodactyline" (exemplo, Savage 1987; Bogart & Hedges 1995). Entretanto, a partir da década de 70, essas variações já indicavam o possível parafiletismo deste grande táxon (ver, Bogart 1970, 1974; Bogart & Hedges 1995). Nesse sentido, uma análise dos dados citogenéticos dos Terrarana, levando em conta o número cromossômico e a localização geográfica dessas espécies, indica que esses dados são coerentes com os estudos moleculares de Frost *et al*. (2006), Heinicke *et al*. (2007) e Hedges *et al*. (2008), e que a maioria dos agrupamentos recém-formados tem reduzido muito a diferença em relação aos números diplóides atribuídos aos Terrarana. Um exemplo disso é a recente retirada de *Diasporus diastema,* com 2n=18 (DeWeese 1975), do gênero *Pristimantis* (Hedges *et al*. 2008) cujas espécies amostradas no trabalho de Hedges *et al*. (2008) têm número cromossômico de 2n=30, 32 e 34.

Para as espécies brasileiras de Terrarana, a literatura se restringe a dados cariotípicos de apenas sete espécies do sul e sudeste: *I. holti* e grupo *Ischnocnema lactea* (2n=20) (De Lucca *et al*. 1974; De Lucca & Jim 1974), *I. guentheri, I. parva, I. juipoca, H. binotatus* e *B. ternetzi* (Beçak 1968; Brum-Zorrilla & Saez 1968; Beçak & Beçak 1974; Siqueira *et al*. 2004; Campos *et al*. 2008) (2n=22). Todas essas espécies também foram amostradas por estudos moleculares e indicam, apesar do número cromossômico próximo, que existem problemas de relacionamentos entre estes gêneros. Além disso, de acordo com Hedges *et al*. (2008) existem muitos problemas de relacionamentos intra- e intragenéricos e até mesmo interfamiliares levantados por dados morfológicos, indicando assim a necessidade de se ampliar o número de espécies amostradas, tanto por dados moleculares quanto citogenéticos e outras ferramentas, para o melhor entendimento dos relacionamentos das espécies brasileiras deste grupo de anuros.

Um panorama da distribuição dos números cromossômicos disponíveis para as espécies de Terrarana em função dos clados obtidos no estudo de filogenia molecular de Hedges *et al*. (2008) pode ser visto no Apêndice – item IX).

6. A ultra-estrutura do espermatozóide em Anura

Os estudos de ultra-estrutura de espermatozóides vêm sendo usados desde os anos 80 para

estudos filogenéticos de Platelmintos (Brooks 1989) e Cestódeos (Euzet *et al*. 1981; Bâ & Marchand 1995). A partir dos primeiros estudos em Platelmintos, as características ultra-estruturais dos espermatozóides foram usadas numa grande variedade de grupos animais tais como, peixes (Mattei 1991; Jamieson 1991), répteis (Jamieson 1995; Teixeira *et al*. 1999), aves (Koehler 1995; Asa & Phillips 1987), insetos (Dallai *et al*. 1984; Baó & De Souza 1992) e moluscos (Introíni *et al.* 2004) para a elucidação de questões sistemáticas e taxonômicas.

A análise da ultra-estrutura do espermatozóide em Anura tem se mostrado uma ferramenta valiosa de descrição de caracteres, potencialmente úteis para auxílio em estudos sistemáticos (exemplos, Jamieson 1999; Garda *et al*. 2002; Aguiar *et al*. 2003, 2004; Veiga-Menoncello *et al*. 2007). Segundo Jamieson (1999) tal ferramenta pode indicar sinapomorfias, simplesiomorfias e autapomorfias.

A ultra-estrutura de espermatozóides de aproximadamente 50 espécies das famílias Bufonidae, Hylidae, Leptodactylidae, Ranidae, Microhylidae e Dendrobatidae, além de alguns Archeobatrachia já foi estudada (exemplos, Lee & Jamieson *et al*. 1993; Costa *et al*. 2004; Garda *et al*. 2002; Aguiar *et al*. 2003, 2004, 2006; Veiga-Menoncello *et al*. 2006, 2007).

Estes estudos têm mostrado que a morfologia dos espermatozóides dos anuros é bastante diversificada em nível de Famílias e tem sido útil para esclarecer alguns relacionamentos taxonômicos (exemplos, Jamieson *et al*. 1993; Garda *et al*. 2002; Aguiar *et al*. 2003, 2004; Costa *et al*. 2004). O estudo da ultra-estrutura dos espermatozóides é fundamentado principalmente nas análises de três partes que possuem morfologia variável: cabeça, peça intermediária e flagelo (Lee & Jamieson 1992, 1993; Jamieson *et al*. 1993, Kwon & Lee 1995, Meyer *et al*. 1997; Garda *et al*. 2002).

O tamanho de um espermatozóide em Anura pode variar de 25µm a 180µm, e com poucas exceções pode chegar a 250µm e até 2500µm como é o caso de *Discoglossus pictus* (Furieri 1975; Pugin-Rios 1980; Scheltinga *et al*. 2001). Exceto por um gênero, *Bombina,* (subordem Archaeobatrachia), que tem cauda associada lateralmente ao núcleo, a morfologia do espermatozóide, sob a microscopia de luz, é visivelmente dividida em duas partes: cabeça e cauda, sendo que os dois possuem praticamente o mesmo tamanho e raramente a cauda é menor (Jamieson 2003).

A região da cabeça é composta pelo núcleo e uma vesícula acrossomal que é encontrada na porção anterior, recobrindo grande parte ou apenas o topo do núcleo (Kwon & Lee 1995).

Estruturas associadas (perforatório e cone subacrossomal), são encontradas em combinações variadas nas espécies, além disso, o núcleo pode ter várias formas: cilídrico, fusiforme, e até mesmo, formas peculiares, a exemplo de espécies da família Rhacophoridae que tem o núcleo em forma de "saca-rolhas" (Mainoya 1981; Mizuhira *et al*. 1986; Jamieson 1999).

A peça intermediária compõe o complexo de ligação entre o núcleo e a cauda, normalmente é composta por um centrílo proximal perpendicular ao núcleo e logo abaixo um centríolo distal que dá origem ao axonema; estes centríolos podem estar envoltos por uma matriz protéica e cercados de mitocôndrias. Apesar de possuírem uma forma básica de apresentação, estas estruturas podem variar em sua composição e posição em relação ao núcleo (Jamieson 1999).

A estrutura e organização da cauda também são muito variáveis. O flagelo pode ser formado apenas pelo axonema, a exemplo das famílias Ranidae, Pipidae, Leptodactylidae, ou, a exemplo de outras, como Bufonidae, Leptodactylidae (diversos), Hylidae e Rhinodermatidae, o axonema pode estar ligado um bastão paraxonemal por intermédio de uma membrana ondulante, que pode ser dilatada na extremidade de contato com os microtúbulos, formando a fibra juxta-axonemal. Além disso, podem também apresentar-se aos pares (biflagelados), com dois axomemas, sem bastão ou membrana ondulante, a exemplo de *Telmatobufo australis* (Pugin-Rios 1980), ou dois flagelos completos (*sensu* Aguiar *et al*. 2003), com membrana ondulante e bastão paraxonemal, como encontrado em *Alobates femoralis* e *Alobates brunneus* (Aguiar *et al*. 2003; Veiga-Menoncello *et al*. 2006).

Os estudos filogenéticos recentes que incluíram membros da família Leptodactylidae são um exemplo da importância e coerência dos sinais filogenéticos dos dados de ultra-estrutura dos espermatozóides. Essa família foi uma das mais estudadas através da ultra-estrutura de espermatozóides, com pelo menos 16 espécies analisadas de três subfamílias e 11 gêneros e apresentaram uma grande variação na ultraestrura dos espermatozóides, dentro até mesmo de uma única subfamília. Como exemplo, membros da antiga subfamília Telmatobiinae, como *Telmatobius hauthali* (Pisanó & Adler 1968), *Caudiverbera caudiverbera* e *Telmatobufo australis* (Pugin-Rios 1980), não possuem elementos auxiliares na cauda, enquanto outras espécies da mesma subfamília, como *Odontophrynus cultripes* (Báo *et al*. 1991), *Eupsophus roseus* e *Alsodes vittatus* (Pugin-Rios 1980) possuem estas estruturas. Essas mesmas espécies, após um grande número de reformulações baseadas principalmente em dados moleculares, estão alocadas em famílias separadas mostrando coerência com os dados de ultra-estrutura de

espermatozóides: *Telmatobius hauthali* está alocado na família Ceratophryidae, *Calyptocephalla caudiverbera* e *Telmatobufo australis* na família Calyptocephalellidae, *Eupsophus roseus, Odontophrynus cultripes* e *Alsodes vittatus* na família Cycloramphidae.

Com exceção de *Brachycephalus ephippium,* que teve apenas algumas das características de seus espermatozóides informadas por Garda (2002), nenhuma outra espécie de Terrarana foi analisada até o presente momento, Nesse sentido, os problemas inter- e intragenéricos e até mesmo interfamiliares levantados por dados morfológicos em Hedges *et al*. (2008), indicam a importância da utilização dessa ferramenta nos estudos dos relacionamentos das espécies brasileiras de Terrarana

7. Objetivo

Realizar estudos cromossômicos e da ultra-estrutura dos espermatozóides de espécies dos gêneros: *Pristimantis, Barycholos, Ischnocnema, Brachycephalus* e *Haddadus*, buscando contribuir para o entendimento dos relacionamentos inter- e intragenéricos das espécies de Terrarana do Brasil.

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IV. Artigo 1

Chromosomal analysis of three Brazilian “eleutherodactyline”frogs (Anura: Terrarana), with sugestion of a new species

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Chromosomal analysis of three Brazilian "eleutherodactyline" frogs (Anura: Terrarana), with suggestion of a new species

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Abstract

The karyotypes of four Brazilian "eleutherodactyline" samples were analyzed aiming to provide additional cytogenetic data for future understanding of the evolutionary and systematic relationships of this large anuran group. The populations consisted of *Pristimantis dundeei* (Chapada dos Guimarães and Rondonópolis, Mato Grosso), *Pristimantis* aff. *dundeei* (Aripuanã, Mato Grosso) and *Ischnocnema paulodutrai* (Ilhéus, Bahia). The data revealed that *P. dundeei* and *P.* aff. *dundeei* have 2n=28 chromosomes, whereas *I. paulodutrai* has 2n=30. All pairs of chromosomes were telocentric, except for the subtelocentric pair 5 in *I. paulodutrai.* Differences in Ag-NOR pattern and interstitial heterochromatin positions clearly distinguished *P.* aff. *dundeei* from *P. dundeei*, and differentiated them from *I. paulodutrai.* The specimens of *I. paulodutrai* showed two distinct color patterns, but they did not differ in their cytogenetic characteristics. Karyotypes with 2n=28 and 2n=30 chromosomes have not been previously described for Brazilian "eleutherodactylines" which, to date, had been characterized as 2n=20, 2n=22 and 2n=34. The NOR position differences identified between *P. dundeei* and *P.* aff. *dundeei*, allied to their known distinct behavior and ecological data, suggested that the *P.* aff. *dundeei* from the Aripuanã sampling location is a new species. Similarities between *I. paulodutrai* and species currently assigned to *Pristimantis* are herein discussed on the basis of chromosome number and morphological characteristics.

Key words: *Pristimantis*, *Ischnocnema,* cytogenetics, karyotype, NOR, C-banding

Introduction

Eleutherodactylus was, for a long time, the largest vertebrate genus and had been considered to encompass a high degree of speciation (Bogart & Hedges 1995). However, following recent comprehensive studies (Frost *et al.* 2006; Heinicke *et al.* 2007; Hedges *et al.* 2008), this taxon has undergone a radical restructuring, including partitions into new taxa. Approximately 700 species, formerly included in the subfamily Eleutherodactylinae (Leptodacylidae), were recently split into the new genera *Euhyas* (94 species), *Craugastor* (116 species), *Pelorius* (6 species), *Syrrhopus* (24 species), and *Eleutherodactylus* (492 species), according to Frost *et al.* (2006). They were assigned to the family Brachycephalidae, which contain more than 800 species grouped in 19 genera (Frost *et al.* 2006). In a further reorganization, Heinicke *et al.* (2007) recognized two additional genera, *Pristimantis* (393 species), and *Limnophys* (15 species) and considered *Euhyas, Pelorius* and *Syrrho-*

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pus as subgenera of the genus *Eleutherodactylus*. In addition, the former *Eleutherodactylus* species natives to southeastern Brazil were allocated in the genus *Ischnocnema.*

Hedges *et al.* (2008) placed 882 described "eleutherodactyline" species in a new taxon, Terrarana, based on the analysis of 344 species through molecular characters. Nearly 85 species are reported in Brazil, assigned to the families: Strabomantidae (*Strabomantis*, *Euparkerella*, *Holoaden*, *Noblella*, *Oreobates* and *Pristimantis*), Craugastoridae (*Haddadus*), Eleutherodactylidae (*Adelophryne*, *Phyzelaphryne*) and Brachycephalidae (*Brachycephalus* and *Ischnocnema*).

A high degree of diploid number (2n) variation (2n=18 to 2n=36) and complex sexual chromosome systems have been identified among the more than 100 species of *Eleutherodactylus* (*sensu lato*) thus far analyzed (see Kuramoto 1991, Bogart & Hedges, 1995, Kaiser *et al.* 1995, Schmid *et al.* 2002, Schmid *et al.* 2003, Siqueira *et al.* 2004; Campos *et al.* 2006). Structural rearrangements have been postulated as determinant in their conspicuous chromosome variation (Bogart 1991; Schmid *et al.* 2003).

In Brazil, only seven "eleutherodactyline" species have been cytogenetically analyzed: *E. binotatus* (currently *Haddadus binotatus*) (Beçak & Beçak 1974; Siqueira *et al.* 2004; Campos *et al.* 2008), *E. guentheri* (currently *Ischnocnema guentheri*) (Beçak 1968; Brum-Zorrilla & Saez 1968; Beçak & Beçak 1974; Siqueira *et al.* 2004; Campos *et al.* 2008), *E. holti* (currently *I. holti*) (De Lucca *et al.* 1974), *E. juipoca* (currently *I. juipoca*) (Campos *et al.* 2008), *E. lacteus* (currently *I. lactea*) (De Lucca & Jim 1974), *E. parvus* (currently *I. parva*) (Beçak & Beçak 1974; Siqueira *et al.* 2004; Campos *et al.* 2008) and *E. fenestratus* (currently *Pristimantis fenestratus*) (Siqueira *et al.* unpublished data). The karyotyped species can be discriminated in two groups of distinct diploid chromosome number and morphology. One group is characterized by 2n=20 and 2n=22 chromosomes, most or all of them metacentric and submetacentric, represented by species from the southern and southeastern Brazil (*I. holti*, *I. lactea, I. guentheri, I. juipoca*, *I. parva* and *Haddadus binotatus*). The second group is characterized by 2n=34 chromosomes, all of them telocentric, represented by *E. fenestratus* from the northern and northeastern Brazil.

In the present work, the karyotypes of *Pristimantis dundeei, Ischnocnema paulodutrai,* and a third taxon here nominated *Pristimantis* aff. *dundeei* (based on ecological and behavioral differences) were analyzed aiming at increasing the number of karyotyped Brazilian "eleutherodactyline" species and improving the understanding of their evolutionary relatedness and taxonomic status.

Material and Methods

The Brazilian "eleutherodactyline" frogs analyzed in this work were sampled in four locations, at the central western and northeastern regions, under a permit issued by the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA) (License nº 206/2005 - CGFAU/LIC). *Pristimantis dundeei* was sampled in Mato Grosso State, in the type-locality Chapada dos Guimarães (15° 40' 00" S, 55° 84' 00" W, ten females and two males) and in Rondonópolis (16° 40' 00" S, 54° 44' 00" W, five females and one male). *Pristimantis* aff. *dundeei* were sampled in Aripuanã, Mato Grosso State (10º 15' 00" S, 59º 23' 00" W, two females and three males). *Ischnocnema paulodutrai* was sampled in Bahia State, at the campus of the Universidade Estadual de Santa Cruz (UESC), Ilhéus (14º 47' 00" S 39º 10' 00" W, five males and five females). Two distinct dorsal morphological patterns were identified among the *I. paulodutrai* specimens, characterized by typical dorsal specked staining or brown dorsal stripes. Although it has been observed that *Pristimantis* aff. *dundeei* also exhibit distinct morphological patterns, with or without a median dorsal stripe (C. Strüssmann, personal information), the specimens analyzed in the present work had no stripes.

All voucher specimens were deposited in the Museu de Zoologia "Prof. Adão José Cardoso" (ZUEC), at the Universidade Estadual de Campinas (UNICAMP), and in the Coleção "Célio F. B. Haddad" at the Universidade Estadual Paulista (UNESP) Rio Claro, São Paulo, Brazil, under the accession numbers: ZUEC 14.061

to 14.073 (*P. dundeei*, from Chapada dos Guimarães, MT), ZUEC 14.044 to 14.049 (*P. dundeei*, from Rondonópolis, MT), ZUEC 14.052 to 14.054 (*P.* aff. *dundeei*, from Aripuanã, MT) and CFBH 11964 to 11973 (*I. paulodutrai*, from Ilhéus, BA). The specimens CFBH 11963 (female), 11972 and 11973 (males) had median dorsal stripe.

Mitotic chromosomes were obtained from a suspension of intestinal epithelium and testicular cells of animals pre-treated with 2% colchicine for at least 4h, as described by King & Rofe (1976) and Schmid (1978). Conventional staining with 10% Giemsa solution, Ag-NOR labelling (Howell & Black 1980) and C-banding (Sumner 1972) were used in the chromosome analyses. The C-banding technique was modified by applying (1) a pre-treatment in 50% acetic acid, for 30 minutes, previously to the 0.2N HCl hydrolysis for 30 to 60 minutes, and (2) by incubation at 60ºC for both treatments with 5% $Ba(OH)_2$, for 15 to 20 seconds, and the saline solution (2xSSC), for 15 to 20 minutes.

The slides were examined under a BX60 Olympus microscope, and the images were captured using the software Image Pro-Plus 4.5.1 version. Mitotic chromosomes were measured and classified according to Green & Sessions (1991).

Results

A diploid number (2n) of 28 telocentric chromosomes and a fundamental number (FN) of 28 characterized the *P. dundeei* and *P.* aff. *dundeei* localities. In the *P. dundeei* specimens, the Ag-NOR sites were adjacent to the centromere in the pair 1, coincident with a secondary constriction (Fig. 1a). A small amount of heterochromatin was detected in the centromeric region of almost all chromosomes. C-bands were faint and adjacent to the centromere of the pair 1, and interstitially positioned in the pairs 2, 4 and 11. In pair 1, the heterochromatin was adjacent to the NOR (Fig. 1a, b).

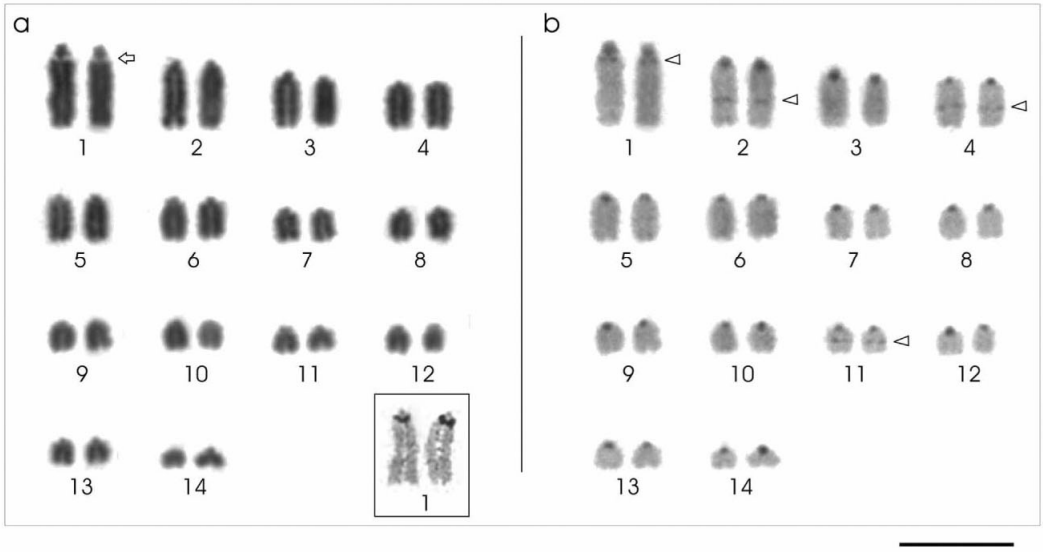

FIGURE 1. Karyotype of *Pristimantis dundeei*: (**a**) Giemsa and Ag-NOR staining; (**b**) C-banding. The arrow indicates secondary constrictions, which are coincident with the NOR position (inset). The arrowheads indicate interstitial heterochromatin. Bar = 10 µm.

In *P.* aff. *dundeei*, the Ag-NOR sites were adjacent to the centromere of the pair 10, coincident with a sec-

ondary constriction (Fig. 2a). A small amount of heterochromatin was detected in the centromeric region of almost all chromosomes, and faint C-bands were observed interstitially in pair 4 as well as adjacent to the centromere of pair 10, coincident to the NOR (Fig. 2a, b).

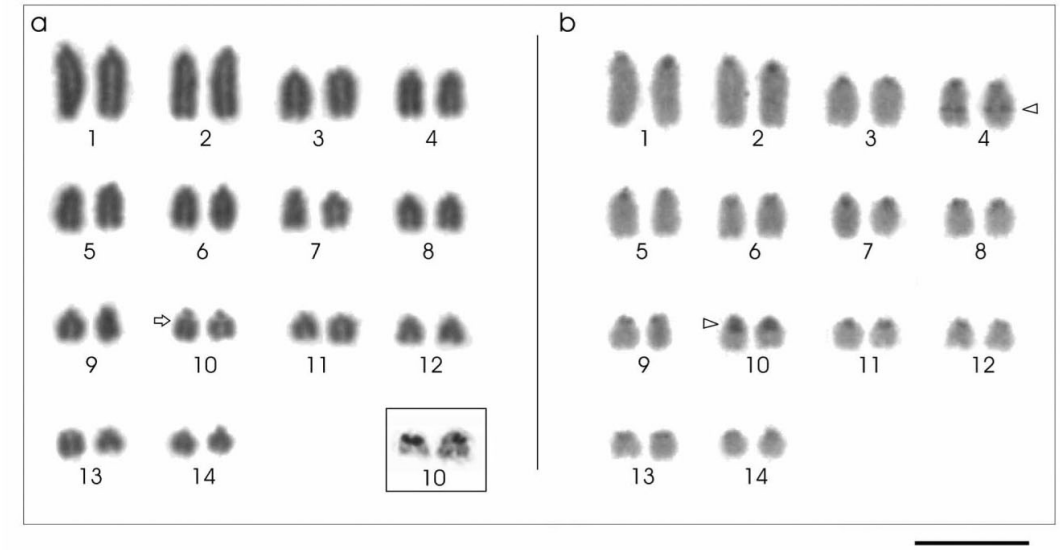

FIGURE 2. Karyotype of *Pristimantis* aff. *dundeei*: (**a**) Giemsa and Ag-NOR staining; (**b**) C-banding. The arrow indicates secondary constrictions, which are coincident with the NOR position (inset). The arrowheads indicate the interstitial heterochromatin. Bar = 10 µm.

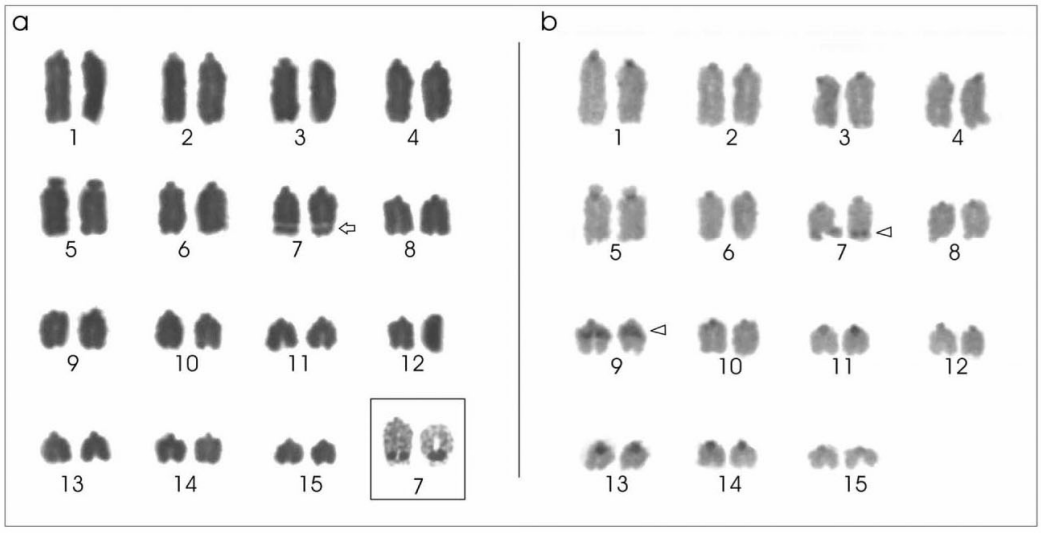

FIGURE 3. Karyotype of *Ischnocnema paulodutrai*: (**a**) Giemsa and Ag-NOR staining; (**b**) C-banding. The arrow indicates secondary constrictions. The arrowheads indicate interstitial heterochromatin. Inset: the Ag-NOR bearing chromosomes. Bar = 10 µm.

The *I. paulodutrai* karyotype consisted of 2n=30 chromosomes and FN 32, with all pairs classified as telocentrics, except for the subtelocentric pair 5 (Fig. 3a, b). The Ag-NOR sites were located at the subtelomeric regions of pair 7, coincident with a secondary constriction (Fig. 3a). A small amount of heterochromatin was detected in the centromeric region of almost all chromosomes. Large blocks of heterochromatin were found interstitially in pair 9, and at the subtelomeric region of pair 7, coincident with the secondary constriction and NOR (Fig. 3a, b). Heterochromatic blocks, similar to the latter ones were also identified in meiotic cells of male specimens, in which 15 bivalents were found (Fig. 4a, b). In the analyzed samples, there were no detectable karyotypical differences between the two distinct morphological types of *I. paulodutrai* specimens. Metaphases with chromosomes associated by thin chromatin bridges, mainly between their telomeric and centromeric regions (Fig. 5a-d), were observed in the specimens of all taxa.

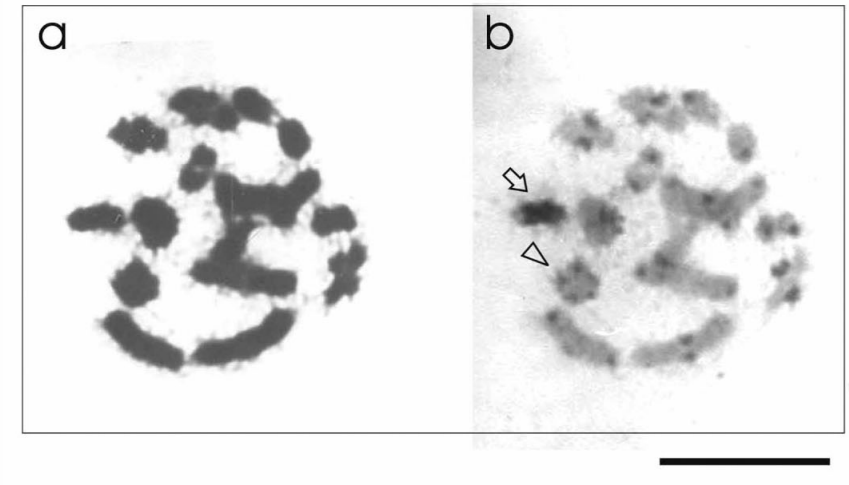

FIGURE 4. Diplotene of *I. paulodutrai*: (**a**) Giemsa- staining; (**b**) C-banding showing bivalents and centromeric heterochromatin in all pairs. The arrow indicates interstitial heterochromatin in the pair 9. The arrowhead indicates the NOR-bearing chromosome pair. Bar = 10 μm.

Discussion

The diploid number of 28 chromosomes, as described for *P. dundeei* and *P.* aff. *dundeei*, has been previously reported in other "eleutherodactylines", comprising ten species from Central America and one from the United States. Of those eleven species, *Eleutherodactylus* (*Euhyas*) *bakeri*, *E.* (*Eleutherodactylus*) *amplinympha*, *E.* (*Eleutherodactylus*) *martinicensis* (Kaiser *et al.* 1994), and *E.* (*Eleutherodactylus*) *johnstonei* (Kaiser *et al.* 1994; Bogart & Hedges 1995) remain in the *Eleutherodactylus* genus (Heinicke *et al.* 2007; Frost, 2007; Hedges *et al.* 2008). The chromosome number 2n=30, found in *I. paulodutrai*, has been described in twelve Central America species of "eleutherodactyline", including one *Pristimantis* species*, P. urichi* (Schmid *et al.* 2002). In Brazilian species, however, this is the first description of 2n=28 and 2n=30 chromosomes.

Despite the coincident chromosome numbers (2n=28 and 2n=30), as well as high number of telocentric ones, the Brazilian and Central America species are clearly differentiated by their chromosome fundamental number (FN). The Brazilian species were characterized by FN=28 in P. dundeei (2n=28) and P. aff. dundeei, and FN=32 in *I. paulodutrai* (2n=30), whereas the Central America *Eleutherodactylus* (either sensu lato or stricto) have been reported as FN=32 in 2n=28 species, and FN=36, 40 or 58 in 2n=30 species (León 1970, Bogart 1970a, 1970b, 1970c, 1981, 1991, DeWeese 1975, Savage & DeWeese 1979, 1980, Miyamoto 1983,

1984, Bogart & Hedges 1995, Kaiser *et al.* 1994, 1995, Schmid *et al.* 2002). Therefore, the data suggest that distinct mechanisms of chromosomal differentiation have originated the 2n=28 and 2n=30 karyotypes of the analyzed Brazilian species. High diploid chromosome numbers and similar chromosome morphology suggested that the *Eleutherodactylus* species in Central America are more closely related to the 2n=34 species native to northern Brazil, than to the species with 2n=20 and 2n=22, of the southern and southeastern Brazilian regions. The karyotypes of *Ischnocnema* species are in agreement with this hypothesis. In addition, the hypothesis is strongly reinforced by the recent assignment of former *Eleutherodactylus* species into the genus *Ischnocnema*, on basis of DNA sequence analyses (Heinicke *et al.* 2007), which included *I. guentheri*, *I. parva* and *I. juipoca* (all characterized by small chromosome number). Therefore, the karyotypes clearly distinguished these three southern *Ischnocnema* species from the *Pristimantis* species found in the Brazilian northern region.

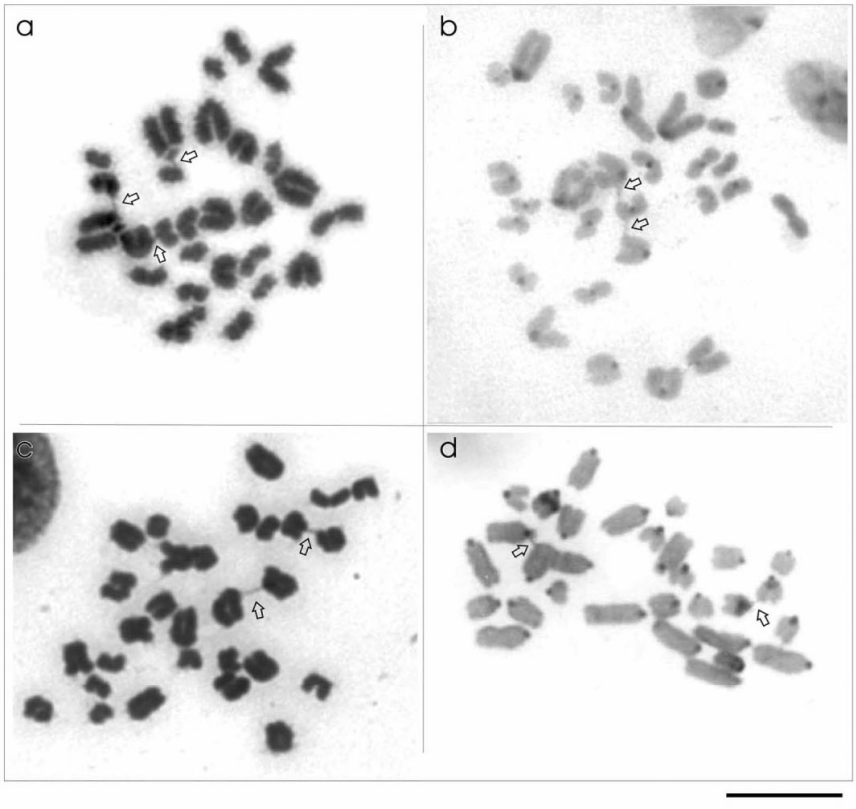

FIGURE 5. Giemsa-stained (**a** and **c**) and C-banded (**b** and **d**) metaphases showing chromosomes associated by thin filaments (arrows) in *P. dundeei* and *I. paulodutrai*, respectively. Bar = 10 µm.

However, in the midwestern region, at Mato Grosso State, *Pristimantis* species already analyzed were characterized by high and small chromosome numbers. The *P. dundeei* species has 2n=28, as described herein, whereas *P. crepitans* has 2n=22 (Siqueira *et al.*, unpublished data).

Ischnocnema paulodutrai is the first northeastern Brazilian species of this genus to be karyotyped. Although it was included in the southeastern group by Heinicke *et al.* (2007) and Hedges *et al.* (2008), its species series, *Ischnocnema ramagii*, occurs in the isolated remnants of Atlantic Coastal Forest in the states of Paraíba, Pernambuco and Bahia in northeastern Brazil.

The karyotype of *I. paulodutrai*, as described in the present work, suggests that it might be more closely related to the *P. dundeei* and *P.* aff. *dundeei* species, than to the remaining congeneric *Ischnocnema* species in Brazil, with low (2n=20 and 22) chromosome numbers. Such variability in "eleutherodactyline" is an elucidative example that high variation in chromosome number indicates polyphyletic groups that deserve a further taxonomic investigation.

The *P.* aff. *dundeei* karyotype showed diverse NOR-bearing and C-band pattern as compared to the *P. dundeei* populations, and constitute strong evidence that most likely they are two distinct species. The chromosome morphology, NOR locations and heterochromatin pattern has been useful to distinguish among different species, as already described for other anurans - e.g. *Physalaemus petersi* (Lourenço *et al.* 1999), *Pseudis minuta* and *Pseudis* aff. *minuta,* currently *Pseudis cardosoi* (Busin *et al.* 2000), *Dendropsophus nanus* and *D. sanborni* (Medeiros *et al*. 2003), *Scythrophrys* species (Lourenço *et al*. 2003a, 2008), *Paratelmatobius* (Lourenço *et al.* 2003b*,* 2008) and *Pristimantis fenestratus* and *Pristimantis* aff. *fenestratus* and *Barycholos ternetzi* and *Barycholos* aff. *ternetzi* (Siqueira *et al.* in preparation).

Such existence of a new species is also supported by the fact that *P.* aff. *dundeei* is out of the area of *P. dundeei* distribution, proposed by Heyer & Muñoz (1999), as well as by their differences in ecological, behavioral and acoustical characteristics (Christine Strüssmann, personal communication). On the other hand, both high similarity in chromosome morphology and undifferentiated interstitial C-band distribution in the pair 4 indicated that *P.* aff. *dundeei* and *P. dundeei* are closely related taxa.

In the analyzed *I. paulodutrai* specimens, the identical karyotypes between those distinguished by dorsal polymorphism reinforce that cytogenetically these two morphotypes belong to the same species. Vila Flor *et al.* (2004) described similar dorsal polymorphism in *Eleutherodactylus* (currently *Ischnocnema*) populations from Salvador, Bahia State, and from Maceió, Alagoas State, both in the northeastern Brazil. However, such polymorphism was not found in an *Ischnocnema* population sampled by those authors in Ilhéus, Bahia State. Based on those data, the authors concluded that the species of Ilhéus, Bahia state, which had only one color pattern, might be *I. paulodutrai* and the one from Salvador and Maceió presenting both patterns, might be *I. ramagii.* However, our specimens sample from Ilhéus (Bahia), the type-locality of the species, exhibited also two color patterns and all of them were here identified as *I. paulodutrai.* Considering these data, we suggest that the color polymorphism, which was previously described only in *I. ramagii*, is also present in *I. paulodutrai* being a case of homologous polymorphism among these *Ischnocnema* species (see De la Riva 1997).

Metaphases of *E.* (*Euhyas*) *glaucorieus, E.* (*Euhyas*) *bakeri*, and *E.* (*Pelorius*) *nortoni*, as reported by Bogart (1991), exhibited interchromosome thread connections in metaphase plates, although the authors did not mention this phenomenon. The observed associations seem very similar to those here described in metaphases of *I. paulodutrai* and *P. dundeei*. Furthermore, in *P. fenestratus* from Rio Branco, Acre state, Brazil, similar interchromosome threads between chromosomes and intra-individual variation in chromosome number have been found (Siqueira *et al.* unpublished data). Although intraspecific variation in chromosome number was not observed within the species *P. dundeei* and *I. paulodutrai*, such thread associations may also have an important role in the chromosome number alteration during the speciation in the "eleutherodactyline" taxa.

The karyotypic data on *P. dundeei*, *P.* aff. *dundeei* and *I. paulodutrai* populations, described in the present work, represent a forward step in the understanding of Brazilian "eleutherodactyline" species regarding their evolutionary relatedness and additional understanding of their taxonomic status. A further analysis of *P.* aff. *dundeei* as well as *I. paulodutrai* is needed to obtain a more solid taxonomic reassessment of these species.

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V. Artigo 2

Unusual karyotypical variation and evidence of cryptic species in Amazonian populations of *Pristimantis* frogs

Unusual karyotypical variation and evidence of cryptic species in Amazonian populations of *Pristimantis* frogs

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Key-words: *Pristimantis*, karyotype, intra-individual variation, telocentric chromosomes

Running title: Cytogenetics of *Pristimantis fenestratus*

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Abstract

We report a cytogenetic analysis of three *Pristimantis fenestratus* frog populations of the Amazon region in Brazil. The specimens were sampled in Borba and Manaus, Amazonas State, and in Rio Branco, Acre State. They were analyzed using Giemsa, silver staining, C-banding and FISH with rDNA probes. The karyotypes of the three populations revealed 2n=34 chromosomes, but they differed in the number and position of Ag-NORs and in the heterochromatin pattern as well. The NOR was located on the pairs 05 and 07 in the Rio Branco specimens, pair 10 in the Manaus specimens, and pair 1 in the Borba specimens. A small C-band was detected on the telomeric region of the pair 05 in the Borba population, while in the Manaus there was a heterochromatic block adjacent to the centromere of pair 9. An unusual intra-individual variation of chromosome number was observed in metaphases of Rio Branco specimens, comprising fundamental numbers of 33, 34 and 35. Additionally, interchromosome thread connections were detected between telomere-telomere, centromere-telomere and centromere-centromere regions, and among chromosomal heterochromatin-rich sites. The NOR sites were also involved in those connections. We hypothesize that this variation is due to chromosome missegregation during mitosis. The inter- and intraindividual variation in chromosome number suggests chromosomal instability in *P. fenestratus*, which has not been detected in any other anuran group. Since Borba is the type-locality of *P. fenestratus*, a taxonomic review of the Manaus and Rio Branco populations should be done, as indicated by the cytogenetic evidence that they could be new species of *Pristimantis*.

Introduction

The "eleutherodactyline" frogs (*sensu* Frost *et al.* 2006) are undergoing nominal restructuring and partitions into new taxa. Recently, Frost *et al.* (2006) divided the genus *Eleutherodactylus* into five genera named *Craugastor* (116 sp.)*, Euhyas* (94 sp.)*, Pelorius* (6 sp.)*, Syrrhopus* (24 sp.) and *Eleutherodactylus* (492 sp.), and assigned them to the family Brachycephalidae. Afterwards, Heinicke *et al.* (2007) proposed about 400 species of *Eleutherodactylus* based on DNA sequences. They were grouped into a large clade, referred to as the South American Clade, for which the new generic name *Pristimantis* was suggested. Recently, Hedges *et al.* (2008) allocated the 882 described "eleutherodactyline" species into a new taxon named Terrarana divided into four families, Eleutherodactylidae, Craugastoridae, Brachycephalidae and Strabomantidae.

Nearly 29 *Pristimantis* species are found, or presumably occur, in the North and Central-Western Brazil (Frost 2008; Heinicke *et al.* 2007). Twenty of the *Pristimantis* species have already been cytogenetically analyzed, including the five Brazilian representatives *P. conspicillatus* (Bogart 1970a, 1973a; DeWeese 1975), *P. lacrimosus* (DeWeese 1975) and *P. altamazonicus* (Bogart 1970a, 1970c, DeWeese 1975), all with 2n=34 chromosomes, and *P. lanthanites* (Bogart 1973b) and *P. ventrimarmoratus* (Bogart 1970a, 1970b), both with 2n=36.

Cytogenetic analysis has been successfully applied to anuran groups as a valuable tool to investigate chromosome evolution, infer species relationships, corroborate suggestion of new species, and differentiate cryptic species (Giaretta and Aguiar 1998; Aguiar *et al.* 2002, Medeiros *et al.* 2003; Lourenço *et al.* 2006; Ananias *et al.* 2007). The understanding of chromosome evolution in "eleutherodactyline" frogs has been limited by a low number of cytogenetic studies, contrasting with the recent advances in molecular taxonomy.

We compared karyotypes of *Pristimantis fenestratus* specimens from its type-locality in the Amazon region of Brazil to other two populations attributed to the same taxon.

Material and Methods

Specimens

The analyzed *Pristimantis fenestratus* populations were sampled in three Amazonian locations . The samples consisted of twelve specimens (5 males and 7 females) from Borba, Amazonas State, which is the species type-locality, plus eighteen specimens (three males and 15 females) from the Reserva Florestal Adolpho Ducke, Manaus, Amazonas State, and thirty-one (12 males and 19 females) from the Parque Zoobotânico of the Universidade Federal do Acre (UFAC), Rio Branco, Acre State. Sampling was done under permission of the Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA - Proc. 02010.000025/2005-51). Voucher specimens from Rio Branco (accession numbers in Table 1), while those from Borba (INPA-H - 20.926-20.937) and Manaus (ZUEC - 13.327-13.338; INPA-H - 20.920-20.9925). Were deposited in the Museu de Zoologia "Prof. Dr. Adão José Cardoso" (ZUEC), at the Universidade Estadual de Campinas (UNICAMP), São Paulo, Brazil, and in the Instituto Nacional de Pesquisa da Amazônia (INPA), Manaus, Amazonas, Brazil.

Chromosome preparation and techniques

Mitotic chromosomes were obtained from intestinal epithelium and testis cell suspensions of frogs previously treated with colchicine 2% for about 4h, as described by King and Rofe (1976) and Schmid (1978). The slides were stained with 10% Giemsa solution and processed for C-banding technique (Sumner 1972), with a modification in the pre-treatment as described by Siqueira *et al.* (2008). In addition, chromosomes were analyzed by Ag-NOR staining (Howell and Black 1980) and fluorescent *in situ* hybridization (FISH) (Viegas-Péquignot 1992) using the HM123 recombinant plasmid containing a rDNA fragment of *Xenopus laevis* (Meunier-Rotival *et al.* 1979) as a probe. Metaphases were examined with a BX60 Olympus microscope and the images captured using Image Pro-Plus 5.1 software. The chromosomes of twenty metaphases from each of the three populations were measured and classified according to Green and Sessions (1991).

Results

Chromosome morphology, NOR and heterochromatin distribution

The karyotypes of the three *P. fenestratus* populations consisted of a diploid number of 34 chromosomes and all pairs were telocentric (figs. 1-3). The fundamental number (FN) was also 34. The Ag-NOR sites were located on the telomeric region of pair 1 in the Borba population, adjacent to the centromere of the pair 10 in the Manaus individuals and on telomeric regions of pairs 5 and 7 in the Rio Branco specimens (figs. 1b, 2b and 3b). In the three populations, small amounts of heterochromatin were detected at the centromeric region of almost all chromosomes (figs. 1c, 2c and 3c). In the Borba specimens there was an interstitial band in pair 6 and a faint additional C-band on the telomeric region of pair 1, which was coincident with the NOR (fig. 1c). In the Manaus population, blocks of heterochromatin were detected adjacent to the centromere in pairs 1 and 10, and interstitially in pair 9 (fig. 2c). The heterochromatic block of pair 10 was coincident with the NOR. In the Rio Branco specimens, heterochromatic blocks were observed on the telomeric region of pairs 1 and interstitially in pairs 5 and 8 (fig. 3c). The telomeric band of pair 5 was coincident with the NOR (figs. 3b and 3c).

Karyotypical variations in the Rio Branco population

Intraindividual variation in the fundamental number (FN=34) was observed in fourteen out of twenty-seven specimens from the Rio Branco population. About 1.8% of the metaphases within nine specimens had FN=35 due to the presence of a small metacentric or an extra telocentric chromosome (figs. 4a, b, 5d; Table 1). In thirteen specimens, 39 (3,8%) of 1028 metaphases had FN=33 due to the absence of one chromosome (monosomy) (fig. 4c; Table 1). The chromosomes were so similar in size and morphology that it was not feasible to identify the telocentric homologues forming metacentric chromosomes, or to determine if the metacentric chromosome arms were composed of homologues from the same pair and precisely recognize the monosomic chromosome.

All *P. fenestratus* male and female specimens showed several metaphases with interchromosome thread connections, which were observed as centromeric-telomeric, telomeric-telomeric and centromeric-centromeric connections between few chromosomes (fig. 5a-h). These connections were obvious in Giemsa stained (fig 5a-f) and C-banded (fig 5g-h) metaphases as well as in the *in situ* hybridization (figs. 6f, g, m, n). Thread connections of sister chromatids were observed between Ag-NOR and rDNA fluorescent sites (fig. 6h, j). Additionally, an intriguing intra-individual variation in the NOR number was detected by silver staining in 23 out of 26 specimens and confirmed by the *in situ* hybridization with the rDNA probe. The number of NOR-bearing chromosomes ranged from three to five (figs. 6a-c, i-k). In 4 individuals, metaphases NOR size heteromorphism was detected between homologues and in the specimens between sister chromatids (fig. 6b-e, l).

Discussion

Karyotypes

The diploid number of 34 telocentric chromosomes, as observed in *P. fenestratus*, was previously reported in the *Pristimantis* species *P. altamazonicus* (Bogart 1970a, Bogart 1970b, DeWeese 1975), *P. bogotensis* (DeWeese 1975), *P. conspicillatus* (Bogart 1970a, 1973a, DeWeese 1975), *P. gaigeae* (Bogart 1973b), *P. lacrimosus* (DeWeese 1975), *P. variabilis* (Bogart 1970a), *P. ridens* (DeWeese 1975, Miyamoto 1984), as well as in *Eleutherodactylus* (*Eleutherodactylus*) *varians* (Bogart 1970a). All the *Pristimantis* 2n=34 karyotypes are highly similar to *P. fenestratus* regarding their chromosome morphology; however, these species differ in the

fundamental number, which is 34 in *P. fenestratus* and 36 in all the other mentioned species, which have one metacentric or one submetacentric pair. Only one species of this genus, *P. crepitans,* showed a lower number of 2n=22 chromosomes and this is also the only species *Pristimantis* living in xeric habitats, which led Siqueira *et al*. (in press) to suggest that this species is not closely related to its congeneric species.

The specimens of the three studied *Pristimantis fenestratus* populations are similar in their phenotype and in chromosome morphology. However, they diverge in number and position of NORs, and in the C-band pattern. Because Borba is the *P. fenestratus* type-locality, we suggest that the individuals from Rio Branco and from Reserva Ducke might be two undescribed species. The cytogenetic data allied to their differences in the acoustical characteristics (Albertina P Lima, personal observation) strongly support this hypothesis and indicate that a reevaluation of the taxon *P. fenestratus* is necessary.

The analyses of chromosome morphology, NOR localization and heterochromatin pattern have been successfully used to distinguish anuran cryptic species. For instance, Medeiros *et al.* (2003) discriminated *Dendropsophus nanus* and *D. sanborni* based on their differences in the number of telocentric and submetacentric chromosomes and primary NOR-bearing chromosomes. These cytogenetic characteristics allowed unambiguous identification of syntopic individuals of those two species that are similar morphologically. In a previous study, two distinct karyotypes, including sexual chromosomes distinguished specimens within a *Physalaemus petersi* population in Acre, Brazil (Lourenço *et al.* 1999). *Scythrophrys* populations were discriminated by karyotypes as well, and the presence of two distinct taxonomic units in those populations was proposed by Lourenço *et al*. (2003a). In *Paratelmatobius*, a new species was described in the *P. cardosoi* group based on differences in the NOR and heterochromatin location (Lourenço *et al.* 2003b).

Interchromosome connections and intra-individual variation

The data described herein represent a first report in anurans of thread connections between chromatids and between chromosomes in mitotic cells, and of intra-individual variation in chromosome number and morphology. In a previous work, Bogart (1991) reported an interindividual chromosome number variation, which was also observed in the *P. fenestratus* populations analyzed in the present study.

The unusual intra-individual variation in NORs and chromosome number of the *P. fenestratus* population from Rio Branco (não entendi o que que voce quer dizer aqui, parece contraditório) is possibly related to the interchromosome thread connections. The presence of an extra free telocentric, a metacentric or a monosomy in mitotic cells might be a consequence of a mitotic missegregation of sister chromatids, since there were telomeric-telomeric thread connections, including the NOR-bearing chromosomes, and centromeric-telomeric thread connections as well. This hypothesis could explain the intra-individual occurrence of metaphases with FN=33 and FN=35, because the metaphases with FN=35 had a free extra telocentric chromosome. Consequently, metaphases with FN=33 may have arisen from the a cell that lost a telocentric through the anomalous segregation during the mitosis. Alternatively, the metacentric (FN=35) could be an isochromosome.

Intra-individual chromosome variation was previoulsy reported in one specimen of the fish *Trichomycterus davisi* (2n=54) that exhibited metaphases ranging from 2n=52 to 2n=56 chromosomes. This variation was attributed to a post-zygotic non-disjunction of a metacentric chromosome, followed by a spontaneous centric fission or to chemical or physical agents, possibly favored by the intense aggression to the urban effluent Iguaçú river in the Paraná State of Brazil (Borin and Martins-Santos 2000). In the present study, the intra-individual chromosome variation of the *Pristimantis fenestratus* population from Rio Branco was observed in 14 (52%) out of the 27 specimens, and there was no evidence an environmental or any other exogenous contributing factor.

The intra-individual variation in number of NOR sites could also be explained by missegregation of the chromatids randomly associated by telomeres, since there were also thread connections between NOR sites at telomeres. Additionally, the chromosome number variation and NOR connections were clearly associated with heterochromatin, as demonstrated by C-banding, indicating that these connections involving telomeric and NOR heterochromatin could be repetitive DNA sequences such as satellite DNA, as observed by Kuznetsova *et al.* (2007) in mouse and human cell lineages.

In human cells, chromosome connections between NOR regions were reported by Tuck-Muller *et al.* (1984) as, most often, associated to chromosomal regions containing constitutive heterochromatin, especially that of centromeric regions. The authors suggested the occurrence of NOR transfer in humans, possibly by transposable genetic elements localized in heterochromatin

sequences. Moreover, they considered that this phenomenon could explain the silver connection associations within heterochromatic regions in different cell lineages of mice and humans, and showed that these connections are composed of satellite DNA of the centromeric and telomeric heterochromatin. We have no conclusive evidence about the composition of the DNA involved in the associations in *P. fenestratus*, but the FISH and C-banding results suggested that the connections might have repetitive sequences related to rDNA and C-banding positive heterochromatin.

Conclusions

The karyotypical differences in the NOR position and banding pattern found in the *Pristimantis fenestratus* populations from Manaus and Rio Branco compared to *P. fenestratus* from the Borba type-locality indicate the existence of previously undescribed d species in this taxon. The population from Rio Branco differed from the others by presenting an unusual intra-individual variation in chromosome number and in NOR number and position, not observed in the Borba and Manaus populations. The intra-individual variation in *P. fenestratus* specimens from Rio Branco suggests chromosomal instability and plasticity not previously reported for anurans. Such chromosomal rearrangements, when occurring in germinative cells, could contribute to karyotype differentiation among species.

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Table 1. Number of metaphases in specimens of *P. fenestratus* from Rio Branco population.

	Specimens (Museum Accession Number)	34T FN=34	33T FN=33	35T FN=35	33T+1M FN=35	Total of analyzed metaphases	% of altered metapahses in each specimen
Female	13300	37	--	--	--	37	--
	13301	58	5	2	--	65	10,8%
	13302	67	5	--	1	73	8,2%
	13307	6	--	--	--	6	--
	13316	18	1	--	2	22	13,6%
	13320	18	2	--	--	20	10,0%
	13322	23	1	--	--	24	4,2%
	13325	25	--	--	--	25	--
	13326	16	2	--	1	19	15,8%
	14103	43	2	1	--	46	6,5%
	14104	56	--	--	--	56	--
	14106	34	--	--	--	34	--
	14107	27	--	--	--	27	--
	14109	19	2	--	1	22	13,6%
Male	13304	5	--	--	--	5	--
	13308	30	--	--	--	30	--
	13309	32	--	--	--	32	--
	13311	60	2	--	--	62	3,2%
	13312	36	3	--	--	39	7,7%
	13314	114	7	4	1	126	9,5%
	13315	56	--	--	--	56	--
	13317	88	--	--	--	88	--
	13321	54	5	4	--	61	11,5%
	13323	6	--	--	--	6	--
	13324	26	2	--	--	28	7,1%
	14105	16	--	1	--	17	5,9%
Total Number		**971**	**39**	**12**	**6**	**1028**	**5,4%**
Total %		**94,4%**	**3,8%**	**1,2%**	**0,6%**	**100,0%**	

Analyzed mitotic metaphases with regular diploid number (2n=34) and with chromosome number variation (33 or 35 telocentrics). T=Telocentic; M=Metacentric; FN= Fundamental Number.

Legends

Figure 1. Karyotypes of *Pristimantis fenestratus* from Borba: (**A**) Giemsa staining; (**B**) Ag-NOR staining; (**C**) C-banding. The arrow indicates the NOR. The arrowheads indicate the interstitial and telomerical heterochromatins. Note the complete metaphase plates beside the karyogram. Bar = 10 μm.

Figure 2. Karyotypes of *Pristimantis fenestratus* from Reserva Ducke: (**A**) Giemsa staining; (**B**) Ag-NOR staining; (**C**) C-banding. The arrow indicates the NOR. The arrowheads indicate the interstitial and pericentromerical heterochromatins. Note the complete metaphase plates beside the karyogram. Bar = 10 μm.

Figure 3. Karyotypes of *Pristimantis fenestratus* from Rio Branco: (**A**) Giemsa staining; (**B**) Ag-NOR staining; (**C**) C-banding. The arrow indicates the NORs. The arrowheads indicate the interstitial and telomerical heterochromatins. Note the complete metaphase plates beside the karyogram. Bar = 10 μm.

Figure 4. Giemsa stained karyotypes of *Pristimantis fenestratus* from Rio Branco; (**A**) 34 chromosomes, FN=35, with a metacentric chromosome; (**B**) 35 chromosomes, FN=35, with an extra telocentric chromosome; (**C**) 33 chromosomes, FN=33 with absence of a chromosome. The arrow indicates the metacentric chromosome in the metaphase plate. Bar = 10 μm.

Figure 5. Metaphases of *Pristimantis fenestratus* from Rio Branco: Giemsa-stained (**A - C**) and C-banded metaphases (**G** and **H**) showing chromosomes associated by thin filaments (arrowheads). The arrow indicates a metacentric chromosome. Bar = 10 μm.

Figure 6. Metaphases of *Pristimantis fenestratus* from Rio Branco: in situ hybridization (**A – H**) and Ag-NOR staining (**I-N**). Note the four NORs (**A, B** and **I**), five NORs (**C** and **K**), and three NORs (**D, E** and **J**) indicated by arrowheads; NOR thread connections (**F–H, J, M** and **N**), NOR heteromorphism between chromosomes (**B, C, E** and **H**) and between sister chromatids (**D, G** and **L**) indicated by arrows.

Figure 1

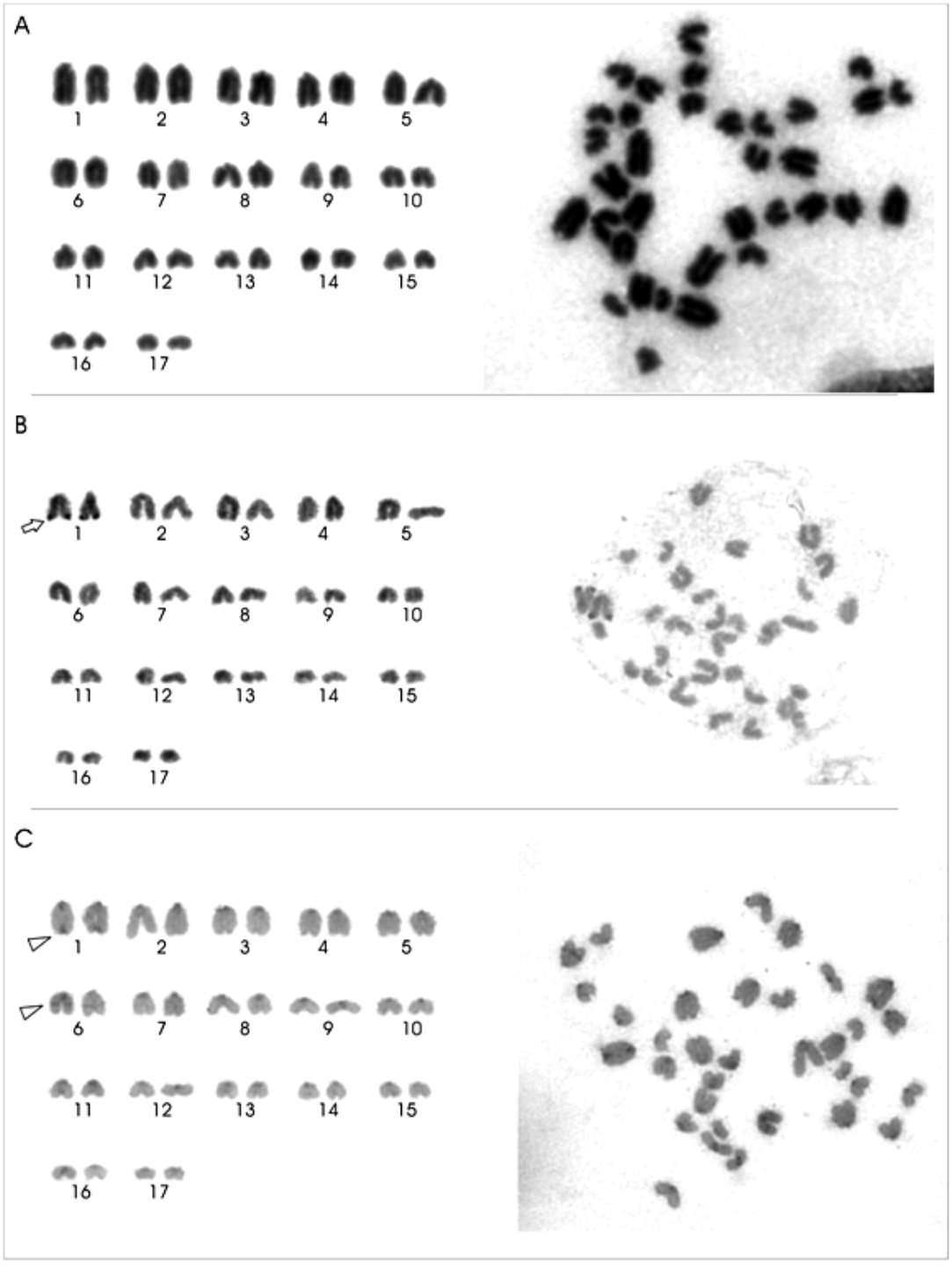

A
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17
B
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17
C
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17

Figure 2

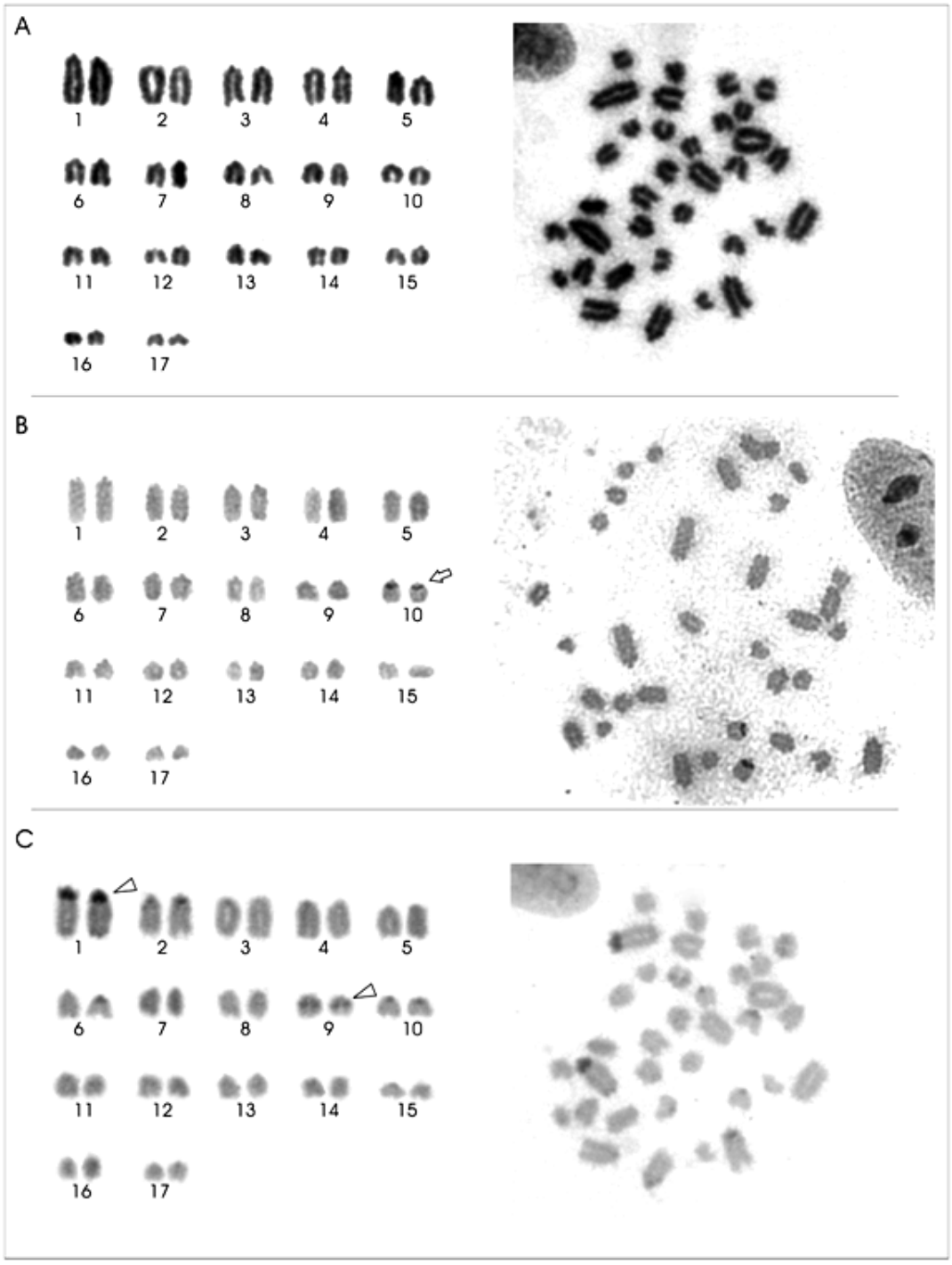

A
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17
B
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17
C
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17

Figure 3

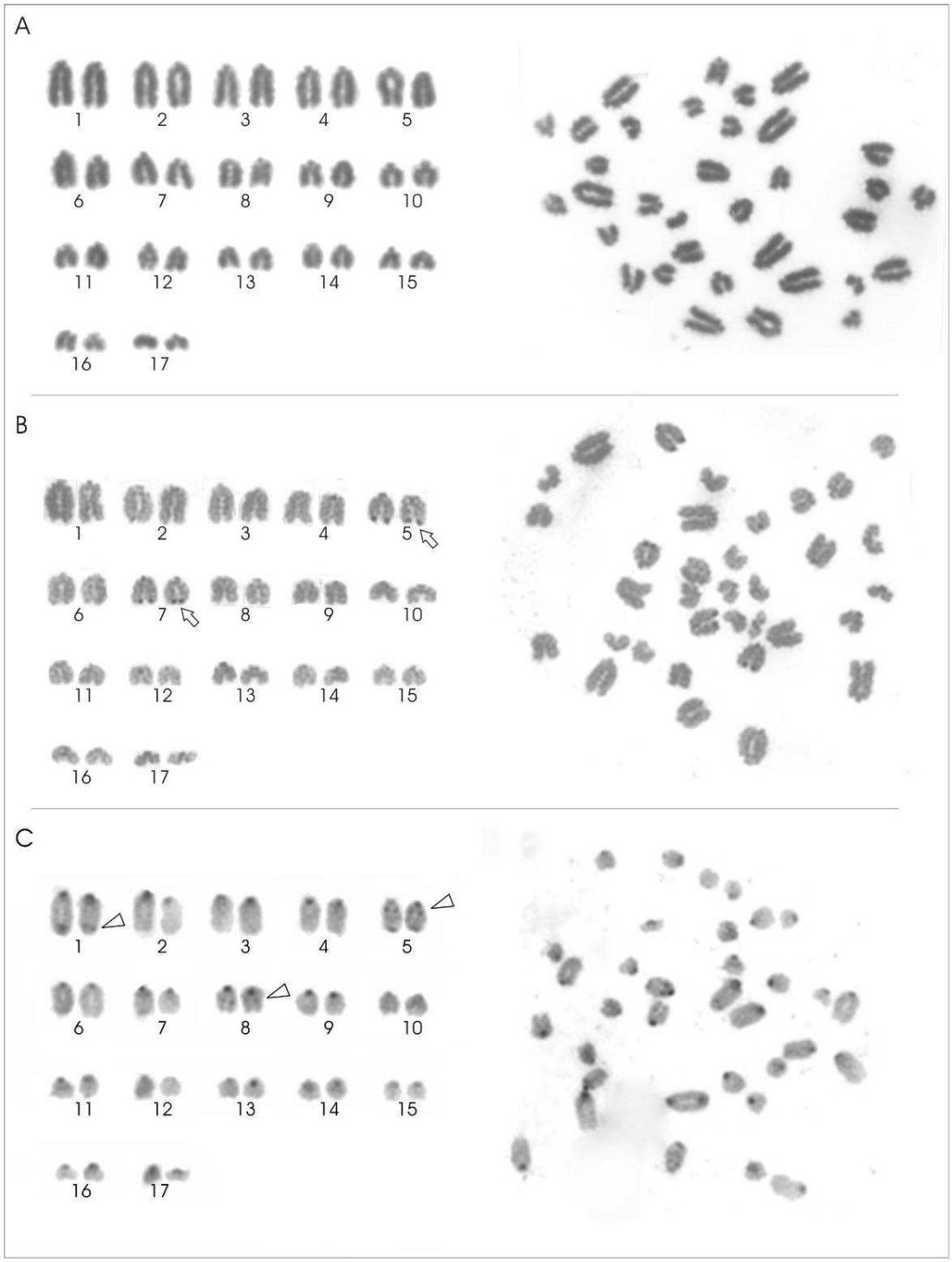

A
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17
B
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17
C
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17

Figure 4

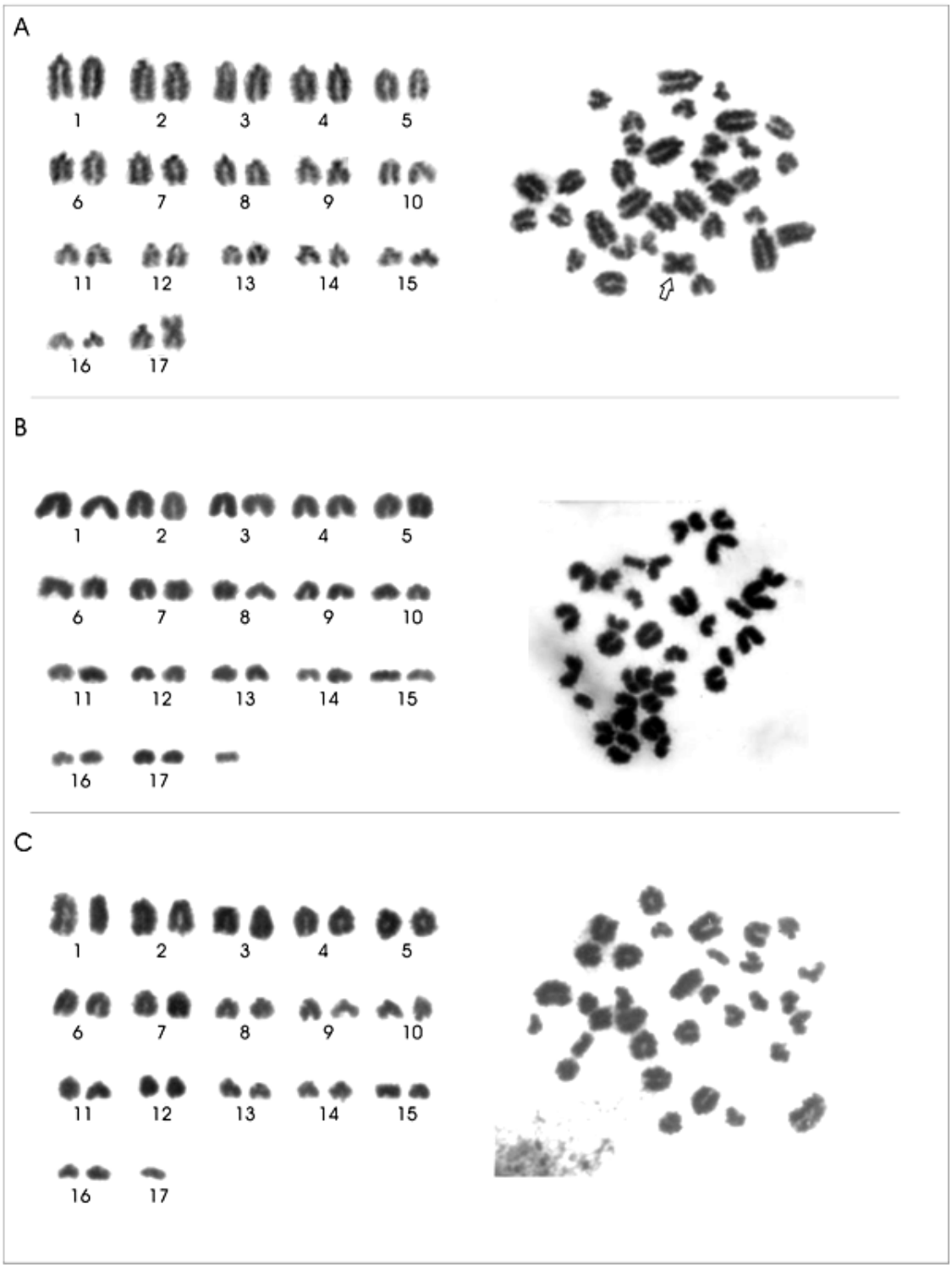

A
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17
B
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17
C
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17

Figure 5

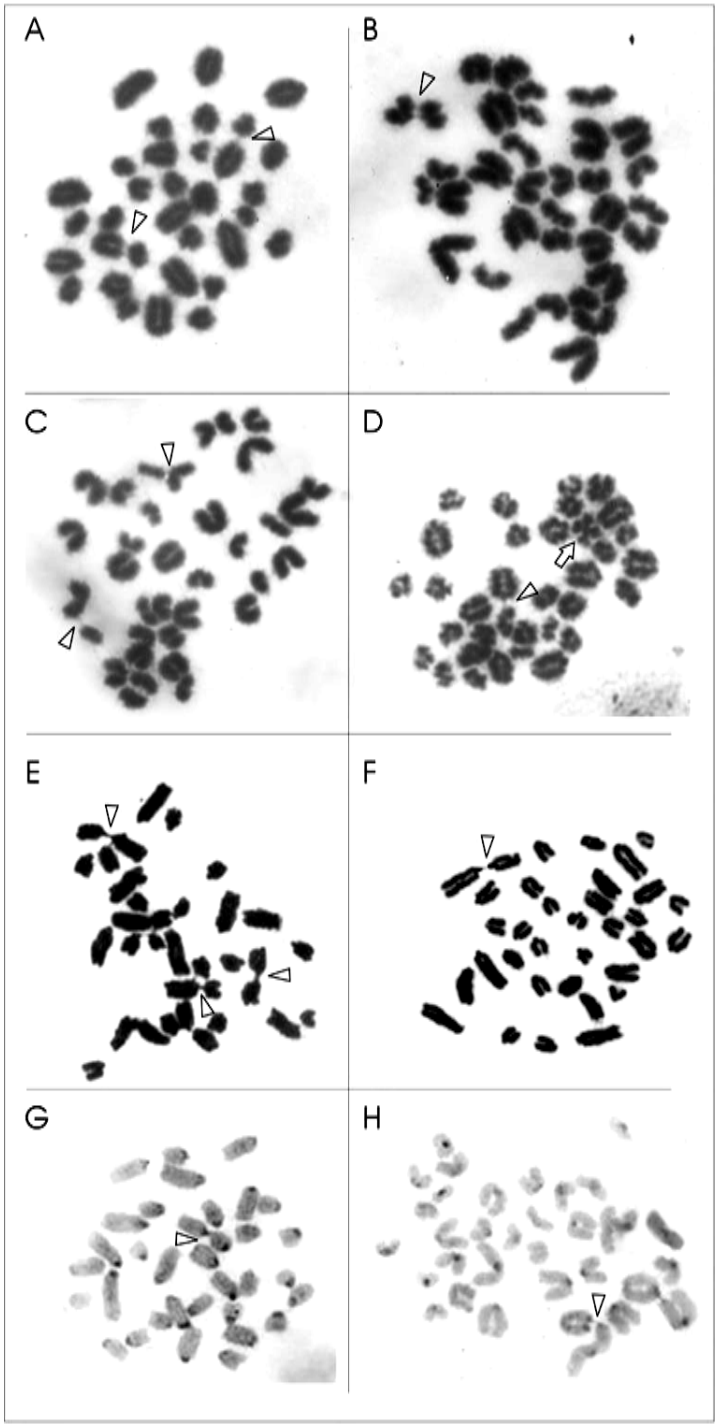
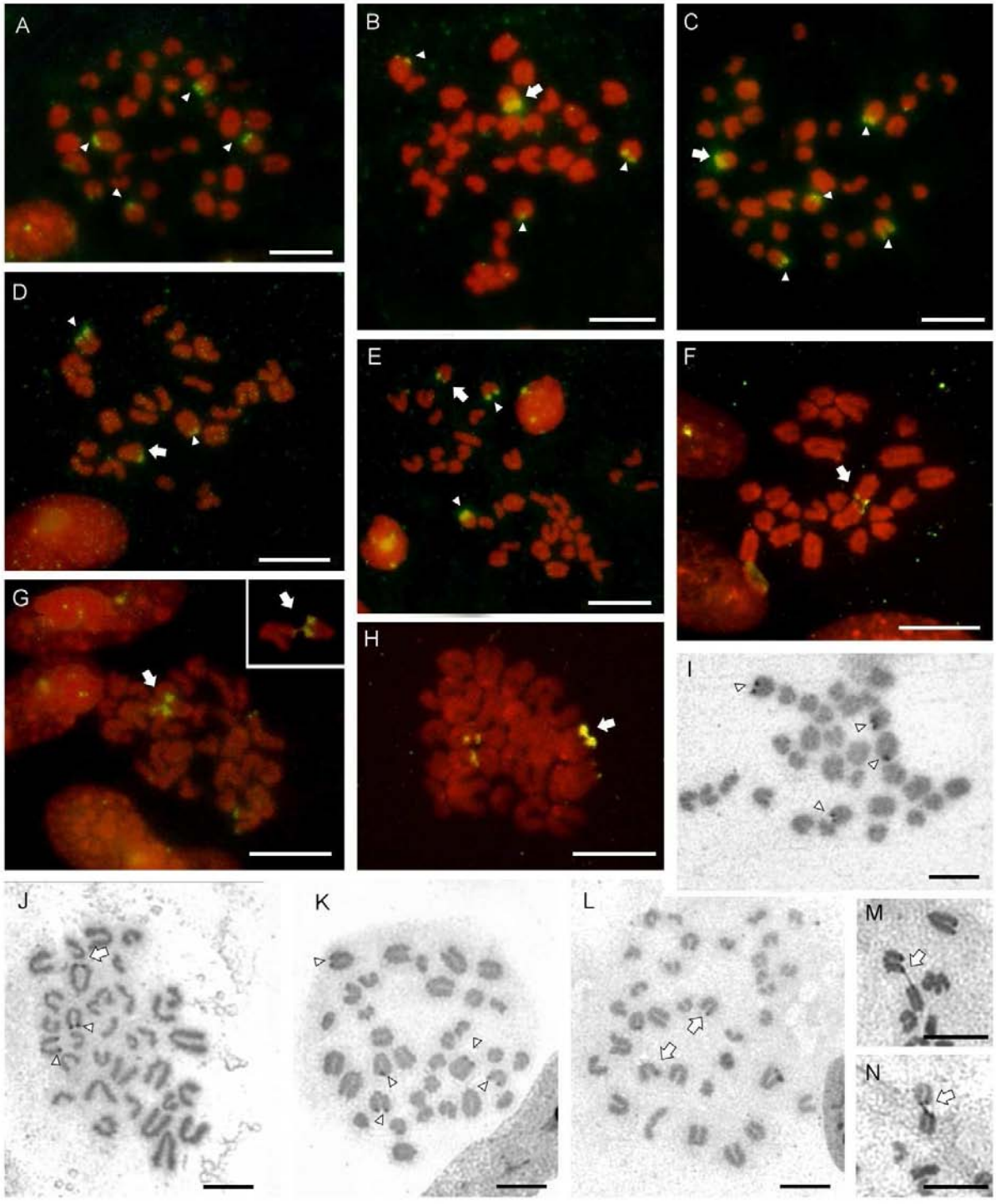


Figure 6



VI. Artigo 3

The karyotype of three Brazilian Terrarana frogs (Amphibia, Anura) with evidence of a new species of *Barycholos*

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The karyotype of three Brazilian Terrarana frogs (Amphibia, Anura) with evidence of a new species of *Barycholos*

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Key-words: Cytogenetics, *Barycholos, Ischnocnema, Pristimantis*, Ag-NOR, C-banding

Running title: Cytogenetics of Brazilian Terrarana frogs

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Abstract

A recent substantial rearrangement of the 882 described species of “eleutherodactyline" frogs has considerably improved the understanding of their systematics. However, many taxonomic aspects of the South America eleutherodactyline species remain unknown and require further investigation using morphological, cytogenetic, and molecular approaches. In this work, the karyotypes of the Brazilian species *Ischnocnema juipoca* (Atibaia and Campos do Jordão, SP), *Barycholos* cf. *ternetzi* (Uberlândia, MG, and Porto Nacional, TO), and *Pristimantis crepitans* (Chapada dos Guimarães and São Vicente, MT) were analyzed using the Giemsa staining, Ag-NOR, and C-banding techniques. All individuals had a diploid number of 22 chromosomes, but the Fundamental Numbers were different among species. The herein described low chromosome number of *Pristimantis crepitans* is unique within this genus, suggesting that cytogenetically this species is not closely related to its congeneric species, and neither to *Ischnocnema.* In addition, karyotypical differences, mainly in the NOR position, clearly distinguished the two *Barycholos* populations and indicated the existence of an undescribed species in this genus. A taxonomic review could clarify the systematic position of *P. crepitans* and verify the hypothetic *Barycholos* new species.

Introduction

Recent taxonomic reviews based on molecular data (Frost *et al.,* 2006; Heinicke *et al.,* 2007; Hedges *et al.,* 2008) dramatically changed the systematics of the long-standing “eleutherodactyline” frogs (*sensu* Frost *et al.*, 2006). Heinicke *et al.* (2007) proposed four major clades to this anuran group, comprising the species from (1) Caribbean (*Eleutherodactylus*), (2) Middle America (*Craugastor*), (3) Northern South America (*Pristimantis*), and (4) Southeastern Brazil (*Ischnocnema*), all of them placed in a single family named Brachycephalidae.

As based on DNA sequences from mitochondrial and nuclear genes of 344 species, Hedges *et al.* (2008) placed the 882 described species of Brachycephalidae into a new taxon, the Terrarana, and distributed them in four families, four subfamilies, 24 genera and 11 subgenera. Of those, two families, three subfamilies, six genera, and two subgenera were proposed and named as new taxa. The genera *Brachycephalus* and *Ischnocnema* remained in the Brachycephalidae, a group restricted to the southeastern region of Brazil, as previously suggested by Heinicke *et al.* (2007). The *Pristimantis* and *Barycholos* were allocated into the family

Strabomantidae, and in the subfamilies Strabomantinae e Holoadeninae, respectively, which are new taxa erected by Hedges *et al.* (2008).

Thus far, more than 100 species of Terrarana have been cytogenetically studied and reported as having high degree of chromosome number variation, ranging from 2n=18 to 36 (Bogart, 1991). Considering the arrangements proposed by Hedges *et al.* (2008), the *Craugastor* genus has 2n=18, 20 and 22 chromosomes, *Diasporus* 2n=18, *Brachycephalus* 2n=22, *Ischnocnema* 2n=20, 22 and 30, *Strabomantis* 2n=20, 22, 34 and 35, while *Haddadus* and *Barycholos* have 2n=22 chromosomes. The *Pristimantis* species have the highest variation of diploid complement, with 2n=26, 30, 32, 34 and 36 chromosomes. The genus *Eleutherodactylus* (*sensu* Hedges *et al.*, 2008) is also highly variable, with 2n=18, 22, 24, 26, 28, 30 and 32 chromosomes. Within the four *Eleutherodactylus* subgenera, *Eleutherodactylus* (*Eleutherodactylus*) have 2n=18, 22, 26, 28 and 30, *Eleutherodactylus* (*Euhyas*) (2n=24–32) and *Eleutherodactylus* (*Pelorius*) (2n=30) and *Eleutherodactylus* (*Syhrophus*) (2n=22 and 30) (Duellman, 1967; Beçak, 1968; Brum-Zorrila and Saez, 1968; Bogart, 1970a, 1970b, 1970c; León, 1970; Bogart, 1973; Beçak and Beçak, 1974; De Lucca *et al.*, 1974; De Lucca and Jim, 1974; DeWeese, 1975; Drewry and Jones, 1976; Savage and DeWeese, 1979, 1980; Green *et al.*, 1980; Bogart,1981; Miyamoto, 1983, 1984; Bogart, 1991; Kaiser *et al.*, 1994, 1995; Bogart and Hedges, 1995; Savage and Myers, 2002; Siqueira *et al.*, 2004; Campos *et al.*, 2008). Additional cytogenetic studies on Terrarana species could help to improve the current taxonomic and evolutionary knowledge in this group.

In the present work we analyzed two samples of *Ischnocnema juipoca*, two of *Barycholos* cf. *ternetzi* and two of *Pristimantis crepitans*. This latter species was not yet included in molecular studies being only the geographic distribution used to include it in the genus *Pristimantis,* in the family Strabomantidae (Hedges *et al.,* 2008). We aimed to increase the number of karyotyped Brazilian Terrarana species and further understand their taxonomy and evolutionary relatedness.

Material and Methods

Specimens of *Ischnocnema juipoca, Barycholos* cf. *ternetzi* and *Pristimantis crepitans* were sampled under a permit (License nº 206/2005 - CGFAU/LIC) issued by the IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis). Voucher specimens were

deposited in the Museu de Zoologia “Prof. Dr. Adão José Cardoso”, at the Universidade Estadual de Campinas (UNICAMP), São Paulo State, Brazil and in the Coleção "Célio F. B. Haddad" at the Universidade Estadual Paulista (UNESP), Rio Claro, São Paulo, Brazil (Table 1). The sampling locations in which the specimens were surveyed are displayed in Figure 1.

Mitotic chromosomes were obtained from suspensions of intestinal epithelium and testicular cells from animals pre-treated with 2% colchicine for at least 4h, as described by King and Rofe (1976) and Schmid (1978). Conventional chromosome staining was done with 10% Giemsa solution, Ag-NOR labelling (Howell and Black, 1980), and C-banding (Sumner, 1972), as modified by Siqueira *et al.* (2008). The slides were examined with a BX60 Olympus microscope and images were captured using the softwares Image Pro-plus 4.5.1 and QCapture 2.81.0. Chromosomes were measured and classified according to Green and Sessions (1991).

Results

In all analyzed individuals the diploid number was 22 chromosomes, but the Fundamental Numbers (FN) discriminated the three species. The FN were determined as 40 in *I. juipoca,* 38 in *Barycholos* cf. *ternetzi* and 44 in *Pristimantis crepitans* (Figures 2-5).

Ischnocnema juipoca

The karyotypes of the two analyzed *I. juipoca* populations consisted of five pairs of metacentric chromosomes (1, 6, 7, 9 and 10), four pairs of submetacentrics (2, 3, 4 and 5) and two pairs of telocentrics (8 and 11) (Figures 2A-C). Secondary constrictions are present in the short arm of pair 3 and occasionally adjacent to the centromere of pair 11 (Figure 2A). Blocks of heterochromatin were detected in the centromeric region of all chromosomes, and in several metaphases a faint C-band was adjacent to the centromere of the pair 11 coinciding with the secondary constriction, and on the telomere of the short arm of pair 2 as well (Figure 2B). In the telocentric pair 11, the Ag-NOR sites were adjacent to the centromere and they coincided with the secondary constriction and the pericentromeric block of heterochromatin (Figures 2A-C).

Unusual size variation of the telocentric pairs was observed among metaphases within specimen and among specimens. The size variation was probably due to differences in chromosome compaction and has hampered the positioning of these chromosomes in the karyogram, since they could be allocated in any position among the last four pairs. Size variation of the telocentric chromosomes was also observed in the *Barycholos* populations.

Barycholos* cf. *ternetzi (Uberlândia, MG)

The karyotype of *Barycholos* cf. *ternetzi* consisted of six metacentric pairs (1, 2, 4, 6, 7 and 9), one submetacentric (pair 3), one subtelocentric (pair 5) and three telocentric pairs (8, 10 and 11) (Figures 3A-E). In several metaphases, secondary constrictions were found on the telomere of the NOR-carrying telocentric chromosome 8 (Figures 3A-E). Three distinct NOR patterns were detected: (1) in three specimens, three Ag-NOR sites were observed on the telomere of one homologue of pair 7, and two labels in one homologue of pair 8, one in the centromere and the other on the telomere (Figure 3C); (2) in four specimens, three Ag-NOR sites were found, two on the telomeres of the pair 7 and one adjacent to the centromere of one chromosome of pair 8 (Figure 3D); and (3) in two specimens, four Ag-NOR were found, one on the telomere of one homologue of the pair 6 and one on the centromere of one homologue of pair 8, and two labels on the centromeres of pair 11 (Figure 3E). Heterochromatic blocks were detected in the centromeric region of all chromosomes, and a faint C-band was observed near the telomere on the long arm of pair 4 (Figure 3B).

Barycholos* cf. *ternetzi (Porto Nacional, TO)

The *Barycholos* cf. *ternetzi* karyotype consisted of six metacentric pairs (1, 2, 4, 6, 7 and 9), one submetacentric (pair 3), one subtelocentric (pair 5) and three telocentric pairs (8, 10 and 11) (Figures 4A-C). Secondary constrictions were found interstitially on the pairs 10 and 11 (Figures 4A-C). The Ag-NORs were interstitially located in the pairs 10 and 11, coincident with the secondary constrictions (Figure 4C). The NORs were heteromorphic in pair 11. Heterochromatic blocks were limited to the centromeric region of all chromosomes (Figure 4B).

Pristimantis crepitans

The *P. crepitans* karyotype consisted of nine pairs of metacentric chromosomes (1, 2, 5, 6, 8, 9, 10 and 11) and three submetacentric pairs (3, 4 and 7). Interstitial secondary constrictions were observed on the long arms of pair 7, where the NOR sites were detected (Figures 5A and C). Blocks of strongly stained heterochromatin were located in the centromeric region of all chromosomes (Figure 5B).

Discussion

The diploid number of 22 chromosomes observed in *I. juipoca, Barycholos* cf. *ternetzi*, and *P. crepitans* has also been described for 28 other species of the Terrarana frogs. In this anuran group, *P. crepitans* is the only species with such low chromosome number and occurring in the Midwestern Brazil. The species with 2n=20 and 22 are typically distributed in the Southeastern and Southern Brazil, while most Brazilian species in the northern and northeastern regions have 2n=30 and 34 (Bogart, 1973; DeWeese, 1975; Siqueira *et al.,* 2004, Siqueira *et al.*, 2008).

The chromosome morphology and C-banding patterns in the karyotype of the *Barycholos* cf. *ternetzi* specimens from Uberlândia was very similar to the one previously described in Gurinhatã specimens (Campos *et al.*, 2008). These two sampling locations are in Minas Gerais State, not very distant from each other. Small karyotypical differences are possibly resulting from the use of different techniques for chromosome preparation, and from the classification methods adopted for karyotype description. In addition, in the *Barycholos* specimens from Uberlândia, the only heterochromatic block detected on the long arm of pair 4 corresponded to a band in the pair 4 of the C-banded karyotype of *Barycholos ternetzi* (Gurinhatã), as shown by Campos *et al.* (2008), although the authors did not explicitly mention this band. In contrast, the karyotype of *Barycholos* cf. *ternetzi* from Uberlândia showed variation in number and position of NORs, as similarly reported for *B. ternetzi* from Gurinhatã by Campos *et al.* (2008). These authors suggested that the fixed NOR should be that of pair 11. In the present work, we found three additional NOR patterns. Of those, only one was observed in pair 11, indicating that the principal NOR-bearing chromosome is still uncertain.

The variation in number and position of NOR-labelling in the two studied *Barycholos* populations, the one by Campos *et al.* (2008) and the *Barycholos* cf. *ternetzi* presented herein, suggest the occurrence of chromosomal rearrangements mainly involving the telomeric regions. In anuran species, transposition of mobile genetic elements, ribosomal cistron amplification, and rDNA reinsertion errors during extra chromosomal amplification of ribosomal cistrons have been suggested as responsible for such NOR variation (Wiley *et al.,* 1989; King *et al.,* 1990; Foote *et al.,* 1991; Schmid *et al.,* 1995; Kaiser, 1996; Lourenço *et al.,* 1998). The variation in the NOR number and location may represent an incipient process of species differentiation and a taxonomic review, including additional methodologies, could confirm if this is indeed a new taxon.

Regarding the *Barycholos* cf. *ternetzi* specimens from Porto Nacional, the Ag-NOR was fixed in two chromosome pairs (10 and 11) and located in an interstitial position, instead of the telomeric position detected in *Barycholos* cf. *ternetzi* from Uberlândia. The four analyzed specimens of *Barycholos* cf. ternetzi from Porto Nacional also differed from *B. ternetzi* from Gurinhatã (Campos *et al.*, 2008). Additionally, the specimens from Porto Nacional did not show the observed heterochromatic block on the long arm of the pair 4.

Despite of the similar chromosome morphology between the two studied *Barycholos* cf. *ternetzi* populations, the karyotypical differences, mainly in the NOR position and C-banding, indicated the possible existence of two species and call attention for the need of a taxonomic review of these populations. The NOR locations and heterochromatin pattern has been useful to distinguish among different species - e.g. *Scythrophrys* species (Lourenço *et al*., 2003a, 2008), *Paratelmatobius* (Lourenço *et al.,* 2003b, 2008) and *Pristimantis dundeei* and *Pristimantis.* aff. *dundeei* (Siqueira *et al.,* 2008). Moreover, since vocalization and chromosomal features from the topotypical population are unknown, none of the already studied populations can be surely assigned to the nominal *Barycholos ternetzi*.

The data obtained for *I. juipoca*, from Atibaia and Campos do Jordão, are in agreement with those described by Campos *et al.* (2008) for populations from Itatiba and Santa Branca, in São Paulo State. The other karyotyped species of *Ischnocnema* and *Brachycephalus* genera, both within Brachycephalidae, were substantially diverse from the data reported herein. Those other species, such as *I. guentheri, I. parva* and *B. ephipppium* with the same chromosome number of *I. juipoca* (2n=22), do not have telocentric chromosomes (Siqueira *et al.*, 2004; Ananias *et al*., 2006), whereas *I. holti* and *I. lactea* present a diploid number of 20 chromosomes (De Lucca *et al.*, 1974; De Lucca and Jim, 1974). In addition, in *B. ephipppium* the NOR was located interstitially in the metacentric pair 8 while in *I. juipoca* it was in the telocentric pair 11.

Pristimantis crepitans was removed from the synonymy of *Eleutherodactylus fenestratus*, where Lynch placed it in 1980 (Heyer and Muñoz, 1999). In 2008, Hedges *et al.* allocated *P. crepitans* in the "*P. peruvianus*" group and J. M. Padial (pers. comm. to Hedges *et al.*, 2008) indicated the possibility that both *P. crepitans* and *P. dundeei* may belong to the "*P. conspicillatus*" group. However, the *P. crepitans* and *P. dundeei* low chromosome number and their ecological characteristics indicate that these species are not closely related to the other *Pristimantis.* The putative taxonomic position of the former in the genus *Pristimantis*, as

proposed by Heinicke *et al.* (2007) and Hedges *et al.* (2008), was based solely on its geographical distribution, since *P. crepitans* was not included in any molecular analysis. The low chromosome number of 2n=22 of *P. crepitans* is commonly found in *Ischnocnema*, and it is highly divergent from other known *Pristimantis* karyotypes, which typically have high chromosome numbers (2n=30 to 34). Unfortunately, the only other *Pristimantis* species with a low diploid number, the *P. altae* with 2n=26 (DeWeese, 1975), was not yet submitted to molecular analysis. On the other hand, the preliminary analysis of spermatozoa ultrastructure indicated great differences between *P. crepitans* and the other *Pristimantis* species as well as the studied *Ischnocnema* species (S. Siqueira S. unpublished data). The *Pristimantis crepitans* is also unique by living in open and xeric habitats in the Cerrado biome, among granitic or arenitic outcrops. Such divergences strongly suggested a need of complementary molecular analysis to reassess the recently proposed allocation of *P. crepitans* in the genus *Pristimantis* (Heinicke *et al.*, 2007; Hedges *et al.*, 2008). Therefore, further studies are necessary to clarify the systematic position of these species.

Concluding remarks

An extensive contribution to the understanding of the molecular phylogeny of the South American eleutherodactyline species was recently done by Frost *et al.* (2006), Heinicke *et al.* (2007) and Hedges *et al.* (2008). However, for Brazilian species there are many scarcely known taxonomic aspects, which require further investigation using a combination of morphological, cytogenetic, and molecular techniques. As based on previously reported molecular data (Frost *et al.,* 2006), behavioral studies (Caramaschi and Pombal, 2001), and karyotypes (Campos *et al.*, 2008) it seems conceivable to infer that *Barycholos* and the karyotyped *Ischnocnema* species are close relatives. However, the additional molecular studies reported by Hedges *et al.* (2008) indicate that *Barycholos* is phylogeneticaly distant from both *Ischnocnema* and *Haddadus binotatus.* Therefore, the observed chromosomal similarities might be simplesiomorphies or resulting of convergence generated by chromosomal rearrangements, and so they do not substantiate a suggestion of close evolutionary relationships between the *I. juipoca* and *Barycholos* species.

In the new systematic arrangement for “eleutherodactyline” frogs proposed by Heinicke *et al.* (2007) and Hedges *et al.* (2008), mostly based on molecular data, there are still indications of divergences in relation to the available karyological data. Most likely, the divergences are due to the lack of molecular analysis of many of the Brazilian karyotyped species, such as *Pristimantis*

crepitans and *P. altae*. Further molecular and chromosome analyses of Terrarana frogs, including these divergent species, should provide a broader understanding of their evolutionary relatedness and systematic status.

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Table 1. Voucher specimens, sampling locations and accession numbers

Species	Male	Female	Sampling Location	ZUEC and CFBH* Accession Numbers
Ischnocnema juipoca	2	1	Atibaia and Campos do Jordão, São Paulo State	13265, 13266, 9904*
Pristimantis crepitans	3	2	Chapada dos Guimarães and Distrito de São Vicente, Cuiabá, Mato Grosso State	14114-14119
Barycholos* cf. *ternetzi	7	2	Uberlândia, Minas Gerais State	13262-13264, 13475, 13476, 14120-14123,
Barycholos* cf. *ternetzi	--	4	Porto Nacional, Tocantins State	14124-14127

Number of specimens and sampling locations of the cytogenetically analyzed Brazilian Terrarana species. Accession numbers refer to the Zoology Museum "Prof. Adão José Cardoso" (ZUEC), UNICAMP.

Legends

Figure 1. Map of Brazil displaying the sampling locations in which the studied frog populations were surveyed. 1: Atibaia, São Paulo State, *Ischnocnema juipoca*; 2: Campos do Jordão, São Paulo State, *I. juipoca*; 3: Chapada dos Guimarães, Mato Grosso State, *Pristimantis crepitans*; 4: São Vicente, Cuiabá, Mato Grosso State, *P. crepitans*; 5: Uberlândia, Minas Gerais State, *Barycholos* cf. *ternetzi*; 6: Porto Nacional, Tocantins Sate, *Barycholos* cf. *ternetzi*.

Figure 2. Karyotype of *Ischnocnema juipoca*: (**a**) Giemsa staining; (**b**) C-banding; (**c**) Ag-NOR labelling. The arrow indicates secondary constrictions. The arrowheads indicate the NOR coincident with secondary constriction. Bar = 10 μm.

Figure 3. Karyotype of *Barycholos* cf. *ternetzi,* Uberlândia, Minas Gerais State: (**a**) Giemsa staining; (**b**) C-banding; (**c - e**) Ag-NOR staining. The arrow indicates the interstitial heterochromatin. The arrowheads indicate the NORs. Note the distinct position and number of NORs in **c, d** and **e**. Bar = 10 μm.

Figure 4. Karyotype of *Barycholos* cf. *ternetzi,* Porto Nacional, Tocantins Sate: (**a**) Giemsa staining; (**b**) C-banding; (**c**) Ag-NOR staining. The arrow indicates the secondary constrictions. The arrowheads indicate the NORs coincident with secondary constriction. Bar = 10 μm.

Figure 5. Karyotype of *Pristimantis crepitans*: (**a**) Giemsa staining; (**b**) C-banding; (**c**) Ag-NOR staining. The arrow indicates the constriction. The arrowheads indicate the NORs coincident with secondary constriction. Bar = 10 μm.

Figure 1

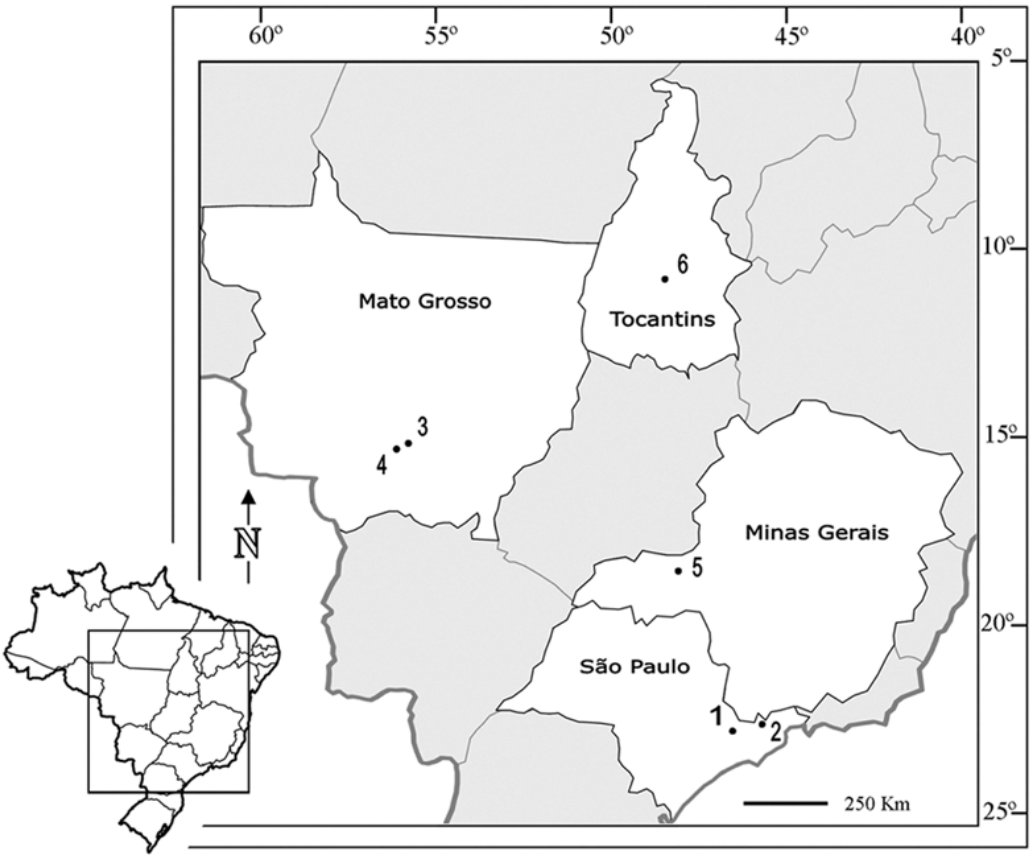
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Figure 2

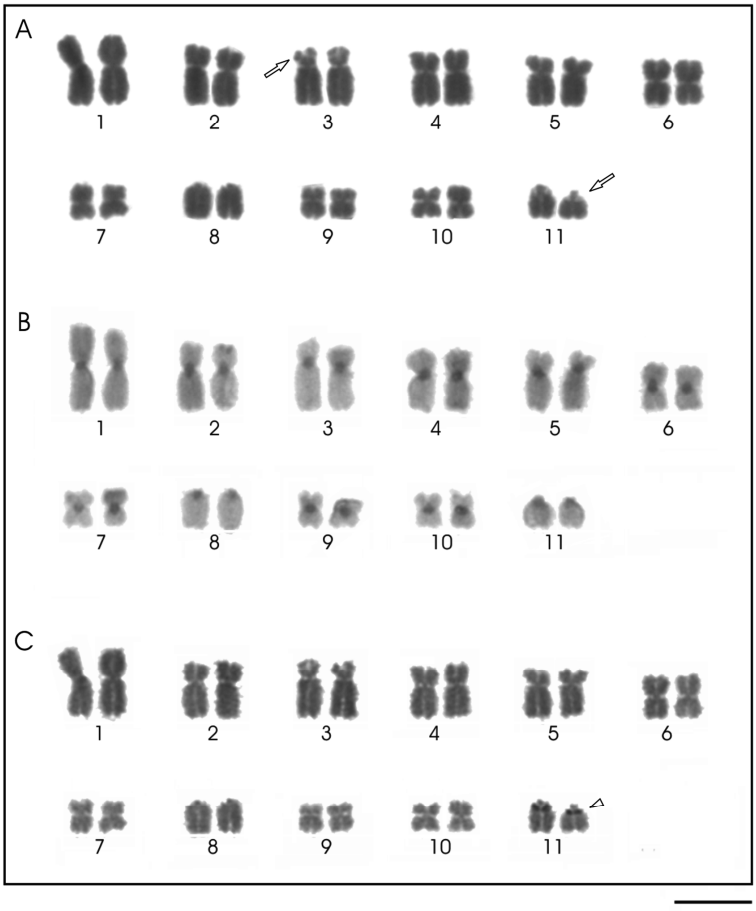

Figure 3

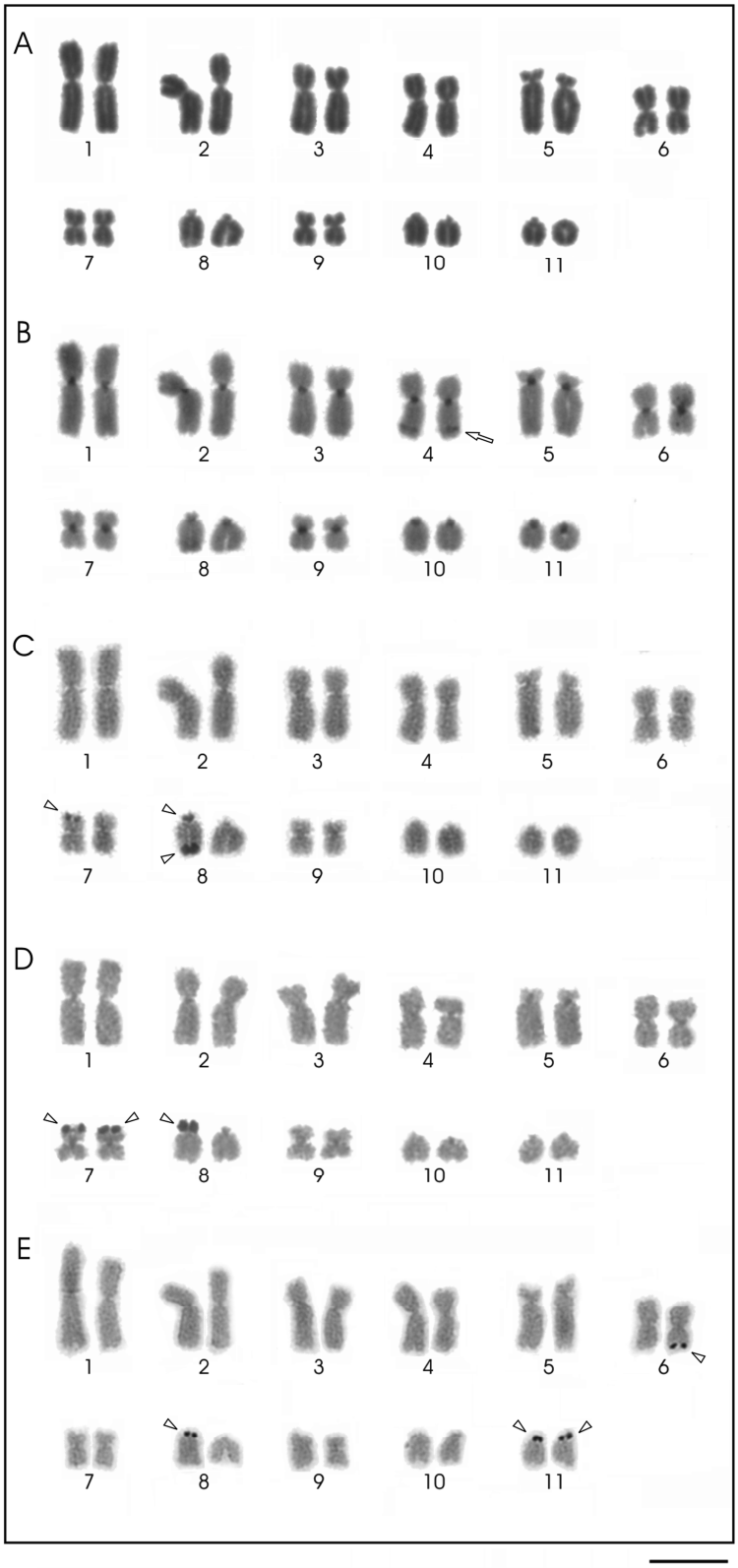
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Figure 4

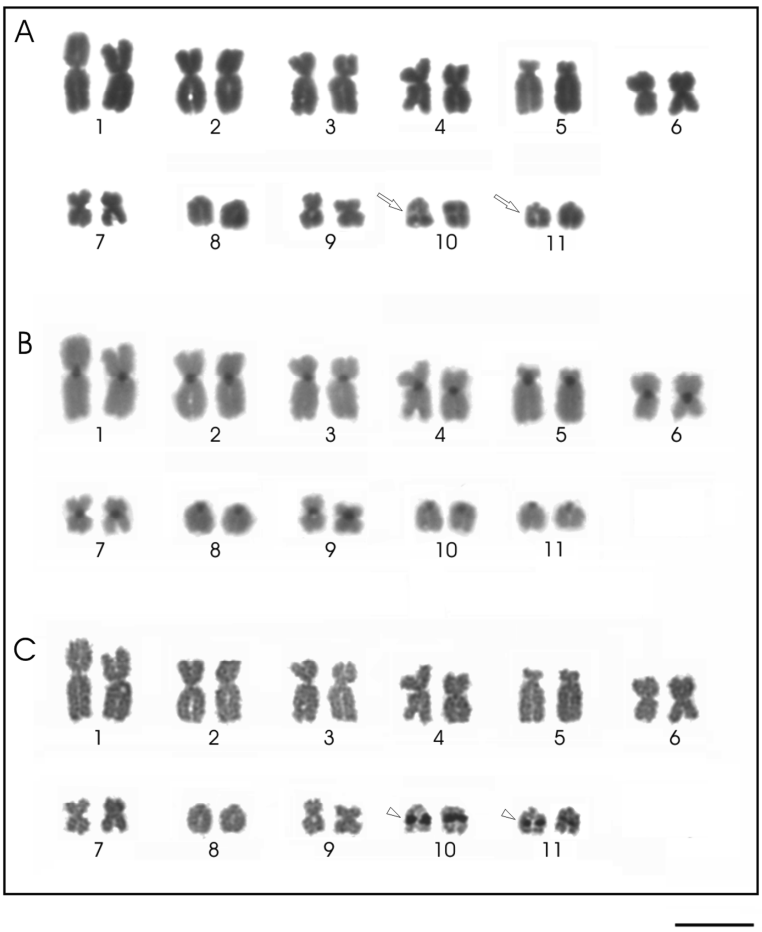
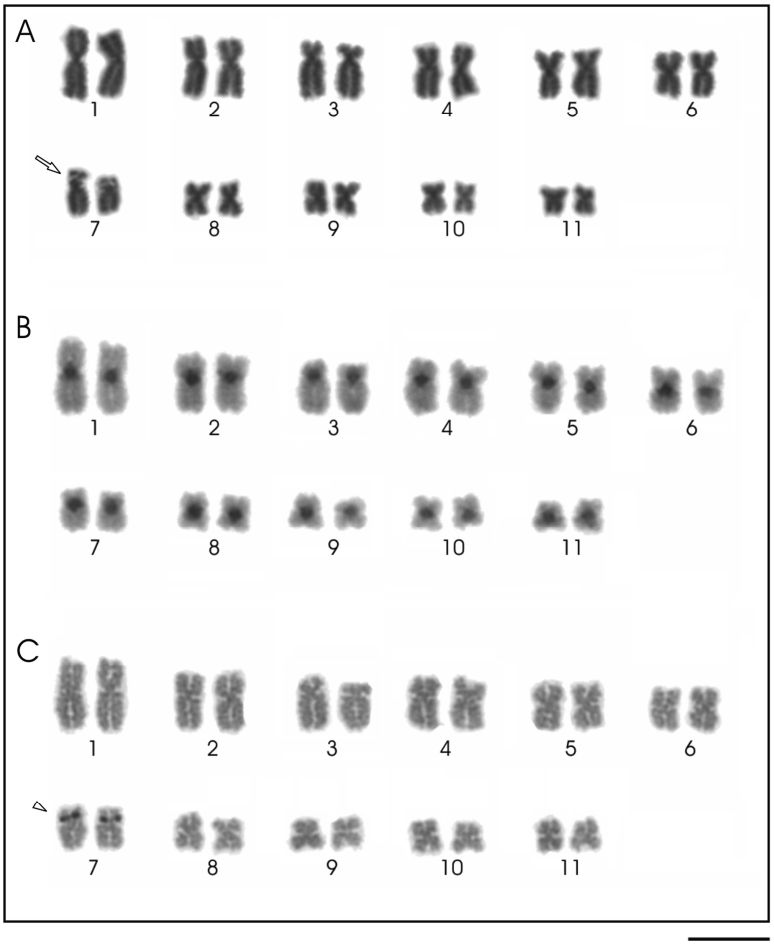


Figure 5



VII. Artigo 4

Sperm ultrastructure of four Brazilian Strabomantidae species (Amphibia, Anura, Terrarana), with taxonomic comments

Sperm ultrastructure of four Brazilian Strabomantidae species (Amphibia, Anura, Terrarana), with taxonomic comments

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Keywords: *Pristimantis, Barycholos,* spermatozoon, Terrarana

Running title: Sperm ultrastructure of Strabomantidae species

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Abstract

The systematics of the Terrarana frogs has been considerably improved by the recent rearrangements of their nearly 900 described species. Nevertheless, the South American species still require further taxonomic investigations. The objective of the present work was to analyze and compare the sperm ultrastructure of the Brazilian species of *Pristimantis fenestratus*, from Borba and Reserva Duke, AM, and from Rio Branco, AC*, P. dundeei* and *P. crepitans* from Chapada dos Guimarães, MT, and *Barycholos ternetzi*, from Uberlândia, MG, aiming at improving the understanding of their systematics. The transmission electron microscopy studies revealed sperm ultrastructural differences among all species, mostly in the acrosome, midpiece and flagellum. Several of the non-conserved ultrastructural characteristics identified within the family Strabomantidae and in the genus *Pristimantis* were capable of distinguishing the studied species. These data suggests that the taxonomic position of *P. crepitans* and *P. dundeei* in relation to *P. fenestratus,* which is strongly suported within the Strabomantidae clade. The data on spermatozoa ultrastructural variation reinforce the need of further taxonomic investigations in the subfamily Strabomantinae and indicated that further studies of sperm ultrastructural characteristics can be an additional suitable approach attempting to understand taxonomic aspects of Terrerana species.

Introduction

In recent molecular phylogeny studies, the nearly 900 anuran species formerly included in the genus *Eleutherodactylus,* family Leptodacylidae, were split into new genera allocated in the Brachycehalidae family (Frost et al., 2006). Of those 900 species, 340 were then separated in four families, comprising four subfamilies, 24 genera, 11 subgenera, 33 species series, 56 species groups, and 11 species subgroups, which were all allocated into a new taxon named Terrarana (Hedges et al., 2008). Based on the molecular data reported by Frost (2008) and Hedges et al. (2008) about 85 species of Terrarana are described in Brazil, which are distributed in four families: Strabomantidae (genera *Strabomantis*, *Euparkerella*, *Holoaden*, *Nobella*, *Oreobates, Barycholos* and *Pristimantis*), Craugastoridae (genus *Haddadus*), Eleutherodactylidae (genera *Adelophryne* and *Phyzelaphryne*), and Brachycephalidae (genera *Brachycephalus* and *Ischnocnema*).

Hedges et al. (2008) considered that South America is the most poorly sampled geographic region, where the clades Strabomantidae and Brachycephalidae can be found. Those authors indicated that in spite of the inclusion of more than 100 *Pristimantis* species in the molecular phylogeny, the intrageneric relationship was not completely elucidated. In addition, they argued that the further inclusion of species and genera in such kind of analysis might improve the classification within Terrarana.

In the Anura analyses using alternative data sets, mainly molecular markers as above mentioned, have added new insights to the systematic problems and also have refuted well morphologically established clades (Hay et al., 1995; Garda et al., 2002). The gaps on existing data sets indicate the need to exploring new types of characters as important ways to improve phylogenetic hypotheses in this Order (Ford and Cannatella, 1993). Studies of the ultrastructural organization of spermatozoa has been used as an additional source of data for systematic purposes in many anuran groups (e.g. Jamieson, 1991; Garda et al., 2002; Aguiar et al., 2006; Veiga-Menoncello et al., 2006; Veiga-Menoncello et al., 2007).. Anuran spermatozoa provide a useful suite of characters that include variation in the head, particularly the acrosomal complex, and in the arrangement of the centrioles and the flagellum structures (Jamieson et al., 1993; Meyer et al., 1997; Costa et al., 2004; Garda et al., 2004).

In the present work we analyzed the sperm ultrastructure of four species from two subfamilies of Strabomantidae, the *Pristimantis fenestratus, P. dundeei*, *P. crepitans* (Strabomantinae) and *Barycholos ternetzi* (Holoadeninae), aiming at further understanding their taxonomic relatedness. The systematics of the *Pristimantis* species is discussed based on sperm differentiating characteristics

Material and Methods

Adult males of *Pristimantis fenestratus, P. dundeei, P. crepitans,* and *Barycholos ternetzi* were sampled as described in Table I. The collections were done under a permit of the Instituto Brasileiro do Meio Ambiente and Recursos Naturais Renováveis - IBAMA (License nº 206/2005 – CGFAU/LIC). Voucher specimens were deposited in the Instituto Nacional de Pesquisa do Amazonas (INPA) in Manaus, AM, and in the Museu de História Natural "Prof. Adão José Cardoso" (ZUEC) of the Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil.

The testes of the specimens were removed, cut into small pieces, and fixed overnight at 4ºC in 0.1M sodium cacodylate buffer, pH 7.2, containing 2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose, and 5 mM $CaCl_2$. Post fixation was done for 1 h in the same buffer containing 1% osmium tetroxide, 1% potassium ferricianide, and 5 mM $CaCl_2$. The tissue samples were subsequently rinsed in sodium cacodylate buffer and incubated for 2 h in 0.5% uranyl acetate. After rinsing in buffer, the samples were dehydrated in an increasing acetone series and embedded in Epon resin. Ultrathin sections, obtained with an ultramicrotome using a diamond knife, were stained with uranyl acetate and lead citrate and examined with a LEO 906 and EM 900, transmission electron microscope.

Results

The spermatozoon basic structure differed among the four examined species regarding the spermatozoa widths, acrosome complex, shape of midpiece, and the presence or absence of axial fiber and undulating membrane.

Spermatozoa of the *P. fenestratus* and *P. dundeei* species were filiform as observed under the light microscopy. The head was curved and the midpiece very long, visible in phase contrast. The tails do not have an undulating membrane or paraxonemal rod (*sensu* Lee and Jamieson, 1993). In *P. crepitans* and *B. ternetzi,* the spermatozoa were also filiform, with a curved head wider than that of *P. fenestratus* and *P. dundeei*. The midpiece was short and not clearly visible, and the tail was long with a conspicuous undulating membrane. The axoneme was seen describing a very sinuous path along an axial fiber.

Electron Microscopy

Acrosomal complex

In *P. fenestratus* and *P. dundeei*, a thin acrosomal vesicle covers the anterior portion of the nucleus and the acrosome complex width varies from 0.4 μm in the apex to 0.5 μm in the basis, having a length of 6.0μm (Fig. 1a, h). In these species a subacrosomal cone was observed as a low electrondense thin lamina attached to the chromatin (Fig. 1a, b, e, f).

In the *P. crepitans*, a conical acrosomal vesicle covers the anterior portion of the nucleus, which is thin and filled with a low electrondense substance while in *B. ternetzi* a thin acrosomal vesicle covers the anterior portion of the nucleus and the acrosome complex (Fig. 1i-l, n-s). In *P.*

crepitans the acrosome complex width 8.2µm long and varies from 0.4 µm in the apex to 1.8 µm in the basis and in *B. ternetzi* the acrosome complex width 8,0µm long and varies from 0.6µm in the apex to 2.0µm in the basis (Fig. 1i, m, n-t). In both species a subacrosomal cone is located behind the acrosomal vesicle (Fig. 1j-l, p-t). In *P. crepitans,* the subacrosomal cone appeared very thin in longitudinal sections and with a fibrilar arrangement in cross sections wile in *B. ternetzi* a subacrosomal cone was observed as a low electrondense thin lamina attached to the chromatin (Fig. 1i, n).

Nucleus

The nucleus of the *P. fenestratus* and *P. dundeei* spermatozoa is thin and circular in all its extension as observed in transverse sections. In longitudinal sections, the nucleus is cylindrical, thin, with similar thickness in both the proximal and posterior portions. The chromatin is highly condensed and electrondense (Fig. 1c, h).

In *P. crepitans* and *B. ternetzi,* the nucleus was circular in transverse sections and conical in the longitudinal ones and he chromatin is highly condensed and electrondenses (Fig. 1m, t). *Pristimantis crepitans* showed a nuclear space below the nuclear envelope (Fig. 1j-k).

Midpiece

In *P. fenestratus* and *P. dundeei*, the posterior portion of the nucleus is curved, forming a discrete nuclear fossa above the proximal centriole and the pericentriolar material. In both species, the proximal centriole lies at an approximate angle of 90° with respect to the longitudinal axis of the spermatozoon (Fig. 2a, f). The proximal and distal centrioles are embedded in a pericentriolar material of low electrondensity (Fig. 2a, f). In the sperm of *P. fenestratus* no mitochondria were observed in the midpiece while in *P. dundeei,* few mitochondria were observed in the midpiece, near the centrioles (Fig 2f).

In *P. crepitans* and *B. ternetzi*, the posterior portion of the nucleus is curved, forming a nuclear fossa that surrounds the proximal centriole and the pericentriolar material, while in the last one the nuclear fossa is deepest (Fig. 2j, k, o, p). In *Pristimantis crepitans*, the proximal centriole was positioned at an approximate angle of 50° with respect to the longitudinal axis, and 65º to the distal centriole, in an oblique arrangement and en *B. ternetzi,* the proximal centriole lies

at an angle of 90° with respect to the longitudinal axis and oblique to the distal centriole (Fig. 2j, k, o). In these two species a paraxonemal rod reachs the proximal centriole and is embedded in the pericentriolar material, which is fibrous and low electrondense (Fig. 2j,k, o). *Pristimantis crepitans* and *B. ternetzi* also showed few mitochondria surrounding the midpiece (Fig. 2k, o).

Tail

In *P. fenestratus* and *P. dundeei,* the tail consists of an axoneme with the usual 9+2 pattern (Fig. 2c,e, h, i). The *P. fenestratus* sperm contained five or six cylindrical mitochondria surrounding the axoneme anterior portion, which were observed in the tail transverse sections (Fig. 2c). While for *P. dundeei*, one or two cylindrical mitochondria surrounded the axoneme, with the usual 9+2 pattern (Fig. 2h). In *P. fenestratus*, these flagellar mitochondria were observed in both immature and advanced sperm maturation stages, as inferred by the surrounding microtubules (Fig. a-c). On the other hand, only tail sections of immature sperm cells were obtained to *P. dundeei* (Fig 2f-h). In both species, only the axoneme was observed at the tip end of the flagellum (Fig 2b-e, g-i).

When observed in transverse sections, the sperm tail of *P. crepitans* and *B. ternetzi* contained an axoneme with the usual 9+2 pattern, a very thin undulating membrane, and an axial fiber. In these two species, the axial fiber is connected to the axoneme by a low electrondense substance within the undulating membrane (Fig. 2l, q). In both cases,the usual juxtaxonemal fiber was not observed, in addition, at the anterior portion of the flagellum, the axial fiber is not distinguishable and, hence, named paraxonemal rod (*sensu* Lee and Jamieson, 1993) (Fig. 2l, q). In transverse sections of *P. crepitan,* four to six cylindrical mitochondria surrounded the axoneme and such pattern was maintained in longitudinal sections of the tail which was also observed in longitudinal sections of sperm at further maturation stages, and also in this species only the axoneme was observed at the tip end of the flagelum (Fig. 2l-n). Flagelar mitochondria were not observed in *B. ternetzi* (Fig. 2o-q).

Discussion

The spermatozoa of Strabomantidae

The divergence in the spermatozoon ultrastructure like those seen among the species here analyzed have been considered an indicative of non-monophyletism, including in anuran groups (see, Amaral et al., 2000; Aguiar et al., 2003; Aguiar et al., 2006; Veiga-Menoncello et al., 2007). For instance, the previously family Leptodactylidae, a recognized non-monophyletic taxon, has diverse sperm ultrastructure. This concerns the uxiliary fibers, which are absent in *Caudiverbera caudiverbera* (Pugin-Rios and Garrido, 1981), currently *Calyptocephalella gayi,* Calyptocephallidae family, but well developed in *Odontophrynus cultripes* (Báo et al., 1991) currently Cycloramphidae. In addition, the previous leptodactylids of the genera *Odontophrynus*, *Pseudopaludicola* and *Telmatobius* have an acrosomal vesicle and a subacrosomal cone in the acrosome complex, in contrast to the genera *Calyptocephala*, *Leptodactylus*, and *Pleurodema*, in which the acrosome complex consists of a single acrosome vesicle. As previously emphasized by Amaral et al. (2000), this variability supports the view that the Leptodactylidae may be a polyphyletic taxon, as suggested by Duellman and Trueb (1994).

The data on sperm reported herein represents a first description of sperm ultrastructure in the family Strabomantidae. Sperm cell description has not been found in the former *Eleutherodactylus* as well. The differences in the acrosome, midpiece and flagellum among the four Strabomantidae species indicate non-conserved sperm characteristics within the subfamily Strabomantinae and in the genus *Pristimantis*. This sperm diversity is not in agreement with the recent taxonomic rearrangements based on molecular data (Hedges et al., 2008). Indeed, in the phylogenetic tree of Terrarana by Hedges et al. (2008), the statistical support of most nodes are low and the relationships between most clades are largely unresolved, mainly concerning the family Strabomantidae.

Accordingly, the the molecular findings of Frost et al. (2006) have shown that most species diverging in sperm ultrastructure are, indeed, in separated families. This example illustrates the feasibility of the sperm ultrastructural data to improve the understanding of the taxonomic relationships among some Anura taxa.

The shared presence of a subacrosomal cone in all the analyzed species could imply in a simplesiomorphy, since according to Scheltinga et al. (2003) the presence of a subacrosomal cone behind a conical acrosomal vesicle is possibly a plesiomorphic characteristic. It has been found in

urodeles, gymnophionans, and in the most primitive anurans, such that it is not suitable to infer relationships among them.

In all species, the midpiece comprised unique characteristics, including the angle of the centrioles and the pericentriolar material density. The centriolar arrangement has been tentatively considered as suitable for phylogenetic information. Kwon and Lee (1995) reported an evolutionary trend in this trait. while Lee and Jamieson (1993) argued that the centrioles lie perpendicular to each other in gymnophionans and urodeles, and that appears to be the plesiomorphic state. In the present work, such plesiomorphy, typical of sperm cell without undulating membrane, is shared by *P. fenestratus and P. dundeei*, not contributing to infer relationships between them. Only *P. crepitans* and *B. ternetzi*, placed in different subfamilies, share the putative apomorphic condition (oblique arrangement) typical of species possessing undulating membrane. It might indicate that these two species are more closely related, when considering this trait, than the two congeneric *Pristimantis*.

None of the four species here analyzed presented the typical collar-like mitochondrial arrangement seen in the bufonoid lineage, to which Terrarana belongs. The cylindrical mitochondria found in the three *Pristimantis* species, surrounding a portion of the axoneme, were already described in *Hyperolius punctatus* (Hyperoliidae), a biflagellate species, *Mantidactylus majory* (Mantellidae), *Cophixalus ornatus,* (Microhylidae) and *Rana temporaria* (Ranidae). Despite of the differences among the tree studied *Pristimantis* species, their shared character of cylindrical mitochondria adjacent to the axoneme indicates close evolutionary relationship among them. It is specially true when the hypothesis by Garda (2002), who scored this state as the most advanced when comparing the conditions where this organelles are present in a collar around the midpiece and the anterior portion of the flagellum or distributed around the axial fiber.

However, the actual contribution of the mitochondria distribution to phylogenetic inferences is not yet clear, since these organelles seem to disappear during the gamete ontogeny in anurans, as reported in Lee and Jamieson (1993). Furthermore, the axial fiber absence in the sperm tail of the *P. fenestratus* and *P. dundeei* species, and the presence of this structure in *P. crepitans* and *B. ternetzi* indicate unclear systematic relationships, mainly regarding the *Pristimantis* species. Considering the argument of Jamieson (2003), that the absence of an axial fiber in Megophryidae, Pelobatidae, Pipidae, Hyperoliidae, Calyptocephallidae, (*Telmatobius* and *Telmatobufo*), Hylidae (*Pseudis paradoxa*), Microhylidae, Ranidae and Rhacophoridae is an

apomorphy, we hypothesized that there are phylogenetic affinities between *P. fenestratus* and *P. dundeei*, which are not shared with *P. crepitans*.

The variability among the analyzed Strabomantidae species included some of the evolutionary trends proposed by Kwon and Lee (1995) and Lee and Jamieson (1993) to the species previously analyzed (Pugin-Rios, 1980; Pugin-Rios and Garrido, 1981; Báo et al., 1991; Amaral et al., 1999; Amaral et al., 2000; Scheltinga et al., 2003). (Lee and Jamieson, 1993) argued that the simplification of spermatozoa structures is an evolutionary trend in anurans, including the absence of tail accessory filaments and the absence of the subacrosomal cone in highly apomorphic taxa. However, only four of the approximately 530 species of Strabomantidae were analyzed in the present work. Further studies increasing the number of studied species will improve the understanding of the evolutionary way of ultrastructural sperm differentiation and its correlation and congruence with molecular data, which currently constitutes the main basis for anuran systematics.

Systematic comments

(Hedges et al., 2008) allocated *P. crepitans* and *P. dundeei* in the *P. peruvianus* group, without molecular data on these two species. Padial (apud Hedges et al., 2008) indicated that possibly *P. crepitans* and *P. dundeei* belong to the *P. conspicillatus* group. However, the differences in sperm ultrastructure, such as number and organization of the mitochondria, indicate unresolved taxonomic relatedness among these *Pristimantis* species, mainly regarding the *P. fenestratus*, a genuine representaive of the genus *Pristimantis*, to which *P. dundeei* share an apomorphy (the absence of tail accessories).

Indeed, *P. crepitans* is unique by living in open, xeric habitats in the Cerrado biome, amongst granitic or arenitic outcrops. The low chromosome number of 2n=22 of this species (Siqueira *et al.*, in press), is commonly found in *Ischnocnema* and indicates high divergence from the other *Pristimantis* karyotypes,with high chromosome numbers (2n=30, 32 and 34), also differing from *P. dundeei* that have 2n=28 chromosomes (Siqueira et al., 2008).

The presence of an axial fiber in *P. crepitans* and the low chromosome number (Siqueira et al., unpublished data), suggest that this species might be misallocated in the genus *Pristimantis*. In agreement to Siqueira *et al*. (unpublished data), such divergence strongly suggest the need of

complementary molecular analyses to reassess the recent allocation of *P. crepitans* in the genus *Pristimantis*, proposed by (Heinicke et al., 2007) and (Hedges et al., 2008).

The species *P. dundeei,* unexpectedly showed spermatozoon morphology similar to *Ischnocnema paulodutrai* (Siqueira *et al*., unpublished data), from Ilhéus, BA, Brazil. *Ischnocnema paulodutrai* also had a similar karyotype of 2n=30 chromosomes (Siqueira *et al.*, 2008), which suggests that this species might be more closely related to the *P. dundeei* than to the remaining congeneric *Ischnocnema* species l, with low (2n=20 and 22) and high (2n=34) chromosome numbers.

The divergence among Terrarana species is an elucidative example of the variation in sperm morphology and high variation in chromosome number are indicatives of polyphyletic groups that deserve a further taxonomic investigation.

In conclusion, a further analysis of *P. dundeei, P. crepitans, B. ternetzi* as well as *I. paulodutrai* and other species of Terrarana frogs are necessary to clarify the systematic position of these species.

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Table I. Specimens of Brazilian Terrarana species analyzed for sperm ultrastructure.

Species	Number of Specimens	Localities	Accession Numbers
Pristimantis fenestratus	5	Borba and Reserva Ducke, Amazonas State and Rio Branco, Acre State	20.926-20.929*
Pristimantis dundeei	4	Chapada dos Guimarães and São Vicente, Mato Grosso State	13.084, 13.087, 14.101,14.102
Pristimantis crepitans	3	Chapada dos Guimarães, Mato Grosso State	14114-14116
Barycholos ternetzi	5	Uberlândia, Minas Gerais State	13262-13264, 13475, 13476

Number of specimens and sampling locations of the analyzed species. Accession numbers refer to the Instituto Nacional de Pesquisa do Amazonas (INPA)* in Manaus, AM and Museu de Zoologia "Prof. Adão José Cardoso" (ZUEC), Unicamp

Legends

Figure 1. Electron micrographs of *Pristimantis fenestratus* (**a-d**), *P. dundeei* (**e-h**), *P. crepitans* (**i-m**) and *Barycholos ternetzi* (**n-t**). (**a,d,e,i,n**) Longitudinal section through the anterior head region showing the acrosomal complex with the acrosomal vesicle (av) and subacrosomal cone (sc). Note the subacrosomal space (ss) behind the subacrosomal cone in *P. crepitans,* above the nucleus (N). (**b,c,f-h,j-m,o-t**) Transverse sections through the head. Note the high degree of chromatin condensation in (**c,h,m,t**).

Figure 2. Electron micrographs of *Pristimantis fenestratus* (**a-e**), *P. dundeei* (**f-i**), *P. crepitans* (**j-n**) and *Barycholos ternetzi* (**o-q**). In (**a-c**), note the mitochondria (m) surrounding the axoneme (ax) in *P. fenestratus,* (**g,h**) besides the axoneme in *P. dundeei,* (**k-m**) surrounding the axoneme in *P. crepitans* and (**p**) in *B. ternetzi* note few mitochondrias besides the midpiece. (**a,f,j,k,o**) Longitudinal section through the posterior most region of the nucleus, midpiece, and proximal portion of the flagellum, showing the nuclear fossa and the axoneme with an adjacent accessory fiber (*i.e.* paraxonemal rod, pr). In (**l,q**), transverse section of the anterior portion of the flagellum showing a conical axial fiber (af), note the very thin undulating membrane (um) connecting the accessory fibers and the axoneme. (**b-e,g-i**) End of the mitochondria region, and only the axoneme (ax) is seen at the tip of the tail shortening. (**l-n,p,q**) Progressive shortening of the undulating membrane (um), and approximation of the accessory fibers and disappearance of the axial fibers.

Figure 1

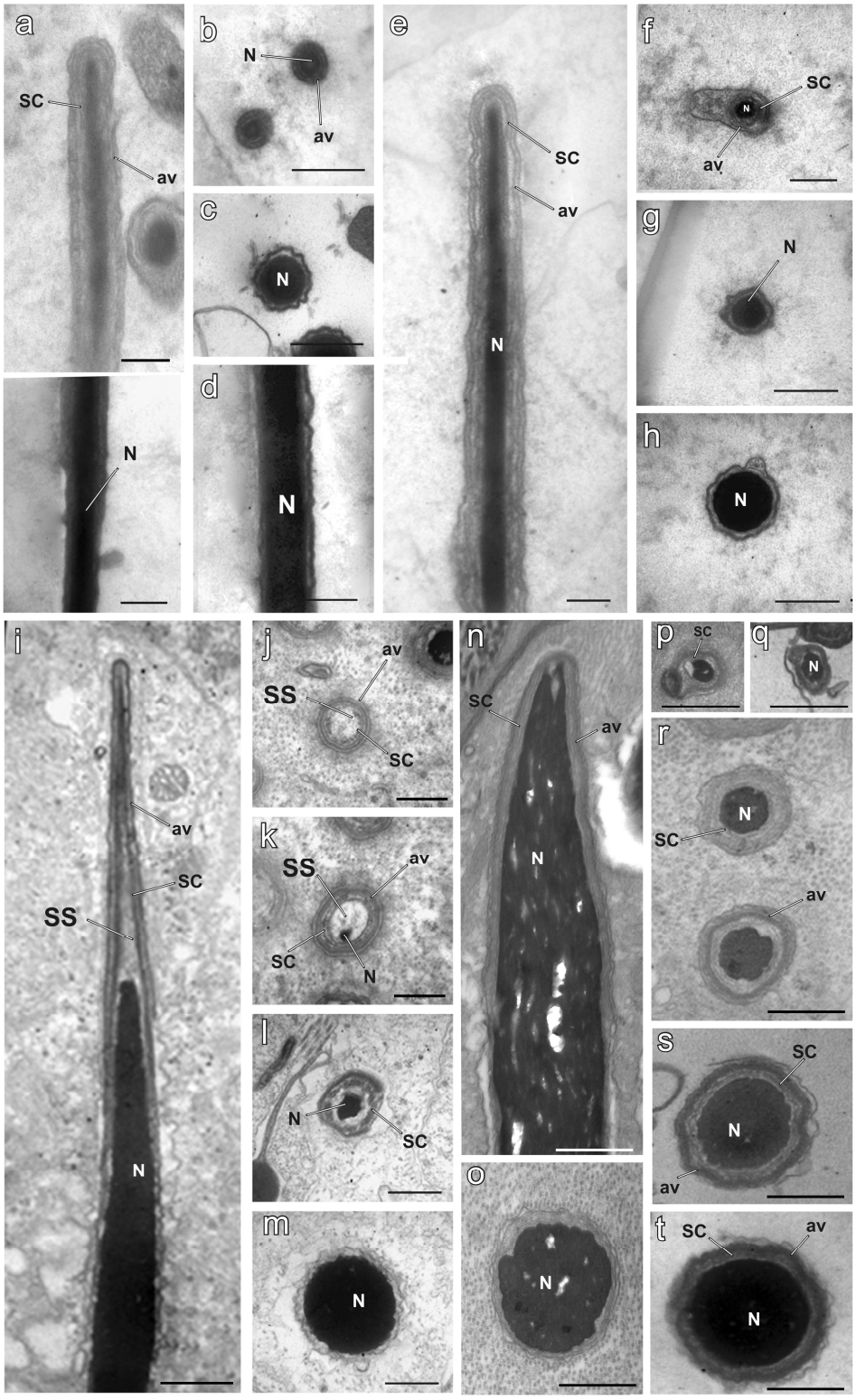
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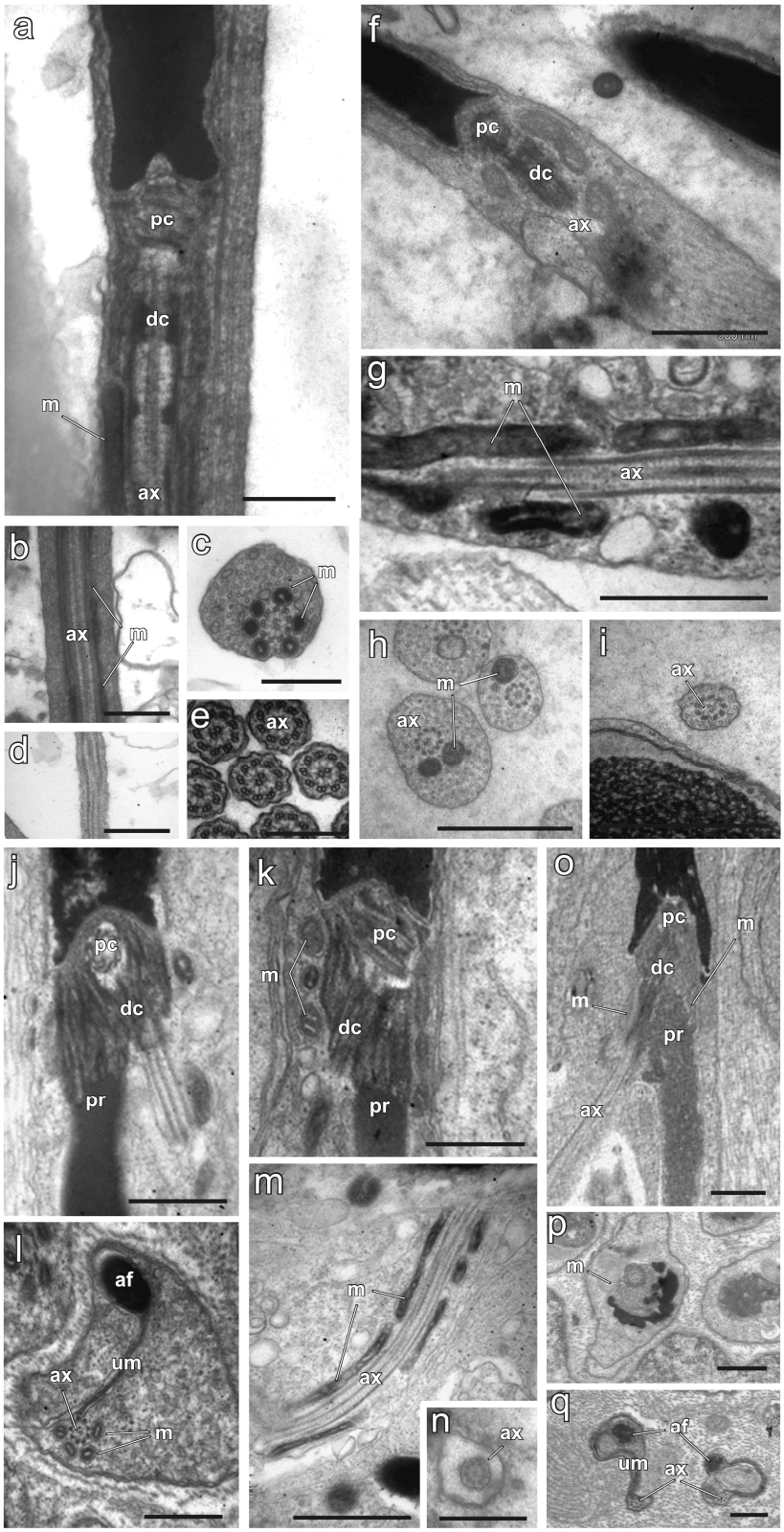
Figure 2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VIII. Artigo 5

Sperm ultrastructure of Brachycephalidae and Craugastoridae species from Brazil (Anura, Terrarana) with comments on sistematics

Sperm ultrastructure of Brachycephalidae and Craugastoridae species from Brazil (Anura, Terrarana) with comments on sistematics

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Key-words: spermatozoon, ultrastructure, *Brachycephalus, Ischnocnema, Haddadus*

Running title: Sperm ultrastructure of Brazilian species of Terrarana

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Abstract

Many taxonomic aspects of the eleutherodactyline frogs, particularly of South American species, remain uncertain despite their recent comprehensive systematic rearrangements based on molecular phylogeny. In this work, six Brazilian eleutherodactyline species of the families Brachycephalidae and Craugastoridae were analyzed for sperm ultrastruture aiming at further understanding their evolutionary and taxonomic relationships. All the studied species are from São Paulo State, except the *Ischnocnema paulodutrai* from Bahia State. The *Ischnocnema guentheri, Ischnocnema parva* and *Ischnocnema juipoca* species were not differentiated by sperm ultrastructure and formed a group distinct of *Brachycephalus ephippiun, Haddadus binotatus and Ischnocnema paulodutrai*, which were discriminated one from the other. The discriminating sperm characteristics were cell width, acrosome complex, midpiece shape and the presence or absence of a paraxonemal rod and undulating membrane in the tail. In *I. guentheri, I. parva, I. juipoca, B. ephippium* and *H. binotatus*, an acrosomal vesicle covers the anterior portion of the nucleus while the typical subacrosomal cone was not observed in the *I. paulodutrai* samples. In tranversion sections, the nucleus of *Ischnocnema* and *H. binotatus* was thin and circular in all its extension. As for *B. ephippium*, the subacrosomal cone form invaginations filled by the chromatin and, consequently, the nucleus shape was irregular and not circular. A conspicuous nuclear fossa was present in the spermatozoa of *B. ephippium* and *H. binotatus* while in all the *Ischnocnema* species the posterior portion of the nucleus form a discrete nuclear fossa above the proximal centriole. The tail of the *Ischnocnema* species consists of a single axoneme with the usual 9+2 pattern. In transversal sections of *I. guentheri, I. parva* and *I. juipoca* samples, nine to eleven flat and contiguous mitochondria were observed below the distal centriole, surrounding the axoneme, unlike the *I. paulodutrai*, which had one or two cylindrical mitochondria at this position. The sperm tail of *B. ephippium* and *H. binotatus* consists of an axoneme with the usual 9+2 pattern, a very thin undulating membrane, and an axial fiber, which is larger and less electrondense in *H. binotatus*. The usual juxtaxonemal fiber was exclusive to *B. ephippium*. The sperm ultrastructure diversity seems to be coherent with observed low support among most clades in the Terrarana phylogenetic tree, including the node clustering *Brachycephalus* and *Ischnocnema* into Brachycephalidae, and the node grouping *Haddadus* into Craugastoridae. The data reported herein represent elucidative evidence that variability in sperm ultrastructure is suitable to evaluate polyphyletic groups that require further taxonomic investigation.

Introduction

More than 340 species constitute the current taxon Terrarana, as proposed by Hedges *et al.* (2008). The taxonomic relationships among the Terrarana species emerged from molecular phylogeny studies, which discriminated four families, four subfamilies, 24 genera, 11 subgenera, 33 species series, 56 species groups, and 11 species subgroups. According to Frost (2008) and Hedges *et al.* (2008), nearly 85 species of Terrarana are found in Brazil, being representatives of the families Strabomantidae, Craugastoridae, Eleutherodactylidae and Brachycephalidae. However, such suggested number of Brazilian species appears to be highly underestimated, mainly for the Amazon region in which more than 300 species were already described in the neighbour countries (Frost, 2008).

In their molecular phylogenetic study, Hedges *et al.* (2008) indicated that the South America is the most poorly sampled region. In addition, most nodes in the phylogenetic tree proposed by those authors are low supported suggesting polytomies and increasing the risk of misinterpretation of species clustering.

The use of molecular analysis as a tool to complement the traditional morphology based taxonomy has added new insights to anuran phylogeny. Such new kinds of characters have been proven important to improve anuran phylogenetic hypotheses since previously well-established clades have been refuted (Ford and Cannatella, 1993; Haty *et al.*, 1995; Garda *et al.*, 2002).

In many anuran groups, several aspects of the spermatozoa ultrastructural organization have also been used as an additional source of data for systematic and taxonomy inferences (e.g. Jamieson, 1991; Garda *et al.*, 2002; Veiga-Menoncello *et al.*, 2007; Aguiar *et al.*, 2006; Siqueira et al, unpublished data). The anuran spermatozoa provide a useful suite of taxonomic discriminating characters, including variations in the head structures, arrangement of the centrioles and flagelle components (Jamieson *et al.*, 1993; Meyer *et al.,* 1997; Costa *et al*., 2004; Garda *et al.*, 2004, Siqueira S., unpublished data). As previously indicated by Garda *et al.* (2002), the anuran sperm ultrastructure must be evaluated under a phylogenetic scope with continued evaluation of distinguishing characters, including the families that have not been studied (e.g. Sooglossidae, Centrolenidae, Rhinophrynidae, Mantellidae, Hyperoliidae, and Brachycephalidae), in order to built a consistent data set that enables parsimony analysis.

In the present work we analyzed the sperm ultrastructure of the Brazilian eleutherodactyline species *Brachycephalus ephippium, Ischnocnema guentheri, I. parva, I. juipoca* and *I.*

paulodutrai, in the family Brachycephalidae, and of *Haddatus binotatus*, in the family Craugastoridae, aiming at further understanding their taxonomic relationships.

Material and Methods

Adult males of *Brachycephalus ephippium, Ischnocnema guentheri, I. parva, I. juipoca, I. paulodutrai* and *Haddadus binotatus* were sampled under a permit issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (License nº 206/2005 - CGFAU/LIC). Voucher specimens were deposited in the Museu de História Natural "Prof. Dr. Adão José Cardoso" (ZUEC) of the Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil. Additional information on the analyzed species and respective accession numbers are shown in Table I.

The specimen testes were removed by dissection, cut into small pieces, and fixed overnight at 4ºC in 0.1M sodium cacodylate buffer, pH 7.2, supplemented with 2% paraformaldehyde, 2% glutaraldehyde, 3% sucrose, and 5 mM CaCl2. Postfixation was done for 1 h in the same buffer, containing 1% osmium tetroxide, 1% potassium ferricianide, and 5 mM CaCl2. The tissue samples were subsequently rinsed in sodium cacodylate buffer and incubated for 2 h in 0.5% uranyl acetate. Subsequently, after rinsing in buffer, the samples were dehydrated in an increasing acetone series and embedded in Epon resin. Ultrathin sections obtained with an ultramicrotome using a diamond knife Diatome were stained with uranyl acetate and lead citrate, and then examined with a LEO 906 transmission electronmicroscope (EM).

Results

The species *Ischnocnema guentheri*, *I. parva* and *I. juipoca* were highly similar in their sperm morphology and distinct from the other examined species. The basic spermatozoon ultrastructure of *I. paulodutrai*, *Brachycephalus ephippium* and *Haddadus binotatus* differed in the sperm cell width, in the acrosome complex, shape of midpiece and presence or absence of axial fiber and undulating membrane in the tail.

Light microscopy

Under light microscopy, the spermatozoa of the four *Ischnocnema* species were filiform with curved head and a very long midpiece that were visible in phase contrast. The tail did not present undulating membrane or paraxonemal rod (*sensu* Lee and Jamieson 1993).

The spermatozoa of *B. ephippium* and *H. binotatus* were filiform, and their head was curved and wider when compared with the other species. The midpiece was short, not clearly visible, and the tail was long with an easily distinguishable undulating membrane.The axoneme was seen describing a very sinuous path along the axial fiber.

Electron Microscopy

Acrosomal complex

In the sperm cells of the *I. guentheri, I. parva* and *I. juipoca* species, a thin acrosomal vesicle covers the anterior portion of the nucleus and the acrosome complex width varies from the apice to the basis and a thin subacrosomal cone is located behind the acrosomal vesicle, consisting of low electrondense substance with fibrilar arrangement (Fig 1a-g). As for *I. parva* and *I. juipoca*, such characteristic can be inferred by the tranverse sections of the nucleus (Fig 1e-g). In the *I. paulodutrai*, the typical subacrosomal cone was not observed in the examined samples, but this structure putatively exists as a low electrondense thin lamina attached to the nuclear envelope and chromatin, as inferred by transverse sections of the head (Fig 1h-l).

In *B. ephippium* and *H. binotatus*, a conical acrosomal vesicle, thin and filled with a substance of low electrondensity, covers the anterior portion of the nucleus (Figs 1n-r, t-x). In both species, the acrosome complex width was the largest of all species, varying from the apice to the basis, and a subacrosomal cone is located behind the acrosomal vesicle. In *B. ephippium*, this structure was fusionate in the apice of the acrosome complex and characterized by a gross fibrillar appearance and in cross sections of intermediate portions, this structure appears as irregular masses of dense material (Fig 1n-r). In *H. binotatus*, the subacrosomal cone consists of a thin substance of more solid/homogeneous appearance and low electrondensity (Fig 1t-v). A subacrosomal space was observed in *H. binotatus* (Fig 1v).

Nucleus

In the sperm of the four *Ischnocnema* species, the nucleus is thin and circular in all its extention when observed in transverse sections and conical in longitudinal sections. As for all other species the nucleous is thin and almost with the same thickness along its entire lenght. In this species the chromatin is highly condensed and electron-dense (Fig. 1d, g, j, m, s, z).

In *B. ephippium* the subacrosomal cone form invaginations filled by the chromatin, which confers a non circular shape to the nucleus (Figs. 1p, r). In the longitudinal section of *Haddadus binottatus* and *B. ephippium* the nucleus is conical and the chromatin is highly condensed and electron-dense. Despite the high degree of condensation, in both species, several small electron-lucent nuclear lacunae are seen and also inclusions can be seen in *B. ephippium* and *H. binotatus* (Fig. 1s, r, t, y,z).

Midpiece

In all *Ischnocnema* species, the posterior portion of the nucleus is curved, forming a discrete nuclear fossa above the proximal centriole and the pericentriolar material (Figs. 2a, f, j, o). In these species, the proximal centriole lies at an approximate angle of 90° with respect to the longitudinal axis of the spermatozoon and the distal centriole (Figs. 2a, f, j, o). Both centrioles are embedded in a pericentriolar material, with low electrondensity and no mitochondria were observed in the midpiece.

In *B. ephippium* and *H. binotatus,* the posterior portion of the nucleus is curved, forming a nuclear fossa surrounding the proximal centriole and the pericentriolar material (Figs. 2r, v). In both species, the proximal centriole lies at an approximate angle of 90° with respect to the longitudinal axis and oblique to the distal centriole (Fig. 2r, v, w). A paraxonemal rod reachs the proximal centriole and is embedded in the pericentriolar material, which is composed by a homogenous and low electrondense material (Fig. 2r, s, v, w). The *Brachycephallus ephippium* species showed numerous small mitochondria surrounding the pericentriolar material from the nuclear base to the end of the distal centriole (fig 2r). *Haddadus binotatus* showed large and numerous mitochondria surrounding the midpiece (Fig. 2v).

Tail

In all *Ischnocnema* species, the tail consists of only an axoneme with the usual 9+2 pattern and in the sperm transversal sections of *I. guentheri, I. parva, I. juipoca,* below the distal centriole, nine to eleven flat and contiguous mitochondria were observed surrounding the axoneme (Fig. 2c, h, l, m) while only one or two cylindrical mitochondria surrounded the axoneme in *I. paulodutrai* (Fig 2p). In longitudinal sections, a continuous layer of mitochondria was seen adjacent to the axoneme of these species (Fig 2b, f, g, j, k, p). These mitochondria did not reach the end of the flagellum, where only the axome was observed. In *I. guentheri*, the mitochondria of the flagellum

were present in sperm at both immature and advanced stages of maturation, as inferred by the surrounding microtubules (Fig 2c). In all *Ischnocnema* species, only the axoneme was seen at the tip end of the flagellum (Fig 2d, e, i, n, q).

The sperm tail of *B. ephippium* and *H. binotatus* consists of an axoneme with the usual 9+2 pattern, a very thin undulating membrane, and an axial fiber which is larger and less electrondense in *H. binotatus*, as observd in transverse sections (Fig 2t, u, x-z). In *B. ephippium*, the axial fiber is connected to the axonema at doublet 3 by an electrondense substance within the undulating membrane – the axial sheet (Fig 2t, u). In adition, in *B. ephippium* the axial fiber is not distinguishable at the anterior portion of the flagellum and, hence, it was named paraxonemal rod (*sensu* Lee and Jamieson 1993) (Fig. 2u).

Discussion

As first descriptions of sperm ultrastructure in species of the families Brachycephalidae and Brazilian Craugastoridae, the data presented herein are complementary to sperm studies in Terrarana frogs by Siqueira S. (unpublished data). The variation in sperm ultrastructure observed among the analyzed Brachycephalidae and Craugastoridae sperm cells included some of the evolutionary trends proposed by Kwon and Lee (1995), Lee and Jamieson (1993) and Jamieson (1999) to the species previously analyzed. Lee and Jamieson (1993) argued that the simplification of spermatozoa structures would be an evolutionary trend in anurans. Such simplifications include the absence of tail accessory filaments and of the subacrosomal cone (Jamieson, 1999; Garda *et al.*, 2002; Aguiar *et al.*, 2004).

The shared lack of tail accessories in *I. guentheri, I parva* and *I. jupoca*, indicates close relationships among these species, and corroborate their clustering in the same genus, as proposed by Hedges *et al.* (2008) based on molecular studies, since the absence of such flagellar accessories is considered as an apomorphic condition, as reported by Scheltinga *et al.* (2003).

Similarly, differences found in the sperm structures of the species here analyzed, are being considered as indicative of non-monophyletism in another anuran groups (e.g. Amaral *et al.*, 2000; Aguiar Jr. *et al.*, 2003, 2006; Veiga-Menoncello *et al.*, 2007; Siqueira S. unpublished data). As an example, diversity regarding the presence or absence of auxiliary fibers in the tail and the presence of the acrosomal vesicle or the acrosomal vesicle plus a subacrosomal cone has been observed in the family Leptodactylidae which is the family where the “eleutherodactyline” frogs

were previously allocated, and currently is recognized as a non-monophyletic taxon (see Pugin-Rios and Garrido 1981; Báo *et al* 1991). Therefore, as emphasized by Amaral *et al.* (2000), the sperm ultrastructral diversity reinforced the supposed polyphyletism in the leptodactylide frogs. Reinforcing such hypothesis Frost *et al.* (2006) have shown by the molecular findings that most of the previously related species which differed in the sperm ultrastructure are in separated into new families.

According to Lee and Jamieson (1993) Scheltinga *et al.* (2003) the abscence of the subacrossomal cone is a possible evolution of the acrosomal complex, since its presence were seen in urodeles, gymnophionans, and the most primitive anurans. In the present work, the subacrosomal cone was absent only in *I. paulodutrai.* Following the statement of Scheltinga *et al.* (2003), lack of the subacrosomal cone could indicate a shared apomorphy between these two species when compared with the remaining analyzed *Ischnocnema*, *H. binotatus* and *B. ephippium* species, which presented a conspicuous subacrossomal cone.

In the midpiece, all species had particular characteristics including the angle of the centrioles and the pericentriolar material density. Kwon and Lee (1995) tentatively inferred phylogenetic information from the centriolar arrangement. They suggested that the simplification of spermatozoa structures would be an evolutionary trend, while Jamieson (1999) argued that in gymnophionans and urodeles the centrioles lie perpendicular to each other, this condition appears to be the plesiomorphic state. The *B. ephippium* and *H. binotatus* species, which belong to different families, share the putative plesiomorphic condition (oblique arrangement), typical of species possessing undulating membrane. The apomorphic condition arrangement of typical sperm cell, without undulating membrane, is shared by all analyzed *Ischnocnema*, what reinforces their status of congeric species.

The typical collar-like mitochondrial arrangement of the bufonoid lineage was not observed in any of the species analyzed. The mitochondria found in the *I. guentheri, I. parva* an *I. juipoca*, surrounding a portion of the axoneme, were previously described in *Hyperolius punctulatus* (Hyperoliidae), a biflagellate species, *Mantidactylus majory* (Mantellidae), *Cophixalus ornatus,* (Microhylidae) and *Rana temporaria* (Ranidae). Other Terrarana frogs of the family Strabomantidae, such as *Pristimantis fenestratus* and *P. crepitans,* share this character, however with five to six circular mitochondria, differing from these *Ischnocnema* species which showed

interspecific differences in number and morphology of this organelle (Siqueira S. unpublished data).

In spite of differences among the *Ischnocnema* and *Pristimantis* species, the shared character of long mitochondria adjacent to the axoneme seems to indicate a close evolutionary relationship among them, in agreement with Hedges *et al.* (2008). Garda (2002) inferred that this condition as the most advanced compared with mitochondria arranged as a collar around the midpiece either surrounding the anterior portion of the flagellum or distributed around the axial fiber.

The mitochondria lying on a cytoplasmic mass around the base of the nucleus, as observed in *H. binotatus*, were previously reported to the Myobatrachidae *Mixophyes* and *Rheobatrachus*, to the Calyptocephallidae *Caudiverbera* and to the Leiuperidae *Pleurodema* (Pugin-Rios and Garrido 1981). However, in these latter four species the mitochondria are lost during the sperm maturation process mature sperm maturity. As stated by Lee and Jamieson (1993), the actual contribution of mitochondia distribution to phylogenetic inferences is not clear because they seem to disappear during the ontogeny of the gamete in anurans. Therefore, the *H. binotatus* taxonomic relationships caanot be inferred based upon this distinctive trait.

The absence of the axial fiber in the sperm tail of the *Ischnocnema* species and the presence of this structure in *B. ephippium* and *H. binotatus*, indicate a noise in the systematic relationships, mainly among the Brachycephalidae species. According to Jamieson (1991), the absence of an axial fiber is an apomorphy in Megophryidae, Pelobatidae, Pipidae, Hyperoliidae, Calyptocephallidae, Ceratophryidae, the hylid *Pseudis paradoxa*, Microhylidae, Ranidae and Racophoridae. Considering the author's argument, we could infer a synapomorphy within the genus *Ischnochnema*, again reinforcing the close relationships among its analyzed species, as well as between *P. fenestratus* (Strabomantidae) and the analyzed *Ischnocnema* species, giving support to an interfamilial relationship within Terrarana as indicated by the molecular analysis of Hedges *et al.* (2008).

Although sperm differences were identified among the species analyzed in the present work, further studies are necessary, with an increased number of species, in order to better understand the evolutionary significance of ultrastructural sperm characteristics. Moreover, these studies will allow inferences on the correlation and congruence of sperm ultrastructural diversity

with molecular data, since DNA se quences in conjunction with morphological characteristics presently constitutes the basis of anuran systematics (see Frost, 2008).

Systematic comments

Observing the phylogenetic tree proposed by Hedges *et al.* (2008), the support of most nodes is low revealing unsolved relationships between some of the clades. The low supported relatedness includes the node clustering *Brachycephalus* and *Ischnocnema* in the family Brachycephalidae, and the node clustering *Haddadus* in the family Craugastoridae. In addition, the species *I. paulodutrai* were not included in the molecular studies, hampering the assessment of its position in relation to other *Ischnocnema,* as well as in Brachycephalidae. However, considering the apomorphic absence of a subacrosomal cone in the sperm head, it is feasible to infer relationships between *I. paulodutrai* and the congeneric *Ischnocnema*.

The ultrastructural differences between the sperm of *B. ephippium* and the *Ischnocnema* species corroborate remaining doubts regarding the grouping of these taxa in the same family, as conisdered by Hedges *et al.* (2008).

The *H. binotatus* and *I. guenteri* sperm ultrastructure, mainly that of the flagellar apparatus, and the chromosome data described by Siqueira *et al.* (2004) are in agreement with the molecular phylogeny reported by Hedges *et al.* (2008), which removes the previously "*Eleutherodactylus" binotatus* from the genus *Ischocnema,* as previoulsy proposed by Heinicke *et al.* (2007). According to Hedges *et al.* (2008), the genus *Haddadus* and the group of *Ischnocnema ramagii*, that include *I. paulodutrai,* share morphological characters and could be closely related. However, the authors maintained the respective genera. The sperm differences described herein and the high chromosome number in *I. paulodutrai* (2n=30; Siqueira *et al.* 2008) support the status of *Haddadus* and *Ischnocnema* as distinct genera.

Considering the molecular data, only the family Craugastoridae was a well-supported clade, coincidently with the lowest range in chromosome number variation (2n=18, 20 and 22; see Kuramoto, 1990). On the other hand, Hedges *et al.* (2008) included *Haddadus* in the family Craugastoridae, but in a low-supported node, which can indicate the necessity of further studies in order to test this clustering. In terms of sperm ultrastructure, however, *Haddadus binotatus* share sperm plesiomorphies with *B. ephippium*, *i. e.* presence of a subacrosomal cone and

accessory tail fibers, and the former characteristic is also shared with *I. juipoca* and *I. guentheri*. However, this shared plesiomorphy does not indicate phylogenetic relationships.

The inclusion of *Ischnocnema paulodutrai* in the genus *Ischnocnema* seems controversial, as inferred by the differences in the acrosome complex and in the number and form of the mitochondria. This argument is reinforced by the divergence in chromosome number, which in I. paulodutrai is 2n=30 while the common numbers in the other *Ischnocnema* species are 2n=20 and 22. Moreover, the *I. paulodutrai* spermatozoon morphology was similar to the *Pristimantis dundeei* from Chapada dos Guimarães, Mato Grosso State, studied by Siqueira *et al.* (unpublished data). *Ischnochnema paulodutrai* and *Pristimantis dundeei* were also similar in their karyotypes consisting of 2n=30 and 2n=28, respectively (Siqueira *et al*. 2008). Apparently, these two species are more closely related to each other than to the remaining congeneric *Ischnocnema* species in Brazil, which are characterezid by lower (2n=20 and 22) and higher (2n=34) chromosome numbers (Siqueira *et al.* unpublish data). However, the divergences among those species identified thus far do not permit taxonomic inferences, since *I. paulodutrai* and *P. dundeei*.were not analyzed in molecular studies.

The data on the Terrarana species represent an elucidative example that variation in sperm morphology, in conjunction with chromosome number variation, are indicative of polyphyletic groups. Further sperm ultrasctructural and karyotypical analysis of *I. paulodutrai* and *P. dundeei*, as well as of other species of Brazilian Terrarana frogs, can clarify aspects of the taxonomic relationships of these species.

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Table I. Specimens analyzed for sperm ultrastructure

Species	Number of specimens	Localities	Accession Numbers
Ischnocnema guentheri	5	Mogi das Cruzes, São Paulo State	104.1-104.5
Ischnocnema parva	3	Mogi das Cruzes, São Paulo State	103.1-103.3
Ischnocnema juipoca	4	Atibaia, São Paulo State	224.1-224.4
Ischnocnema paulodutrai	11	Ilhéus, Bahia State	254.1-254.11
Brachycephalus ephippium	9	Mogi das Cruzes and Atibaia, São Paulo State	55.1-55.9
Haddadus binotatus	3	Mogi das Cruzes and Ilha Bela, São Paulo State	82.1-82.3

Number of specimens and sampling locations of the analyzed species. Accession numbers refer to the Coleção "Célio F. B. Haddad" at the Universidade Estadual Paulista (UNESP) Rio Claro, São Paulo, Brazil and Museu de Zoologia “Prof. Dr. Adão José Cardoso” (ZUEC), Unicamp

Legends

Figure 1. Electron micrographs of *Ischnocnema guentheri, I. parva* and *I. juipocai* (**a-g**), *I. paulodutrai* (**h-m**), *Brachycephalus ephippium* (**n-s**) and *Haddadus binotatus* (**t-z**). (**a, h, i, n, t**) Longitudinal section through the anterior head region showing the acrosomal complex with the acrosomal vesicle (av) and the subacrosomal cone (sc). Note the subacrosomal space (ss) behind the subacrosomal cone in *I. guentheri, I. parva, I. juipoca* and *H. binotatus,* above the nucleus (N). (**b-g, j-m, o-s, u-z**) Transverse sections through the head. In (**d, g, m, s, z**), note the high degree of chromatin condensation.

Figure 2. Electron micrographs of *Ischnocnema guentheri* (**a-e**)*, I. parva* (**f-i**), *I. juipocai* (**j-n**), *I. paulodutrai* (**o-q**), *Brachycephalus ephippium* (**r-u**) and *Haddadus binotatus* (**v-z**). In (**c, h, m**), note the mitochondria (m) surrounding the axoneme (ax)*,* and (**p**) beside the axoneme in *I. paulodutrai.* In (**r, v,w**) *B. ephippium* and *H. binotatus*, note the mitochondria surrounding the basal portion of the nucleus. (**a, f, j, r, v, w**) Longitudinal section through the posteriormost region of the nucleus, midpiece, and proximal portion of the flagellum, showing the nuclear fossa and the axoneme, with an adjacent accessory fiber in *B. ephippium* and *H. binotatus* (i.e. paraxonemal rod, pr). In (**t, u, x-z**), transverse section of the anterior portion of the flagellum showing a conical axial fiber (af). In (**t-v, x-z**), note the very thin undulating membrane (um) connecting the accessory fibers and the axoneme. (**b-e, g-i, k-n, p-q**) End of the mitochondria region and only the axoneme (ax) is seen at the tip of the tail shortening. (**t-u, x-z**) Progressive shortening of the undulating membrane (um) and approximation of the accessory fibers, and disappearance of the axial fibers.

Figure 1

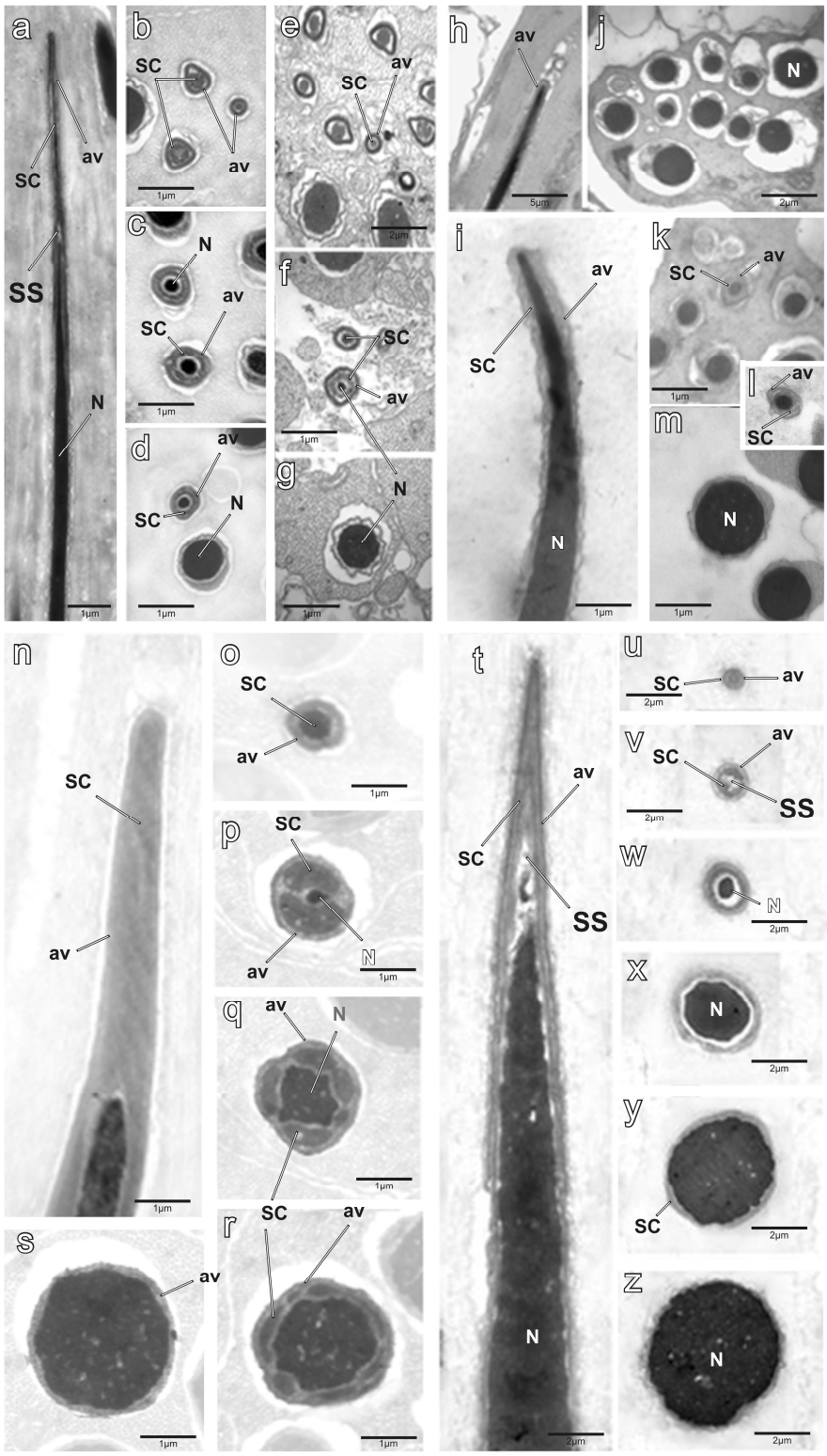
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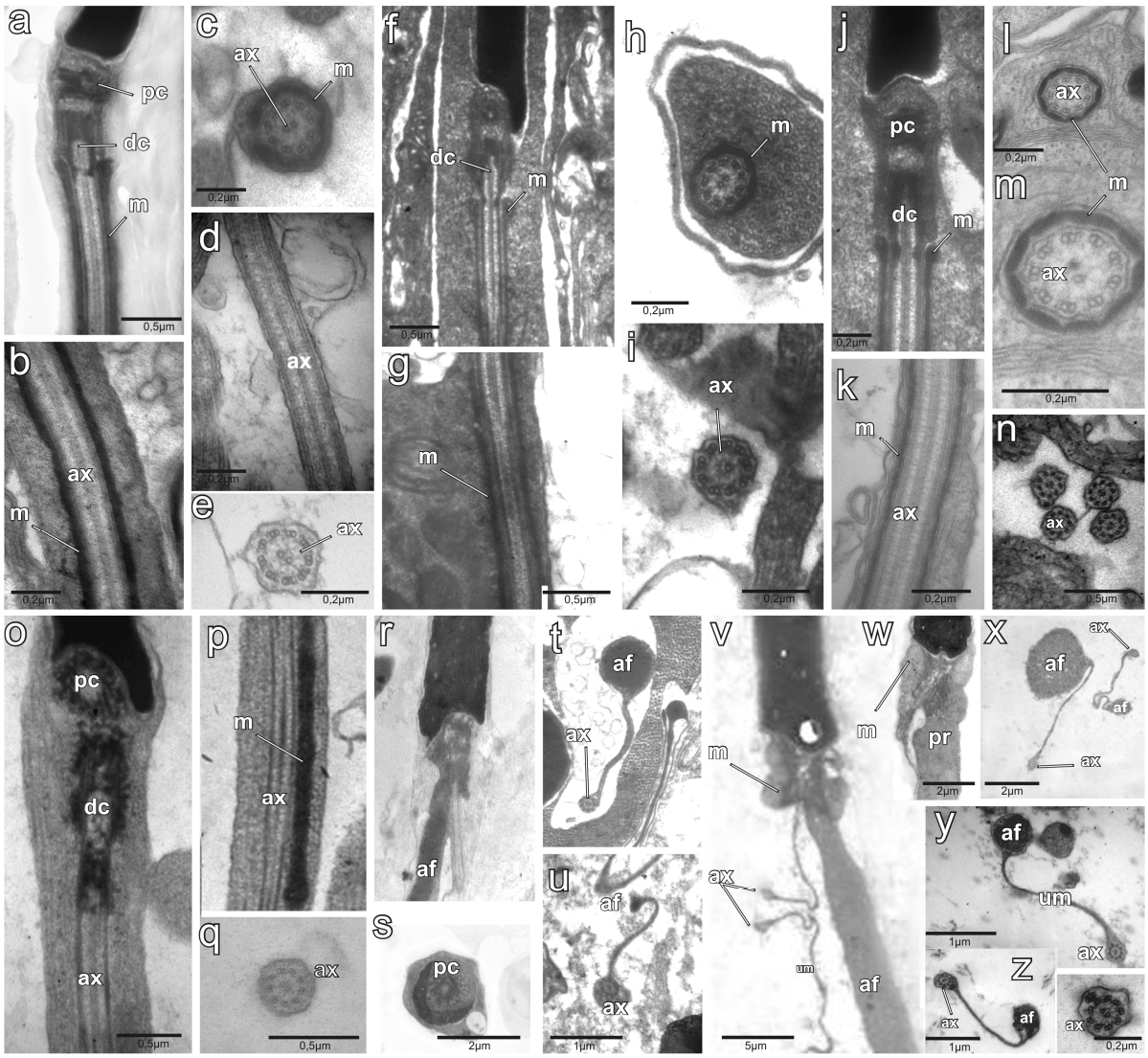
Figure 2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IX. Conclusões

No presente trabalho, tanto os dados citogenéticos, quanto os primeiros dados da ultra-estrutura dos espermatozóides dos Terrarana do Brasil apresentaram características intra- e intergenéricas não conservadas. Nesse sentido, estes estudos contribuíram de modo significativo para a compreensão de alguns dados de estudos anteriores, assim como, para o levantamento de algumas problemáticas.

Neste estudo, os dados citogenéticos foram importantes na caracterização cromossômica de espécies ainda não amostradas como *Pristimantis fenestratus* 2n=34, *P. crepitans* 2n=22, e ainda dois novos números cromossômicos para os Terrarana da América do Sul, 2n=28 em *P. dundeei* e 2n=30 em *Iscnocnema paulodutrai.* Além disso, os marcadores cromossômicos, bandamento C e Ag-NOR, indicaram a existência de novas espécies em populações de *P. fenestratus, P. dundeei* e *Barycholos ternetzi.* Os estudos cromossômicos em *P. fenestratus* revelaram ainda a existência de um caso de variação intra-individual cromossômica somática, possivelmente gerada pela segregação anômala de cromátides irmãs na divisão celular. Os dados de ultra-estrutura dos espermatozóides mostraram variação na estrutura do complexo acrossomal, sendo que apenas *P. dundeei* e *I. paulodutrai* não apresentaram um cone subacrossomal. Ainda, como característica mais marcante, a existência de um bastão axonemal em *Haddadus binotatus, Brachycephalus ephippium, Brarycholos ternetzi* e *P. crepitans* e a ausência desta estrutura em *P. fenestratus, P. dundeei* e todos os *Ischnocnema* amostrados.

Esses dados são coerentes com alguns dos rearranjos propostos por Frost *et al*. (2006), Heinicke *et al*. (2007) e Hedges *et al*. (2008): (1) as análises de ultra estrutura de espermatozóides de *Haddadus* e de *Ischnocnema* mostram um bastão axonemal muito espesso em *H. binotatus* e a ausência desta estrutura em *Ischnocnema.* Essas espécies, antes dos trabalhos de Heinicke *et al*. (2007) e Hedges *et al*. (2008) eram alocadas no mesmo gênero (ver Frost *et al*., 2006); (2) *Ischnocnema parva* que agrupa com duas espécies da série de *I. guentheri* de acordo com a proposta de Heinicke *et al*. (2007) e Hedges *et al*. (2008), apresenta número diplóide 2n=22 cromossomos (Siqueira *et al*., 2004); (3) apesar das espécies da série de *I. ramagii* e *H. binotatus* compartilharem um caráter singular de morfologia digital, que poderia alocar estas espécies no gênero *Haddadus,* a não inserção das espécies da série de *Ischnocnema ramagii* no gênero *Haddadus* poderia ser corroborada por dados citogenéticos de *I. paulodutrai,* já que o número diplóide dessa espécie é 2n=30 e o de *H. binotatus* é 2n=22 (Beçak & Beçak, 1974, Siqueira *et al*., em preparação), e por dados de morfologia da ultra-estrutura de espermatozóides, pois o flagelo de espermatozóide de *I. paulodutrai* não possui bastão axonemal, enquanto o de *H.*

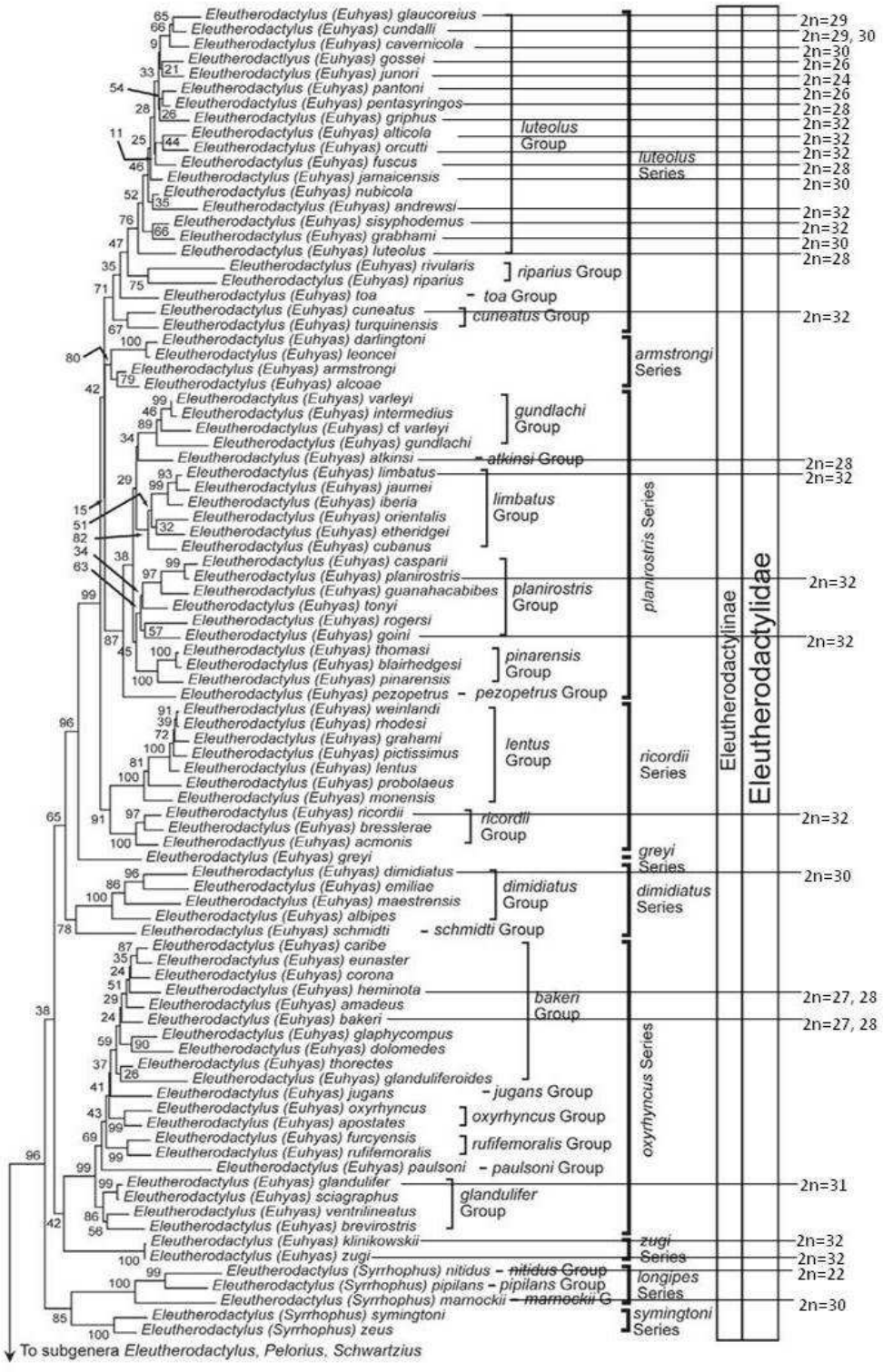
binotatus possui um bastão muito espesso.

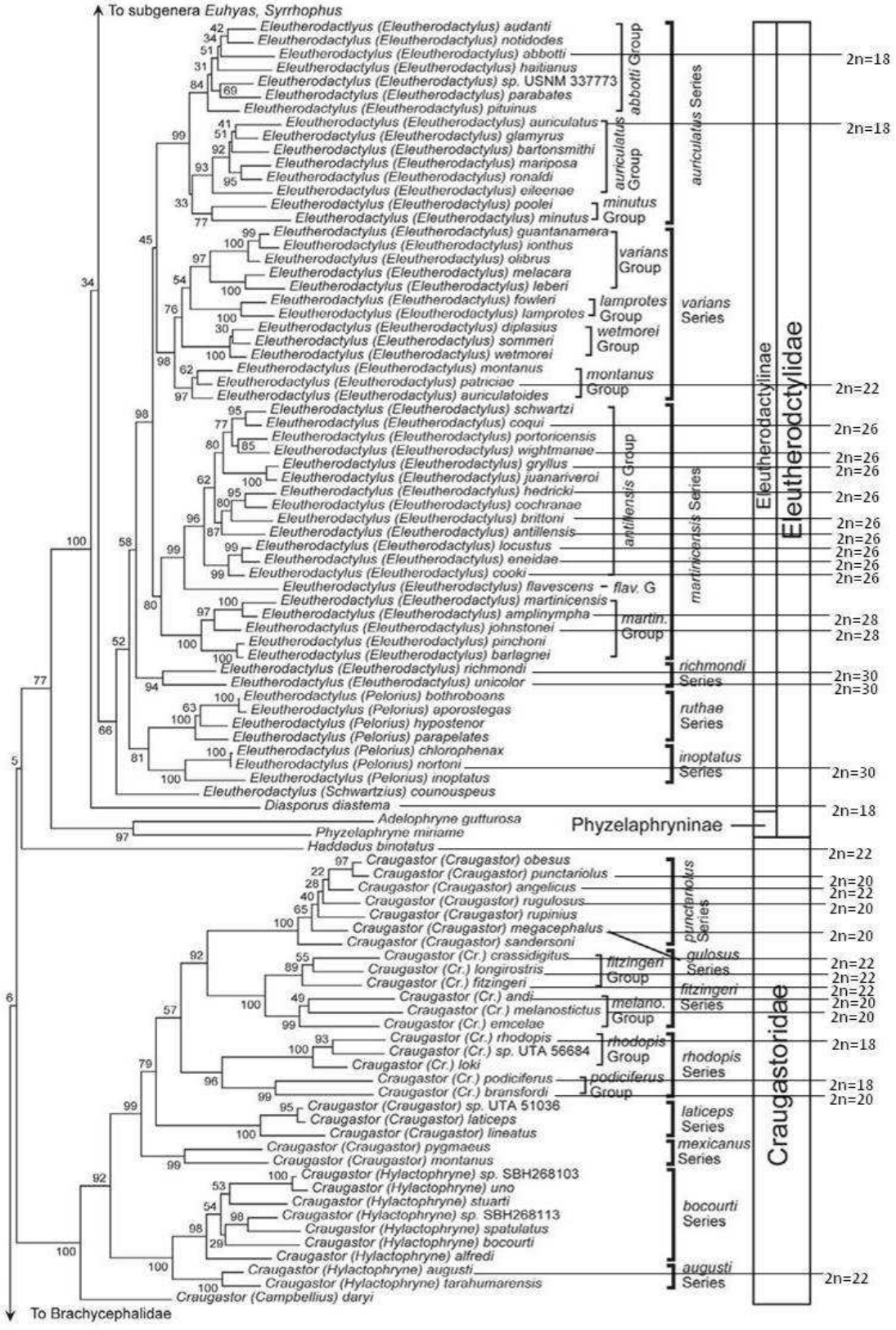
Por outro lado, estes mesmos estudos citogenéticos e de ultra-estrutura de espermatozóides também levantaram algumas questões sobre os relacionamentos de espécies que não foram amostradas nos trabalhos de Heinicke *et al*. (2007) e Hedges *et al*. (2008): (1) Hedges *et al*. (2008) indicam certa inconsistência no agrupamento de *Ischnocnema* e *Brachycephalus* em uma mesma família e as análises de ultra-estrutura de espermatozóides de *I. guentheri, I. parva, I. juipoca* e *B. ephippium* mostram características muito distintas nesse último, cujo espermatozóide possui bastão axonemal, estrutura ausente no espermatozóide das espécies de *Ischnocnema*; (2) apesar da série de *I. ramagii* ter sido alocada dentro do gênero *Ischnocnema*, o número diplóide encontrado para *I. paulodutrai* (serie de *I. ramagii*) é de 2n=30 cromossomos, em contrapartida os números comuns para outras espécies de *Ischnocnema* são de 2n=20 e 22 cromossomos, indicando que podem existir problemas de relacionamento entre a série de *I. ramagii* em relação a outros *Ischnocnema*; (3) estudos cromossômicos de *I. paulodutrai*, 2n=30 e *Pristimantis dundeei*, 2n=28 cromossomos, e de ultra-estrutura de espermatozóides, que mostram que ambas as espécies apresentam flagelo sem bastão axonemal, e a presença de uma ou duas mitocôndrias na peça intermediária, uma característica exclusivamente encontrada nessas espécies até o momento, podem indicar um relacionamento próximo entre essas espécies, apesar de estarem em gêneros separados; (5) *Pristimantis crepitans* (grupo *peruvianus*) diferiu drasticamente nas análises citogenéticas e de ultra-estrutura de espermatozóides em relação a outros *Pristimantis,* apresentando um bastão axonemal, não encontrado em *P. fenestratus* ou *P. dundeei* (grupo de *P. peruvianus*), indicando que *P. crepitans* pode estar alocado dentro do gênero *Pristimantis* equivocadamente.

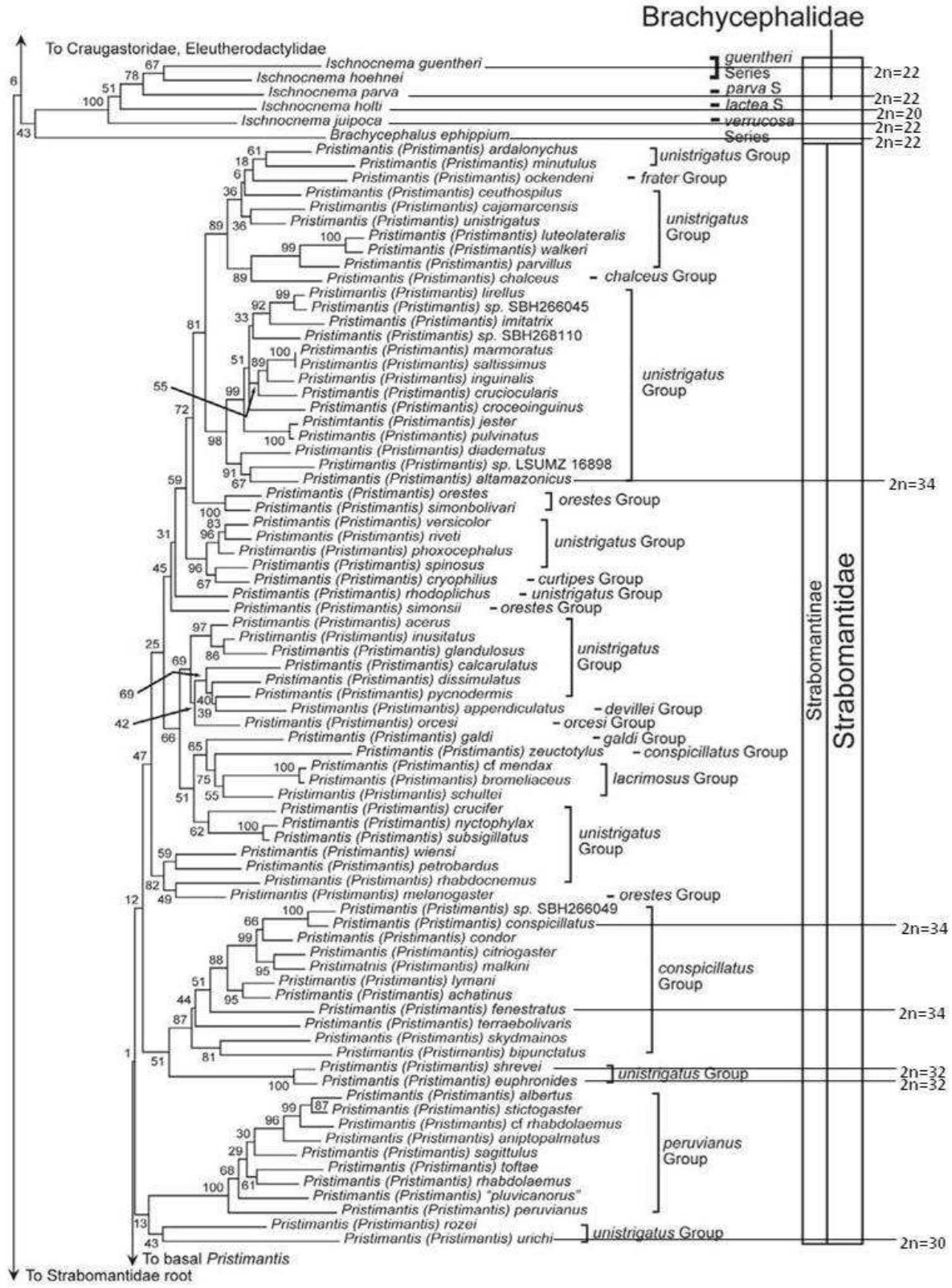
O grande número de diferenças encontradas entre as espécies aqui analisadas por dados citogenéticos e da ultra-estrutura dos espermatozóides, poderiam indicar direções evolutivas e um melhor entendimento dos aspectos filogenéticos destas espécies, entretanto o número de espécies aqui amostradas é muito pequeno e muitas delas ainda não foram amostradas pela filogenia molecular. Este fato indica a necessidade na ampliação do número de táxons amostrados, incluindo dados morfológicos, citogenéticos, da ultra-estrutura dos espermatozóides e moleculares para o entendimento dos relacionamentos inter- e intragenéricos entre os Terrarana do Brasil, além da compreensão dos pocessos evolutivos citogenéticos e dos espermatozóides destas espécies.

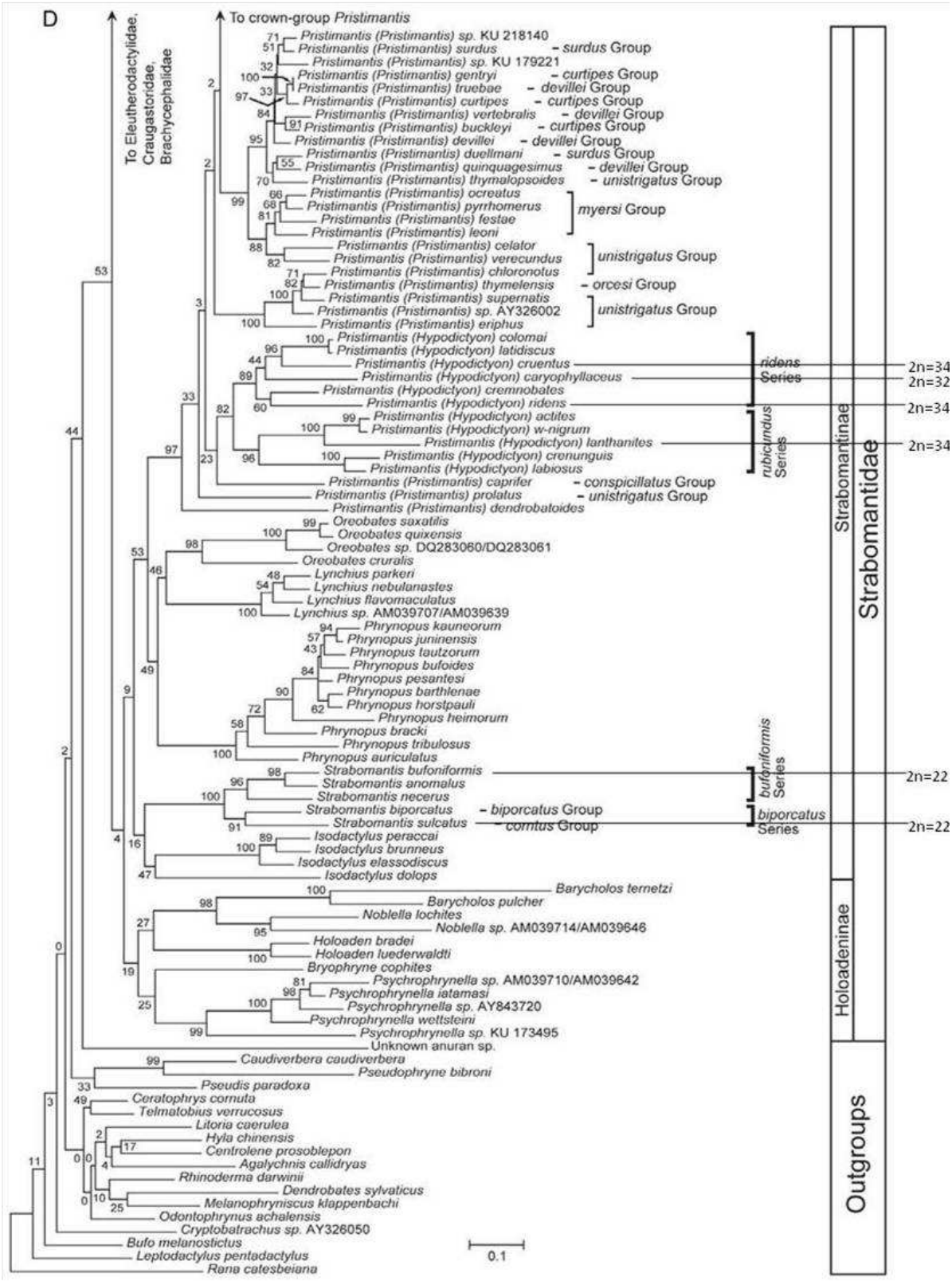
X. Apêndice

Números cromossômicos de espécies presentes nos clados aplicados a árvore filogenética de Hedges *et al*. (2008). Os números sobre os ramos indicam o suporte (bootstrap)




To subgenera Euhyas, Syrrhophus
Eleutherodactlyus (Eleutherodactylus) audanti
Eleutherodactylus (Eleutherodactylus) notidodes
Eleutherodactylus (Eleutherodactylus) abbotti
Eleutherodactylus (Eleutherodactylus) haitianus
Eleutherodactylus (Eleutherodactylus) sp. USNM 337773
Eleutherodactylus (Eleutherodactylus) parabates
Eleutherodactylus (Eleutherodactylus) pituinus
abbotti Group
Eleutherodactylus (Eleutherodactylus) auriculatus
Eleutherodactylus (Eleutherodactylus) glamyrus
Eleutherodactylus (Eleutherodactylus) bartonsmithi
Eleutherodactylus (Eleutherodactylus) mariposa
Eleutherodactylus (Eleutherodactylus) ronaldi
Eleutherodactylus (Eleutherodactylus) eileenae
auriculatus Group
Eleutherodactylus (Eleutherodactylus) poolei
Eleutherodactylus (Eleutherodactylus) minutus
minutus Group
auriculatus Series
Eleutherodactylus (Eleutherodactylus) guantanamera
Eleutherodactylus (Eleutherodactylus) ionthus
Eleutherodactylus (Eleutherodactylus) olibrus
Eleutherodactylus (Eleutherodactylus) melacara
Eleutherodactylus (Eleutherodactylus) leberi
varians Group
Eleutherodactylus (Eleutherodactylus) fowleri
Eleutherodactylus (Eleutherodactylus) lamprotes
lamprotes Group
Eleutherodactylus (Eleutherodactylus) diplasius
Eleutherodactylus (Eleutherodactylus) sommeri
Eleutherodactylus (Eleutherodactylus) wetmorei
wetmorei Group
varians Series
Eleutherodactylus (Eleutherodactylus) montanus
Eleutherodactylus (Eleutherodactylus) patriciae
Eleutherodactylus (Eleutherodactylus) auriculatoides
montanus Group
2n=22
Eleutherodactylus (Eleutherodactylus) schwartzi
Eleutherodactylus (Eleutherodactylus) coqui
2n=26
Eleutherodactylus (Eleutherodactylus) portoricensis
Eleutherodactylus (Eleutherodactylus) wightmanae
2n=26
Eleutherodactylus (Eleutherodactylus) gryllus
2n=26
Eleutherodactylus (Eleutherodactylus) juanariveroi
Eleutherodactylus (Eleutherodactylus) hedricki
2n=26
Eleutherodactylus (Eleutherodactylus) cochranae
Eleutherodactylus (Eleutherodactylus) brittoni
2n=26
Eleutherodactylus (Eleutherodactylus) antillensis
2n=26
Eleutherodactylus (Eleutherodactylus) locustus
2n=26
Eleutherodactylus (Eleutherodactylus) eneidae
2n=26
Eleutherodactylus (Eleutherodactylus) cooki
2n=26
antillensis Group
Eleutherodactylus (Eleutherodactylus) flavescens
flav. G
martinicensis Series
Eleutherodactylus (Eleutherodactylus) martinicensis
Eleutherodactylus (Eleutherodactylus) amplinympha
2n=28
Eleutherodactylus (Eleutherodactylus) johnstonei
2n=28
Eleutherodactylus (Eleutherodactylus) pinchoni
Eleutherodactylus (Eleutherodactylus) barlagnei
martin. Group
Eleutherodactylus (Eleutherodactylus) richmondi
richmondi Series
2n=30
Eleutherodactylus (Eleutherodactylus) unicolor
2n=30
Eleutherodactylus (Pelorius) bothroboans
Eleutherodactylus (Pelorius) aporostegas
Eleutherodactylus (Pelorius) hypostenor
Eleutherodactylus (Pelorius) parapelates
ruthae Series
Eleutherodactylus (Pelorius) chlorophenax
Eleutherodactylus (Pelorius) nortoni
Eleutherodactylus (Pelorius) inoptatus
inoptatus Series
2n=30
Eleutherodactylus (Schwartzius) counouspeus
Diasporus diastema
2n=18
Adelophryne gutturosa
Phyzelaphryne miriame
Phyzelaphryninae
Haddadus binotatus
2n=22
Eleutherodactylinae
Eleutherodactylidae
Craugastor (Craugastor) obesus
Craugastor (Craugastor) punctariolus
2n=20
Craugastor (Craugastor) angelicus
2n=22
Craugastor (Craugastor) rugulosus
2n=20
Craugastor (Craugastor) rupinius
Craugastor (Craugastor) megacephalus
2n=20
Craugastor (Craugastor) sandersoni
punctariolus Series
Craugastor (Cr.) crassidigitus
Craugastor (Cr.) longirostris
Craugastor (Cr.) fitzingeri
fitzingeri Group
gulosus Series
2n=22
2n=22
fitzingeri Series
2n=22
Craugastor (Cr.) andi
Craugastor (Cr.) melanostictus
Craugastor (Cr.) emcelae
melano. Group
melano. Series
2n=20
2n=20
Craugastor (Cr.) rhodopis
Craugastor (Cr.) sp. UTA 56684
Craugastor (Cr.) loki
rhodopis Group
2n=18
rhodopis Series
Craugastor (Cr.) podiciferus
Craugastor (Cr.) bransfordi
podiciferus Group
2n=18
2n=20
Craugastor (Craugastor) sp. UTA 51036
Craugastor (Craugastor) laticeps
Craugastor (Craugastor) lineatus
laticeps Series
Craugastor (Craugastor) pygmaeus
Craugastor (Craugastor) montanus
mexicanus Series
Craugastor (Hylactophryne) sp. SBH268103
Craugastor (Hylactophryne) uno
Craugastor (Hylactophryne) stuarti
Craugastor (Hylactophryne) sp. SBH268113
Craugastor (Hylactophryne) spatulatus
Craugastor (Hylactophryne) bocourti
Craugastor (Hylactophryne) alfredi
bocourti Series
Craugastor (Hylactophryne) augusti
Craugastor (Hylactophryne) tarahumarensis
augusti Series
2n=22
Craugastor (Campbellius) daryi
Craugastoridae
To Brachycephalidae


Brachycephalidae
To Craugastoridae, Eleutherodactylidae
Ischnocnema guentheri
Ischnocnema hoehnei
Ischnocnema parva
Ischnocnema holti
Ischnocnema juipoca
Brachycephalus ephippium
guentheri Series
parva S
lactea S
verrucosa Series
2n=22
2n=22
2n=20
2n=22
2n=22
Pristimantis (Pristimantis) ardalonychus
Pristimantis (Pristimantis) minutulus
Pristimantis (Pristimantis) ockendeni
unistrigatus Group
frater Group
Pristimantis (Pristimantis) ceuthospilus
Pristimantis (Pristimantis) cajamarcensis
Pristimantis (Pristimantis) unistrigatus
Pristimantis (Pristimantis) luteolateralis
Pristimantis (Pristimantis) walkeri
Pristimantis (Pristimantis) parvillus
unistrigatus Group
Pristimantis (Pristimantis) chalceus
chalceus Group
Pristimantis (Pristimantis) lirellus
Pristimantis (Pristimantis) sp. SBH266045
Pristimantis (Pristimantis) imitatrix
Pristimantis (Pristimantis) sp. SBH268110
Pristimantis (Pristimantis) marmoratus
Pristimantis (Pristimantis) saltissimus
Pristimantis (Pristimantis) inguinalis
Pristimantis (Pristimantis) cruciocularis
Pristimantis (Pristimantis) croceoinguinus
Pristimtantis (Pristimantis) jester
Pristimantis (Pristimantis) pulvinatus
Pristimantis (Pristimantis) diadematus
Pristimantis (Pristimantis) sp. LSUMZ 16898
Pristimantis (Pristimantis) altamazonicus
unistrigatus Group
2n=34
Pristimantis (Pristimantis) orestes
Pristimantis (Pristimantis) simonbolivari
orestes Group
Pristimantis (Pristimantis) versicolor
Pristimantis (Pristimantis) riveti
Pristimantis (Pristimantis) phoxocephalus
Pristimantis (Pristimantis) spinosus
unistrigatus Group
Pristimantis (Pristimantis) cryophilius
curtipes Group
Pristimantis (Pristimantis) rhodoplichus
unistrigatus Group
Pristimantis (Pristimantis) simonsii
orestes Group
Pristimantis (Pristimantis) acerus
Pristimantis (Pristimantis) inusitatus
Pristimantis (Pristimantis) glandulosus
Pristimantis (Pristimantis) calcarulatus
Pristimantis (Pristimantis) dissimulatus
Pristimantis (Pristimantis) pycnodermis
unistrigatus Group
Pristimantis (Pristimantis) appendiculatus
devillei Group
Pristimantis (Pristimantis) orcesi
orcesi Group
Pristimantis (Pristimantis) galdi
galdi Group
Pristimantis (Pristimantis) zeuctotylus
conspicillatus Group
Pristimantis (Pristimantis) cf mendax
Pristimantis (Pristimantis) bromeliaceus
lacrimosus Group
Pristimantis (Pristimantis) schultei
Pristimantis (Pristimantis) crucifer
Pristimantis (Pristimantis) nyctophylax
Pristimantis (Pristimantis) subsigillatus
unistrigatus Group
Pristimantis (Pristimantis) wiensi
Pristimantis (Pristimantis) petrobardus
Pristimantis (Pristimantis) rhabdocnemus
Pristimantis (Pristimantis) melanogaster
orestes Group
Pristimantis (Pristimantis) sp. SBH266049
Pristimantis (Pristimantis) conspicillatus
Pristimantis (Pristimantis) condor
Pristimantis (Pristimantis) citriogaster
Pristimatnis (Pristimantis) malkini
Pristimantis (Pristimantis) lymani
Pristimantis (Pristimantis) achatinus
Pristimantis (Pristimantis) fenestratus
Pristimantis (Pristimantis) terraebolivaris
Pristimantis (Pristimantis) skydmainos
Pristimantis (Pristimantis) bipunctatus
conspicillatus Group
2n=34
2n=34
Pristimantis (Pristimantis) shrevei
Pristimantis (Pristimantis) euphronides
unistrigatus Group
2n=32
2n=32
Pristimantis (Pristimantis) albertus
Pristimantis (Pristimantis) stictogaster
Pristimantis (Pristimantis) cf rhabdolaemus
Pristimantis (Pristimantis) aniptopalmatus
Pristimantis (Pristimantis) sagittulus
Pristimantis (Pristimantis) toftae
Pristimantis (Pristimantis) rhabdolaemus
Pristimantis (Pristimantis) "pluvicanorus"
Pristimantis (Pristimantis) peruvianus
peruvianus Group
Pristimantis (Pristimantis) rozei
Pristimantis (Pristimantis) urichi
unistrigatus Group
2n=30
To basal Pristimantis
To Strabomantidae root
Strabomantinae
Strabomantidae


D
To Eleutherodactylidae, Craugastoridae, Brachycephalidae
To crown-group Pristimantis
Pristimantis (Pristimantis) sp. KU 218140
Pristimantis (Pristimantis) surdus – surdus Group
Pristimantis (Pristimantis) sp. KU 179221
Pristimantis (Pristimantis) gentryi – curtipes Group
Pristimantis (Pristimantis) truebae – devillei Group
Pristimantis (Pristimantis) curtipes – curtipes Group
Pristimantis (Pristimantis) vertebralis – devillei Group
Pristimantis (Pristimantis) buckleyi – curtipes Group
Pristimantis (Pristimantis) devillei – devillei Group
Pristimantis (Pristimantis) duellmani – surdus Group
Pristimantis (Pristimantis) quinquagesimus – devillei Group
Pristimantis (Pristimantis) thymalopsoides – unistrigatus Group
Pristimantis (Pristimantis) ocreatus
Pristimantis (Pristimantis) pyrrhomerus
Pristimantis (Pristimantis) festae
Pristimantis (Pristimantis) leoni
myersi Group
Pristimantis (Pristimantis) celator
Pristimantis (Pristimantis) verecundus
unistrigatus Group
Pristimantis (Pristimantis) chloronotus
Pristimantis (Pristimantis) thymelensis – orcesi Group
Pristimantis (Pristimantis) supernatis
Pristimantis (Pristimantis) sp. AY326002
unistrigatus Group
Pristimantis (Pristimantis) eriphus
Pristimantis (Hypodictyon) colomai
Pristimantis (Hypodictyon) latidiscus
Pristimantis (Hypodictyon) cruentus
Pristimantis (Hypodictyon) caryophyllaceus
Pristimantis (Hypodictyon) cremnobates
Pristimantis (Hypodictyon) ridens
ridens Series
2n=34
2n=32
2n=34
Pristimantis (Hypodictyon) actites
Pristimantis (Hypodictyon) w-nigrum
Pristimantis (Hypodictyon) lanthanites
Pristimantis (Hypodictyon) crenunguis
Pristimantis (Hypodictyon) labiosus
rubicundus Series
2n=34
Pristimantis (Pristimantis) caprifer – conspicillatus Group
Pristimantis (Pristimantis) prolatus – unistrigatus Group
Pristimantis (Pristimantis) dendrobatoides
Oreobates saxatilis
Oreobates quixensis
Oreobates sp. DQ283060/DQ283061
Oreobates cruralis
Lynchius parkeri
Lynchius nebulanastes
Lynchius flavomaculatus
Lynchius sp. AM039707/AM039639
Phrynopus kauneorum
Phrynopus juninensis
Phrynopus tautzorum
Phrynopus bufoides
Phrynopus pesantesi
Phrynopus barthlenae
Phrynopus horstpauli
Phrynopus heimorum
Phrynopus bracki
Phrynopus tribulosus
Phrynopus auriculatus
Strabomantis bufoniformis
Strabomantis anomalus
Strabomantis necerus
bufoniformis Series
2n=22
Strabomantis biporcatus – biporcatus Group
Strabomantis sulcatus – cornutus Group
biporcatus Series
2n=22
Isodactylus peraccai
Isodactylus brunneus
Isodactylus elassodiscus
Isodactylus dolops
Barycholos ternetzi
Barycholos pulcher
Noblella lochites
Noblella sp. AM039714/AM039646
Holoaden bradei
Holoaden luederwaldti
Bryophryne cophites
Psychrophrynella sp. AM039710/AM039642
Psychrophrynella iatamasi
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Psychrophrynella wettsteini
Psychrophrynella sp. KU 173495
Unknown anuran sp.
Caudiverbera caudiverbera
Pseudophryne bibroni
Pseudis paradoxa
Ceratophrys cornuta
Telmatobius verrucosus
Litoria caerulea
Hyla chinensis
Centrolene prosoblepon
Agalychnis callidryas
Rhinoderma darwinii
Dendrobates sylvaticus
Melanophryniscus klappenbachi
Odontophrynus achalensis
Cryptobatrachus sp. AY326050
Bufo melanostictus
Leptodactylus pentadactylus
Rana catesbeiana
0.1
Strabomantinae
Strabomantidae
Holoadeninae
Outgroups