UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

CÍNTIA PELEGRINETI TARGUETA DE AZEVEDO BRITO

"ESTUDO CITOGENÉTICO E DAS RELAÇÕES FILOGENÉTICAS DE *Engystomops petersi* E *Engystomops* sp. (ANURA, LEIUPERIDAE)"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Cíntia Pelegrineti T. de Azevedo Brito

e aprovada pela Comissão Julgadora.

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

Orientadora: Profa. Dra. Luciana Bolsoni Lourenço Morandini

Campinas, 2009

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

B777e

Brito, Cíntia Pelegrineti Targueta de Azevedo
Estudo citogenético e das relações filogenéticas de *Engystomops petersi* e *Engystomops* sp. (Anura, Leiuperidae) / Cíntia Pelegrineti Targueta de Azevedo Brito. – Campinas, SP: [s.n.], 2009.

Orientadora: Luciana Bolsoni Lourenço Morandini.
Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. *Engystomops*. 2. Anura. 3. Amphibia. 4. Citogenética. 5. Filogenia. I. Morandini, Luciana Bolsoni Lourenço. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(scs/ib)

Título em inglês: Cytogenetics and phylogenetics studies of *Engystomops petersi* and *Engystomops* sp. (Anura, Leiuperidae).
Palavras-chave em inglês: *Engystomops*; Anura; Amphibia; Cytogenetics; Phylogeny.
Área de concentração: Biologia Celular.
Titulação: Mestre em Biologia Celular e Estrutural.
Banca examinadora: Luciana Bolsoni Lourenço Morandini, Ana Paula Zampieri Silva de Pietri, João Miguel de Barros Alexandrino.
Data da defesa: 16/02/2009.
Programa de Pós-Graduação: Biologia Celular e Estrutural.

Campinas, 16 de fevereiro de 2009

BANCA EXAMINADORA

Profa. Dra. Luciana Bolsoni Lourenço Morandini (Orientadora) _____
Assinatura

Profa. Dra. Ana Paula Zampieri Silva de Pietri _____
Assinatura

Prof. Dr. João Miguel de Barros Alexandrino _____
Assinatura

Profa. Dra. Carmen Sílvia Busin _____
Assinatura

Profa. Dra. Ana Cristina Prado Veiga Menoncello _____
Assinatura

Dedico minha tese a três pessoas importantes da minha vida: Pedro, Daniel e o nenem que vem vindo. Por tornar minha vida cada dia mais feliz.

Agradecimentos

Agradeço primeiramente duas pessoas que tornam minha vida cada dia mais feliz: Pedro e Daniel. Eles foram essenciais em todos os momentos do meu mestrado, assim como são essenciais em cada momento da minha vida. Dando-me força, mas principalmente me trazendo muita alegria. Cada dia meu amor por eles cresce mais. Obrigada por todos os sorrisos.

Agora, não posso deixar de agradecer ao novo sorrisinho que está crescendo na minha barriga e que já me faz tão feliz, por pensar que daqui a alguns meses, ele se juntará aos dois aí de cima pra tornar minha vida repleta de amor e felicidade.

Agradeço aos meus pais por sempre me apoiar, mesmo nas minhas decisões mais malucas e por acreditarem sempre em mim. Aos meus irmãos pelas diversões, visitas e músicas!

Aos meus sogros e cunhadas por também apoiarem cada passo tomado e pelo carinho que recebo deles. Aos avós, tios e primos por todo carinho.

Às minhas amigas, que de tão longe, ainda assim parecem que estão aqui do meu lado...

À minha orientadora, Profa. Luciana, por ter acreditado que eu conseguiria pegar toda prática das novas técnicas, por ter me incentivado com novas perguntas e novos experimentos. Obrigada por todo o conhecimento que tem me passado.

À Profa. Shirlei por disponibilizar o acesso ao laboratório e aos equipamentos que foram essenciais para o desenvolvimento do trabalho.

À Miryan Rivera, Mônica Guerra e Moisés Barbosa pelo apoio prestado, pelas coletas e identificação dos exemplares utilizados na dissertação e discussão dos resultados obtidos.

Obrigada aos amigos do laboratório e do departamento, por tornar o ambiente de trabalho mais divertido e menos estressante.

Agradeço ao Programa de Pós-graduação em Biologia Celular e Estrutural pela oportunidade de realizar o mestrado.

Agradeço à Capes e FAPESP pelo auxílio financeiro.

Obrigada de coração!!!

SUMÁRIO

Resumo

O anuro *Engystomops petersi* está amplamente distribuído na bacia Amazônia, compreendendo países como o Brasil, Equador, Peru e Bolívia. Estudos prévios já descreveram variações no canto e também variações genéticas nesse anuro. Nós apresentamos aqui uma análise citogenética da população de Puyo (Equador), um sítio que inclui a região descrita como sendo a localidade-tipo dessa espécie, duas outras populações Equatorianas que apresentam isolamento pré-zigótico (Yasuní e La Selva), e uma população Brasileira do Estado do Acre. Todos os espécimes analisados citogeneticamente foram também incluídos em estudos filogenéticos utilizando seqüências de DNA mitocondrial. A sequência de parte de um gene nuclear (rodopsina) também foi utilizada neste trabalho. Uma enorme variação citogenética foi encontrada e muitos padrões cariotípicos puderam ser observados, mesmo entre populações que não apresentam isolamento pré-zigótico. Este resultado nos permite sugerir que dados cariotípicos podem ter participado no processo de especiação de *Engystomops*. Populações de Puyo e do Acre apresentaram cromossomos sexuais heteromórficos compondo o sistema XX/XY, o que não foi identificado nas outras populações Equatorianas. Já que as populações Equatorianas estão localizadas em um clado separado das populações do Acre, nós sugerimos a hipótese de que a presença dos cromossomos sexuais X e Y seja um caráter plesiomórfico neste grupo. A análise do gene nuclear permitiu ainda identificar seis SNPs (Polimorfismos de simples nucleotídeos) e alguns espécimes heterozigotos para esses sítios. As variações cariotípicos e moleculares aqui apresentados, em conjunto com os dados previamente descritos, corroboram a hipótese de que *Engystomops petersi* seja um complexo de espécies e sugerem que eventos de hibridação e introgressão possam ter ocorrido entre as populações desse grupo.

Abstract

The frog *Engystomops petersi* is widely distributed in the Amazon basin. Genetic divergence and speciation mechanisms of *E. petersi* populations have been inferred mainly from mitochondria DNA (mtDNA) and microsatellite polymorphisms, male advertisement call and sexual selection. In spite of the significant progress resulting from these studies, many cytogenetic, taxonomic and evolutionary aspects of *E. petersi* populations remain unclear. In the present study, cytogenetic analysis and nucleotide divergence in mtDNA and in the rhodopsin nuclear gene of *E. petersi* populations are described in an attempt to contribute to the understanding of this anuran. High cytogenetic variation distinguished karyotypic patterns, which included two populations with no prezygotic isolation. The Puyo and Acre populations showed heteromorphic sexual chromosomes (XX/XY), which were not identified in the other Equatorial populations. Since all the Equatorial populations were placed in a clade separated from the Acre populations, we hypothesize that the sex chromosomes X and Y is a plesiomorphic condition in this group. The results from mtDNA found here were very similar to those found in previous studies. In the rhodopsine nucleotide sequences, 6 SNPs (Single Nucleotide Polymorphism) were identified and various specimens were heterozygous for these nucleotide sites. The karyotypic and molecular data presented herein, in conjunction with previously reported data, corroborate the hypothesis that *Engystomops petersi* is a complex of distinct species and suggested hybridization and introgression events between populations. Moreover, tha data indicated that karyotypic evolution patterns may have played a substantial role in the *Engystomops* speciation.

Introdução

Em geral, a análise de anuros baseada estritamente em dados morfológicos não tem revelado muitos caracteres informativos para estudos evolutivos, já que muitos dos caracteres morfológicos encontram-se conservados nesse grupo (Hillis, 1991; Ford & Cannatella, 1993; Cannatella *et al.*, 1998). Além disso, muitos caracteres morfológicos são polimórficos e, portanto, de difícil interpretação nas análises filogenéticas (ver Hoffman & Blouin, 2000). Por essa razão, cada vez mais, dados citogenéticos, moleculares e comportamentais têm sido pesquisados nesse grupo. É interessante notar que estudos mais conclusivos acerca de importantes questões taxonômicas e evolutivas têm resultado da combinação de caracteres de diversas naturezas (exemplos em Scott, 2005 e Faivovich *et al.*, 2005).

Estudos **citogenéticos** de anuros têm descrito interessantes e diversos fenômenos cromossômicos. Enquanto alguns grupos apresentam evolução cariotípica bastante conservativa, como é o caso de algumas espécies de *Hypsiboas (=Hyla*) com 24 cromossomos em seu complemento diplóide (Ananias *et al.*, 2004), existem gêneros altamente variáveis, seja em relação ao número diplóide [por exemplo, *Pseudis* (Busin *et al.*, 2001) e *Megaelosia* (Rosa *et al.*, 2003)], em relação à morfologia dos cromossomos ou à distribuição de regiões organizadoras de nucléolo e da heterocromatina [como em *Epipedobates*, (Aguiar-Jr. *et al.*, 2002)]. Em alguns grupos, a diversificação cariotípica supera a morfológica, fazendo da análise citogenética uma importante ferramenta para a distinção de espécies morfologicamente muito parecidas, como é o caso de *Dendropsophus nanus* (=*Hyla nana*) e *Dendropsophus sanborni* (=*Hyla sanborni*) (Medeiros, 2000) e de duas espécies do gênero *Scythrophrys* (Lourenço *et al.*, 2003a).

Em grupos mais estudados do ponto de vista citogenético, sinapomorfias cromossômicas são reconhecidas e permitem a elaboração de propostas para a evolução cariotípica das espécies em estudo. Esse é o caso dos gêneros *Alsodes* (Cuevas e Formas, 2003), *Eupsophus* (Iturra e Veloso, 1989; Cuevas e Formas, 1996; Veloso *et al.*, 2005), *Mantella* (Odierna *et al.*, 2001) e das espécies de *Paratelmatobius* e *Scythrophrys* (Lourenço *et al.*, 2003a,b; 2007). Para as espécies do gênero *Alsodes*, por exemplo, foi sugerido que 2n=26 com ausência de cromossomos telocêntricos seria um caráter primitivo. A presença de pares cromossômicos telocêntricos ou cariótipo com número diplóide menor (2n=22) em outras espécies do mesmo gênero seriam consideradas como sinapomorfias, provavelmente relacionadas à translocações cromossômicas (Cuevas e Formas, 2003). Entretanto, mesmo nesses casos, estudos utilizando outros marcadores citogenéticos ainda são necessários para o completo entendimento da evolução cariotípica nos grupos.

Atualmente, os marcadores citogenéticos mais utilizados no estudo de anuros são as regiões organizadoras de nucléolos (NORs) e as bandas heterocromáticas. As regiões de heterocromatina têm recebido especial atenção. Inicialmente a heterocromatina foi descrita como a região do genoma de uma célula que estava em constante estado de condensação durante todo o ciclo celular e se comportava diferente da eucromatina. Posteriormente, seqüências repetitivas de DNA foram detectadas nessas regiões de heterocromatina. Hoje, já se sabe que as regiões heterocromáticas são formadas por vários componentes específicos, inclusive protéicos e de RNA, que são classificados de acordo com suas funções, dentre elas a metilação do DNA, a metilação de histonas e a repressão transcricional, e atividades relativas à etapa de replicação do DNA (ver revisão de Craig, 2004). As principais regiões de concentração da heterocromatina são as regiões centroméricas e teloméricas dos cromossomos, que estão arranjadas em "cromocentros"

durante a intérfase (para referências, ver revisão de Grewal e Jia, 2007). Algumas das características dessas regiões podem ser analisadas e utilizadas como marcadores citogenéticos.

Uma análise preliminar das seqüências repetitivas de DNA presentes nas regiões heterocromáticas pode ser realizada pela coloração dos cromossomos por fluorocromos base-específicos. A detecção das regiões ricas em bases AT é realizada, por exemplo, por meio da utilização do fluorocromo DAPI (4,6-diamidino-2-fenilindole). Já regiões ricas em bases GC podem ser detectadas pela cromomicina A3 (CMA3) ou mitramicina (MM).

O uso de corantes GC-específicos evidencia também as regiões organizadoras do nucléolo (NOR) em espécies de diferentes grupos, tais como peixes, anfíbios e insetos. Ao contrário da técnica de Ag-NOR, os fluorocromos identificam NORs mesmo que estas estivessem inativas durante a interfase prévia e ainda identificam heteromorfismos de NORs, como por exemplo, em peixes (Amemiya *et al.*, 1986). No entanto, as técnicas mais precisas e mais utilizadas para a detecção de NORs em estudos citogenéticos são a impregnação por prata (método Ag-NOR) e a hibridação *in situ* (Lourenço *et al.*, 1998; Medeiros *et al.*, 2003; Busin *et al.*, 2006; Silva *et al.*, 2006).

O uso seqüencial desses fluorocromos na coloração de cromossomos foi primeiramente descrito por Schweizer (1976) e representa uma interessante abordagem para estudos cromossômicos. Desde então vem sendo utilizado para caracterização citogenética em diferentes espécies desde vertebrados (Schmid, 1978; Schweizer, 1980; Amemiya *et al.*, 1986; Odierna *et al.*, 1999; Kasahara *et al.*, 2003) a insetos (Bione *et al.*, 2005; Brito *et al.*,2003; Golub *et al.*, 2004) e plantas (Zoldos *et al.*, 1999).

Marcadores citogenéticos ligados ao sexo podem vir a representar outra importante ferramenta para estudos cariotípicos, embora ainda não tenham sido devidamente isolados

para esse fim. Dentre as espécies de anuros citogeneticamente estudadas, apenas cerca de 10-15% que equivalem a aproximadamente 30 espécies apresentam cromossomos sexuais heteromórficos e em vários desses casos, o heteromorfismo só é detectado em cariótipos submetidos a alguma técnica de bandamento cromossômico (Chakrabarti *et al.*, 1983; Iturra, *et al.,* 1989; Mahony, 1991; ver revisão de Schmid *et al.,* 1991; Schmid *et al.*, 1992; Nishioka *et al.,* 1993; Miura, 1994a,b; Cuevas & Formas, 1996; Lourenço *et al.*, 1999; Ryuzaki *et al.*, 1999; Hanada, 2002; Schmid *et al.*, 2002, Schmid *et al.,* 2003; Wiley, 2003; Odierna, *et al*., 2007; Ananias *et al.,* 2007; Busin *et al*., 2008). Tanto sistemas cromossômicos XX/XY, como ZZ/ZW (ver referências em Schmid *et al.*, 1991) e também sistemas múltiplos, como o de *Eleutherodactylus biporcatus* (previamente descrito como *E. maussi*; Schmid *et al.*, 1992) já foram descritos para anuros pertencentes a diversas famílias, o que evidencia que a diferenciação de cromossomos sexuais ocorreu diversas vezes após a origem dos anuros.

Os mecanismos que levaram à diferenciação de cromossomos sexuais ainda são pouco conhecidos em Anura. A heterocromatinização como evento responsável pela assincronia no padrão de replicação do DNA de dois cromossomos homólogos, implicando uma redução na freqüência de recombinação entre esses, foi inicialmente sugerida por Singh *et al.* (1976) como o primeiro passo para a diferenciação de cromossomos sexuais, com base em observações em Ophidia. Dentre as espécies de Anura que apresentam cromossomos sexuais diferenciados, apenas os cromossomos de *Pyxicephalus adspersus* (Ranidae) (Schmid, 1980) e *Gastrotheca riobambae* (Hylidae) (Schmid *et al.*, 1983) se adequam a essa hipótese. King (1991), analisando os dados de Green (1988a) sobre *Leiopelma hochstetteri* (Leiopelmatidae), sugere que as variações na quantidade de heterocromatina dos diferentes W encontrados nessa espécie constituem outro exemplo

inequívoco de transformação da eucromatina, embora Green não interprete seus dados dessa maneira, se referindo apenas a uma variação geográfica. Schempp & Schmid (1981), em estudo com o ranídeo *Pelophylax ridibundus* (= *Rana esculenta*), discutem a possibilidade de haver uma concentração diferencial de DNA satélite entre os cromossomos sexuais mesmo que o bandamento C não revele regiões de heterocromatina sexo-específicas.

Por outro lado, o ganho e a perda de heterocromatina, e a perda de DNA ribossomal também já foram considerados fenômenos desencadeadores da diferenciação de cromossomos sexuais em alguns grupos de anuros (Green, 1988b; Iturra & Veloso, 1989; Mahony, 1991; Schmid *et al.*, 1990; Schmid *et al.*, 1993). Outros dois eventos considerados por John (1988) como possíveis responsáveis pela redução de recombinação entre cromossomos homomórficos ancestrais de cromossomos sexuais são: a localização de quiasma determinada genotipicamente e rearranjos estruturais, especialmente inversão pericêntrica. Em Anura, esses mecanismos ainda não foram demonstrados como eventos iniciais na evolução de cromossomos sexuais.

A caracterização dos cromossomos sexuais em espécies proximamente relacionadas poderá auxiliar na inferência de possíveis fenômenos relevantes no processo de acúmulo de diferenças entre os cromossomos sexuais. Já que a diferenciação de cromossomos sexuais é um importante fenômeno na divergência cariotípica interespecífica de anuros, seu estudo pode elucidar importantes eventos de evolução cromossomômica, podendo representar valiosos marcadores para estudos filogenéticos.

Em relação aos **dados moleculares**, embora estudos de alozimas e análises de sítios de restrição presentes em determinadas seqüências dos genomas nuclear e mitocondrial já tenham sido utilizadas com sucesso em inferências filogenéticas de espécies de anuros

(exemplos em Cannatella *et al.*, 1998, Macey *et al.*, 2001; e Hillis & Davis, 1987, Sumida, 1997, respectivamente), a maioria das informações utilizadas em reconstruções filogenéticas consiste em seqüências de DNA, principalmente aquelas localizadas no genoma mitocondrial (exemplos em Hay *et al.*, 1995; Ruvinsky & Maxson, 1996; Lehr *et al.*, 2005; Crawford & Smith, 2005; Chen *et al.*, 2005; Ron *et al.*, 2006).

Algumas características do genoma mitocondrial facilitam sua utilização em estudos filogenéticos e justificam a prevalência dessas inferências em relação àquelas feitas com seqüências do genoma nuclear. Uma delas refere-se à ausência ou à baixa freqüência de eventos de recombinação no DNA mitocondrial (DNAmt). Embora várias evidências da ocorrência de rearranjos na molécula de DNAmt já tenham sido descritas para protistas, fungos e plantas (ver revisão de Gray, 1989), no reino animal tais rearranjos são considerados pouco freqüentes, já que a ordem dos genes mitocondriais não varia muito dentro das grandes linhagens (ver revisões de Boore, 1999, e Pereira, 2000). Tal fato justifica a escassez de seqüências parálogas (pseudogenes, por exemplo) nesse genoma, o que está diretamente relacionado a uma menor produção de possíveis ruídos em inferências filogenéticas realizadas com DNAmt.

A presença de múltiplas cópias do DNAmt por célula (Michaels *et al.*, 1982; Robin & Wong, 1988) é outra particularidade importante desse genoma, pois facilita sua manipulação e a obtenção das seqüências nucleotídicas utilizadas nos estudos.

A terceira relevante característica do genoma mitocondrial é sua elevada taxa evolutiva, estimada em 5-10 vezes maior do que a do genoma nuclear (Brown *et al.*, 1979, 1982; Miyata *et al.*, 1982). Isso possibilita a detecção de sinais filogenéticos mesmo em estudos de táxons menos abrangentes.

Alguns fatores que aparentemente contribuem para a rápida taxa evolutiva observada no genoma mitocondrial são mencionados a seguir: a) elevada exposição a danos oxidativos; b) sistema de replicação falho; c) ausência ou deficiência de mecanismos de reparo; d) eventos de recombinação pouco freqüentes e, portanto, menor chance de eliminar mutações pouco deletérias; e) rápida renovação das moléculas de DNA mitocondrial (revisão de Gray, 1989).

Assim como no genoma nuclear, diferentes regiões do genoma mitocondrial apresentam diferentes taxas evolutivas, sendo as seqüências não-codificadoras as que apresentam maior variabilidade. No genoma mitocondrial animal existem dois genes que codificam RNAs ribossomais, os genes 12S e 16S. Apenas uma cópia de cada um desses genes está presente em cada genoma e eles não contêm regiões espaçadoras. Diferentes taxas evolutivas podem ser observadas ao longo dos genes ribossomais, sendo os sítios mais conservativos aqueles relacionados à associação do ribossomo com proteínas, RNAm e RNAt (Hillis & Dixon, 1991).

Em vertebrados estão presentes 22 genes codificadores de RNAt no genoma mitocondrial. Tais regiões evoluem mais rapidamente do que os genes de RNAt nucleares. Assim como ocorre com os genes ribossomais, as regiões codificadoras de RNAt também apresentam diferentes graus de conservação intramoleculares. Em cada molécula de RNAt, a região codificadora da alça portadora do anticódon é a mais conservada evolutivamente. Alguns estudos sugerem, ainda, que a porção 3' desses genes tenha menor taxa evolutiva do que a porção 5' (revisões de Simon *et al.*, 1994; Pereira, 2000).

As variações intramoleculares da taxa de divergência dos nucleotídeos ao longo do tempo permitem que os genes ribossomais e aqueles codificadores de RNAt sejam capazes de fornecer informações filogenéticas para diferentes categorias taxonômicas. Por isso,

esses genes mitocondriais são utilizados em inferências filogenéticas tanto de espécies proximamente relacionadas (exemplos em Cannatella *et al.*, 1998; Richards & Moore, 1998; Ron *et al.*, 2006), como de grandes grupos taxonômicos (exemplos em Hay *et al.*, 1995, Ruvinsky & Maxson, 1996, Feller & Hedges, 1998, Emerson *et al.*, 2000; Bossuyt & Milinkovitch, 2000; Darst & Cannatella, 2004), embora nesse caso nem sempre os sinais filogenéticos são facilmente recuperados (ver Hertwig *et al.*, 2004).

Ao contrário dos genes mitocondriais, que apresentam herança materna, os genes nucleares apresentam herança dupla, materna e paterna, e menor taxa de substituição nucleotídica (Springer *et al.*, 2001). A utilização de genes nucleares para análises moleculares e filogenéticas pode aumentar o desempenho das seqüências mitocondriais em reconstruir a relações antepassadas (Springer *et al.*, 2001; Hoegg *et al.,* 2004).

Engystomops da Amazônia - o objeto de estudo

O gênero *Engystomops* foi recentemente revalidado por Nascimento *et al.* (2005), com base na análise fenética de dados morfológicos, para abrigar as sete espécies anteriormente reunidas no grupo *Physalaemus pustulosus*: *E. coloradorum* (Cannatella & Duellman, 1984), *E. guayaco* (Ron *et al.*, 2005), *E montubio* (Ron *et al.*, 2004), *E. petersi* Jiménez-de-la-Espada, 1872, *E. pustulatus* (Shreve, 1941), *E. pustulosus* (Cope, 1864) e *E. randi* (Ron *et al.*, 2004). Outras duas espécies ainda não descritas já foram reconhecidas e alocadas nesse gênero, que se mostrou monofilético em análises conduzidas por Ron *et al.* (2006), com base em aproximadamente 2,4kb de DNA mitocondrial, que abrangia os genes ribossomais 12S e 16S e o gene codificador do RNAt-Val.

No entanto, a revalidação de *Engystomops* é ainda motivo de controvérsia. Funk *et al.* (2007) argumentaram que os dados apresentados por Nascimento *et al.* (2005) não apóiam a revalidação desse gênero e defenderam o uso do nome genérico *Physalaemus* para as espécies atribuídas a ele. Até o momento não há nenhuma análise filogenética que permita testar a hipótese de Nascimento *et al.* (2005). Optamos, no presente projeto, por seguir a revalidação proposta por Nascimento *et al.* (2005), assim como Frost *et al.* (2007) e Ron *et al.* (2006), embora acreditemos que novos estudos sejam necessários para uma reavaliação taxonômica desse grupo.

Outra informação taxonômica controversa na literatura refere-se ao táxon *E. freibergi*. Barros (1969) descreveu a espécie *E. freibergi* com base na análise de espécimes de Rurrenabaque (Rio Beni), Bolívia, mas esta foi sinonimizada a *E. petersi* por Cannatella & Duellman (1984). O nome *E. freibergi* ressurgiu, no entanto, no trabalho de Cannatella *et al.* (1998), identificando espécimes do sul do Peru. Na última revisão sobre *Egystomops* apresentada por Ron *et al.* (2006), os autores identificaram um espécime coletado no Rio Tejo, na Reserva Extrativista do Alto Juruá, no Estado do Acre (Brasil) como *E.* cf. *freibergi*, devido à proximidade geográfica desta região com a localidade-tipo de *E. freibergi* e à divergência genética desse indivíduo em relação a populações próximas à localidade-tipo de *E. petersi* (oriente do Equador, provavelmente Napo-Pastaza – ver referência em Cannatella & Duellman, 1984). Funk *et al.* (2007), com base em análises de seqüências moleculares, novamente sugerem a possibilidade de revalidação de *E. freibergi*. No entanto, nenhum estudo com espécimes da localidade-tipo de *E. freibergi* foi realizado e a revalidação desse táxon ainda não foi formalmente proposta.

Apesar dos problemas taxonômicos mencionados, dois clados podem ser claramente definidos dentre as espécies de *Engystomops*. Um deles é composto por *E. coloradorum*, *E.*

guayaco, *E. montubio*, *E. pustulatus*, *E. randi* e as duas espécies ainda não descritas identificadas por Ron *et al.* (2006), todas distribuídas em baixas altitudes a oeste da Cordilheira dos Andes, no Equador e no norte do Peru. O segundo clado inclui *E. pustulosus*, que ocorre na América Central e norte da América do Sul, e os anuros da Amazônia, *E. petersi* e *E.* cf. *freibergi* (Ron *et al.*, 2006).

A análise citogenética de espécimes coletados na mesma localidade do indivíduo identificado como *E.* cf. *freibergi* por Ron *et al.* (2006) distinguiu facilmente dois citótipos ocorrendo em simpatria, evidenciando a existência de espécies crípticas naquela região (Lourenço *et al.*, 1998, 1999). Além dessa importante implicação taxonômica, os estudos citogenéticos citados revelaram fenômenos muito interessantes dentre os espécimes portadores do citótipo I, como intenso polimorfismo de NORs e de blocos heterocromáticos e a presença de cromossomos sexuais heteromórficos (X e Y). Devido à ampla distribuição geográfica de *Engystomops* (Figura 1), a análise citogenética de um grande número de populações pode revelar outros fenômenos cromossômicos e auxiliar na identificação taxonômica do gênero.

Figura 1. Mapa parcial da América do Sul, com a indicação da possível distribuição geográfica de *Engystomops petersi* (extraído de http://www.puce.edu.ec/zoologia/vertebrados/amphibiawebec/especies/anura/leiuperidae/petersi/petersi).

O trabalho de Lourenço *et al.* (1998) foi a primeira descrição cariotípica para *Engystomops* da Amazônia e a segunda disponível para o gênero, uma vez que o número e a morfologia dos cromossomos de *E. pustulosus* foram apresentados por Morescalchi & Gargiulo (1968). No referido trabalho, no entanto, embora uma metáfase mitótica de *E. pustulosus* corada com Giemsa tenha sido apresentada, não há uma descrição cariotípica minuciosa, o que impossibilita a análise da presença de cromossomos sexuais heteromórficos. Já que os autores não mencionam a existência de heteromorfismos, considera-se que cromossomos sexuais não tenham sido detectados. Recentemente, Rivera (2006) caracterizou citogeneticamente mais uma espécie do gênero: *Engystomops sp.* D. De

acordo com os autores, essa espécie apresentou 2n=20, diferindo das outras duas anteriormente citadas (2n=22), a NOR se localizava no par 9 e também nenhum polimorfismo em relação a cromossomos sexuais foi descrito. Além disso, dentre as espécies de *Physalaemus* já estudadas citogeneticamente, nenhum caso de heteromorfismo de cromossomos sexuais foi detectado. Isso sugere que a diferenciação de cromossomos sexuais seja um evento recente na história evolutiva desses anuros, uma vez que *Physalaemus* é considerado proximamente relacionado a *Engystomops* (ver Nascimento *et al.*, 2005). Conseqüentemente, a análise citogenética nos gêneros citados pode revelar caracteres informativos para estudos evolutivos.

Grande divergência também foi detectada entre as populações de *E. petersi* do Equador, tanto em relação ao canto (Boul & Ryan, 2004; Boul *et al.*, 2007) quanto em análises genéticas (Ron *et al.*, 2006; Boul *et al.*, 2007). Com base nessas variações, Ron *et al.* (2006) sugeriram a existência de um complexo de espécies dentre os *Engystomops* da Amazônia. Ron *et al.,* (2006), e Boul *et al.* (2007) atribuíram à seleção sexual por cantos complexos a divergência observada em relação ao canto e consideraram que tal fenômeno resultou no isolamento reprodutivo de diversas populações, levando a um conseqüente processo de especiação incipiente. Com base em novos experimentos de fonotaxia, Guerra & Ron (2008) corroboraram a proposta de envolvimento de seleção sexual na divergência dos *Engystomops* da Amazônia e sugeriram que outros fatores também estejam envolvidos na divergência de caracteres relacionados ao reconhecimento e/ou preferência de parceiros para cruzamento. Tal processo (denominado de "reinforcement") resulta da seleção natural contra híbridos e pode melhor explicar o fato de fêmeas de Puyo discriminarem o canto de machos de La Selva (isolamento pré-zigótico), já que em ambas as populações o mesmo tipo de canto simples é observado.

Funk *et al.* (2007) testaram a hipótese de que barreiras impostas por rio e a de que gradiente elevacional estejam envolvidos no surgimento da diversidade encontrada dentre os anuros atualmente identificados como *Engystomops petersi*. Os autores encontraram suporte apenas para a hipótese do rio Madre de Dios ter representado uma importante barreira no surgimento de dois grupos de *E. petersi* no Peru. No entanto, nem mesmo nesse caso os autores descartam a hipótese de que a variação observada nessa região seja resultante de um contato secundário entre populações já distintas. Dentre o que foi sugerido pelos autores, a existência de um complexo de espécies é novamente colocada em questão.

Objetivos

Já que conspícua variação cromossômica foi encontrada dentre anuros do Acre identificados como *E. petersi* (= *E.* cf. *freibergi* em Ron *et al.*, 2006) (Lourenço *et al.*, 1998, 1999), a análise citogenética de outras populações de *Engystomops* da Amazônia parece representar valiosa ferramenta para o estudo desse grupo, que compreende espécies crípticas e cuja taxonomia permanece pouco compreendida. Considerando ainda que a identificação desses exemplares do Acre permanece duvidosa (ver discussão em Ron *et al.*, 2006), o presente trabalho tem como objetivos:

1. O estudo citogenético de espécimes provenientes de Puyo, localizado na Província de Pastaza, sítio que provavelmente está incluído na região descrita como a localidade-tipo de *E. petersi* (Napo-Pastaza; ver referência em Cannatella & Duellman, 1984).

2. O estudo citogenético de espécimes da Estación Científica Yasuní, localizada no oriente do Equador, próximo a Napo-Pastaza, e de La Selva, no Equador, populações que

apresentam canto complexo e simples, respectivamente e que apresentam isolamento pré-zigótico.

3. O estudo citogenético da população de *Engystomops* encontrada no Parque Zoobotânico da Universidade Federal do Acre, localizado a cerca de 30 km da Reserva de Humaitá, com o intuito de aumentar a amostra de populações dessa região.

4. A análise filogenética dos *Engystomops* da Amazônia, incluindo espécimes analisados citogeneticamente, e exemplares do Parque Zoobotânico da Universidade Federal do Acre, população não amostrada na análise conduzida por Funk *et al.* (2007).

5. Comparar os dados cromossômicos com os de canto disponíveis na literatura e com as inferências filogenéticas.

Referências Bibliográficas

Aguiar-Jr., O., Lima, A.P., Giaretta, A.A., Recco-Pimentel, S.M. Cytogenetic analysis of four dart-poison frogs of the *Epipedobates* genus (Anura, Dendrobatidae). **Herpetologica 58**: 293-303, 2002.

Amemiya, C.T., Gold J.R. Chromomycin A3 Stains Nucleolus Organizer Regions of Fish Chromosomes. Copeia, 1986, 1: 226-231, 1986.

Ananias, F., Garcia, P.C.A. Recco-Pimentel, S.M. Conserved karyotypes in the *Hyla pulchella* species group (Anura, Hylidae). **Hereditas 140**: 42-48, 2004.

Ananias, F., Modesto, A.D.S., Mendes, S.C., Napoli, M.F. Unusual primitive heteromorphic ZZ/ZW sex chromosomes in *Proceratophrys boiei* (Anura, Cycloramphidae, Alsodinae), with description of C-band interpopulational polymorphism. **Hereditas, 144**: 206-212, 2007.

Bione, E., Moura, R.C., Carvalho, R., Souza, M.J. Karyotype, C-and fluorescence banding pattern, NOR location and FISH study of Five Scarabaeidae (Coleoptera) species. **Genetics and Molecular Biology, 28**, 3: 376-381, 2005.

Boore, J.L. Animal mitochondrial genomes. **Nucl. Acids Res. 27**: 1767-1780, 1999.

Bossuyt, F., Milinkovitch, M.C. Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. **Proc. Natl. Acad. Sci. USA 97**: 6585-6590, 2000.

Boul, K.E., Ryan, M.J. Population variation of complex advertisement calls in *Physalaemus petersi* and comparative laryngeal morphology. **Copeia 2004**: 624-631, 2004.

Boul, K.E., Funk, W.C., Darst, C.R., Cannatella, D.C., Ryan, M.J. Sexual selection drives speciation in an Amazonian frog. **Proc. R. Soc. B: Biol. Sci. 274**: 399-406, 2007.

Brito, R.M., Caixeiro, A.P.A., Pompolo, S.G., Azevedo, G.G. Cytogenetic data of Partamona peckolti (Hymenoptera, Apidae, Meliponini) by C banding and fluorochrome staining with DA/CMA3 and DA/DAPI. **Genetics and Molecular Biology, 26**, 1: 53-57, 2003.

Brown, W.M., George, M.Jr., Wilson, A.C. Rapid evolution of animal mitochondrial DNA. **Proc. Natl. Acad. Sci USA 76**: 1967-1971, 1979.

Brown, W.M., Prager, E.M., Wang, A., Wilson, A.C. Mitochondrial DNA sequences of primates: tempo and mode of evolution. **J. Mol. Ecol. 18**: 225-239, 1982.

Busin, C.S., Vinciprova, G., Recco-Pimentel, S.M. Chromosomal rearrangements as the source of variation in the number of chromosomes in *Pseudis* (Amphibia, Anura). **Genetica 110**: 131-141, 2001.

Busin, C.S., Lima, A.P., Prado, C.P.A., Strüsmann, C., Siqueira, S.J., Recco-Pimentel, S.M. Chromosomal differentiation of populations of *Lysapsus limellus limellus*, *L.l. bolivianus*, and of *Lysapsus caraya* (Hylinae, Hylidae). **Micron, 37**: 355-362, 2006.

Busin, C.S., Andrade, G.V., Bertoldo, J., Del Grande, M.L., Uetanabaro, M., Recco-Pimentel, S.M. Cytogenetic analysis of four species of *Pseudis* (Anura, Hylidae), with the description of ZZ/ZW sex chromosomes in *P.tocantins*. **Genetica, 133**:119-127, 2008.

Cannatella, D.C.; Duellman, W. Leptodactylid frogs of the *Physalaemus pustulosus* group. **Copeia 1984**: 902-921, 1984.

Cannatella, D.C., Hillis, D.M., Chippindale, P.T., Weigt, L., Rand, A.S., Ryan, M. Phylogeny of frogs of the *Physalaemus pustulosus* species group, with an examination of data incongruence. **Syst. Biol. 47**: 311-335, 1998.

Chakrabarti, S., Banerjee, S.N., Neogi, L.N., Roy-Choudhuri, S. C-band positive W chromosome in the female Indian frog**. Experientia, 39**: 321-322, 1983.

Chen, L.Q., Murphy, R.W., Lathrop, A., Ngo, A., Orlov, N.L., Ho, C.T., Somorjai, I.L.M. Taxonomic chaos in Asian ranid frogs: An initial phylogenetic resolution. **Herpetol. J. 15**: 231-243, 2005.

Crawford, A.J., Smith, E.N. Cenozoic biogeography and evolution in direct-developing frogs of Central America (Leptodactylidae : *Eleutherodactylus*) as inferred from a phylogenetic analysis of nuclear and mitochondrial genes. **Mol. Phylogenet. Evol. 35**: 536-555, 2005.

Craig, J. Heterochromatin – many flavours, common themes. **BioEssays 27**: 17-28, 2004.

Cuevas, C.C.; Formas, J.R. Heteromorphic sex chromosomes in *Eupsophus insularis* (Amphibia: Anura: Leptodactylidae). **Chrom. Res. 4**: 467-470, 1996.

Cuevas, C.C.; Formas, J.R. Cytogenetic analysis of four species of the genus *Alsodes* (Anura : Leptodactylidae) with comments about the karyological evolution of the genus. **Hereditas 138**: 138-147, 2003.

Darst, C.R., Cannatella, D.C. Novel relation-ships among hylold frogs inferred from 12S and 16S mitochondrial DNA sequences. **Mol. Phylogenet. Evol. 31**: 462-475, 2004.

Emerson, S.R., Richards, C., Drewes, R.C., Kjer, K.M. On the relationships among ranoid frogs: a review of the evidence. **Herpetologica 56**: 209-230, 2000.

Faivovich, J., Haddad, C.F.B., Garcia, P.C.A., Frost, D.R., Campbell, J.A., Wheeler, W.C. Systematic review of the frog family hylidae, with special reference to hylinae: phylogenetic analysis and taxonomic revision. **Bull. Amer. Mus. Nat. Hist. 294**: 6-228, 2005.

Feller, A.E., Hedges, S.B. Molecular evidence for the early history of living amphibians. **Mol. Phylogenet. Evol. 9**: 509-516, 1998.

Ford, L.S., Cannatella, D.C. The major clades of frogs. **Herpetol. Monogr. 7**: 94-117, 1993.

Frost, D.R. Amphibian species of the world: an online reference. Version 5.2 (15 July 2007). Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index..html. American Museum of Natural History, New York, USA, 2008.

Funk, W.C., Caldwell, J.P., Peden, C.E., Padial, J.M., De la Riva, I., Cannatella, D.C., Tests of Biogeografic hypotheses for diversification in the Amazonian Forest frog, *Physalaemus petersi*. **Molecular Phylogenetics and Evolution 44**: 825-837, 2007.

Golub, N.V., Nokalla, S., Kuznetsova, V.G. Holocentric chromosome of Psocids (Insecta, Psocoptera) analysed by C-banding, silver impregnation and sequence specific fluorochromes CMA3 and DAPI. **Folia Biologica (Kraków) 52**, 3-4: 143-149, 2004.

Gray, M.W. Origin and evolution of mitochondrial DNA. **Annu. Ver. Cell Biol. 5**: 25-50, 1989.

Green, D.M. Cytogenetics of the endemic New Zealand frog, *Leiopelma hochstetteri*: extraordinary supernumerary chromosome variation and a unique sex-chromosome system. **Chromosoma 97**:55-70, 1988a.

Green, D.M. Heteromorphic sex chromosomes in the rare and primitive frog *Leiopelma hamiltoni* from New Zealand. **J. Hered. 79**: 165-169, 1988b.

Grewal, S.I.S.; Jia, S. Heterochromatin revisited. **Nature Reviews Genetics 8**: 35-46, 2007.

Guerra, M.A. and Ron, S.R. Mate choice and courtship signal differentiation promotes speciation in an Amazonian frog. **Behavioral Ecology** 1-8, 2008.

Hanada, H. G and C banding show structural differences between the Z and W chromosomes in the frog *Buergeria buergeri*. **Hereditas, 136**: 151-154, 2002.

Hay, J.M., Ruvinsky, I., Hedges, S.B., Maxson, L.R. Phylogenetic relationships of amphibian families inferred from DNA sequences of mitochondrial 12S and 16S ribosomal RNA genes. **Mol. Biol. Evol. 12**; 928-937, 1995.

Hertwig, S. de Sá, R.O., Haas, A. Phylogenetic signal and the utility of 12S and 16S mtDNA in frog phylogeny. **J. Zool. Syst. Evol. Research 42**: 2-18, 2004.

Hillis, D.M., Davis, S.K. Evolution of the 28S ribosomal RNA gene in anurans: regions of variability and their phylogenetic implications. **Mol. Biol. Evol. 4**: 117-125, 1987.

Hillis, D.M., Dixon, M.T. Ribosomal DNA: molecular evolution and phylogenetic inference. **Q. Ver. Biol. 66**: 411-453, 1991.

Hoegg, S., Vences, M., Brinkmann, H., Meyer, A. Phylogeny and Comparative Substitution rates of Frogs Inferred from Sequences of three Nuclear Genes. **Molecular Biology and Evolution, 21**, 7: 1188-1200, 2004.

Hoffman, E.A., Blouin, M.S. A review of colour and pattern polymorphisms in anurans. **Biol. J. Lin. Soc.70**: 633-663, 2000.

Iturra, P.; Veloso, A. Further evidence for early sex chromosome differentiation of anuran species. **Genetica 78**: 25-31, 1989.

John, B. The biology of heterochromatin. In **Heterochromatin: molecular and structural aspects** (R.S. Verma, ed.), pp. 1-128, Cambridge Univ. Press, Cambridge, 1988.

Kasahara, S., Zampieri Silva, A.P., Gruber, S.L., Haddad, C.F.B. Comparative cytogenetic analysis an four tree frog species (Anura, Hylidae, Hylinae) from Brazil. **Cytogenet Genome Res 103**: 155-162, 2003.

King, M. The evolution of the heterochromatin in the amphibian genome. In **Amphibian Cytogentics and Evolution** (D.M. Green and S.K. Sessions eds), pp.359-391, Academic Press, San Diego, 1991.

Lehr, E., Fritzsch, G., Müller, A. Analysis of Andes frogs (*Phrynopus*, Leptodactylidae, Anura) phylogeny based on 12S and 16S mitochondrial rDNA sequences. **Zool. Scripta 34**: 593-603, 2005.

Lourenço, L.B., Recco-Pimentel, S.M., Cardoso, A.J. Polymorphism of the nucleolus organizer regions (NORs) in *Physalaemus petersi* (Amphibia, Anura, Leptodactylidae) detected by silver-staining and fluorescence *in situ* hibridization. **Chrom**. **Res**.**6**: 621-628, 1998.

Lourenço, L.B., Recco-Pimentel, S.M., Cardoso, A.J. Two karyotypes and heteromorphic sex chromosomes in *Physalaemus petersi* (Anura, Leptodactylidae). **Can. J. Zool. 77**: 624-631, 1999.

Lourenço, L.B., Garcia, P.C.A., Recco-Pimentel, S.M. Intrageneric karyotypic divergence in *Scythrophrys* (Anura, Leptodactylidae) and new insights on the relationship with the leptodactylid *Paratelmatobius*. **Ital. J. Zool. 70**: 183-190 2003a.

Lourenço, L.B., Garcia, P.C.A., Recco-Pimentel, S.M. Cytogenetics of a new species of *Paratelmatobius cardosoi* group (Anura: Leptodactylidae), with the description of an apparent case of pericentric inversion. **Amphibia-Reptilia 24**: 47-55, 2003b.

Lourenço, L.B., Bacci-Júnior, Martins, V.G., Recco-Pimentel, S.M., Haddad, C.F.B. Molecular phylogeny and karyotype differenciation in *Paratelmatobius* and *Scythrophrys* (Anura, Leptodactylidae). **Genetica 132**: 255-266, 2007.

Macey, J.R., Strasburg, J.L., Brisson, J.A., Vredenburg, V.T., Jennings, M., Larson, A. Molecular phylogenetics of western north American frogs of the *Rana boylii* species group. **Mol. Phyl. Evol. 19**: 131-143, 2001.

Mahony, M.J. Heteromorphic sex chromosomes in the Australian frog *Crinia bilingua* (Anura: Myobatrachidae). **Genome 34**: 334-337, 1991.

Medeiros, L.R. **Estudo citogenético das espécies *Hyla nana* e *Hyla sanborni* (Anura, Hylidae).** Tese de Mestrado - Unicamp, pp. 1-77, 2000.

Medeiros, L.R., Rossa-Feres, D.C., Recco-Pimentel, S.M. Chromosomal differentiation of *Hyla nana* and *Hyla sanborni* (Anura, Hylidae) with a description of NOR polymorphism inn *H. nana*. **Journal of Heredity, 94**(2): 149-154, 2003.

Michaels, G.S., Hauswirth, W.W., Laipis, P.J. Mitochondrial DNA copy number in bovine oocytes and somatic cells. **Dev. Biol. 94**: 246-251, 1982.

Miura, I. Sex chromosome differentiation in the Japanese brown frog *Rana japonica*. II. Sex-linkage analyses of the nucleolar organizer regions in chromosomes no. 4 of the Hiroshima and Saeki populations. **Zool. Sci. 11**: 807-815, 1994a.

Miura, I. Sex chromosome differentiation in the japanese brown frog, *Rana japonica*. I. Sex-related heteromorphism of the distributiion pattern of constitutive heterochromatin in chromosome no 4 of the Nakuya population. **Zool. Sci. 11**: 797-806, 1994b.

Miyata, T., Hayashida, H., Kikuno, R., Hasegawa, M., Kobayashi, M., Koike, K. Molecular clock of silent substitution: at least six-fold preponderance of silent changes in mitochondrial genes over those in nuclear genes. **J. Mol. Ecol. 19**: 28-35, 1982.

Morescalchi, A., Gargiulo, G. Su alcune relazioni cariologiche del genere *Bufo* (Amphibia, Salientia). **Rend. Acc. Sc. Fis. Mat. Napoli S 4 35**: 117-120, 1968.

Nascimento, L.B., Caramaschi, U., Cruz, C.A.G. Taxonomic review of the species groups of the genus *Physalaemus* Fitzinger, 1826 with revalidation of the genera *Engystomops* Jiménez-de-la-Espada, 1872 and *Eupemphix* Steindachner, 1863 (Amphibia, Anura, Leptodactylidae). **Arq. Mus. Nac. Rio de Janeiro 63**: 297-320, 2005.

Nishioka, M.; Miura, I.; Saitoh, K. Sex chromosomes of *Rana rugosa* with special reference to local differences in sex-determining mechanism. **Sci. Rep. Lab. Amphibian Biol. 12**: 55-81, 1993.

Odierna, G., Aprea, G., Capriglione, T., Parisi, P., Arribas, O., Morescalchi, M.A. Chromosomal and molecular analysis of some repeated families in Discoglossus Otth, 1837 (Anura, Discoglossidae): taxonomic and phylogenetic implications. **Ital. J. Zool., 66**: 273-283, 1999.

Odierna, G., Vences, M., Aprea, G., Lotters, S.; Andreone, F. Chromosome data for Malagasy poison frogs (Amphibia: Ranidae: *Mantella*) and their bearing on taxonomy and phylogeny. **Zool. Sci. 18**: 505-514, 2001.

Odierna, G., Aprea, G., Capriglione, T., Castellano, S., Balletto, E. Cytological evidence for population-specific sex chromosome heteromorphism in Palaeartic green toads (Amphibia, Anura). **J. Biosci. 32**,4: 763-768, 2007.

Pereira, S.L. Mitochondrial genomic organization and vertebrate phylogenetics. **Gen. Mol. Biol. 23**: 745-752, 2000.

Richards, C.M., Moore, W.S. A molecular phylogenetic study of the old world treefrog family Rhacophoridae. **Herpetol. J. 8**: 41-46, 1998.

Rivera, M. La Citogenética: un aporte más al conocimiento de los anfíbios ecuatorianos. **Nuestra Ciência,8**: 27-30, 2006.

Robin, E.D., Wong, R. Mitochondrial DNA molecules and virtual number of mitochondrial per cell in mammalian cells. **J. Cell. Physiol. 136**: 507-513, 1988.

Ron, S.R., Cannatella, D.C., Coloma, L.A. Two new species of *Physalaemus* (Anura: Leptodactylidae) from Western Ecuador. **Herpetologica 60**: 261-275, 2004.

Ron, S.R., Coloma, L.A., Cannatella, D.C. A new, cryptic species of *Physalaemus* (Anura: Leptodactylidae) from Western Ecuador with comments on the call structure of the *P. pustulosus* species group. **Herpetologica 61**: 178-198, 2005.

Ron, S.R., Santos, J.C., Cannatella, D.C. Phylogeny of the túngara frog genus *Engystomops* (=*Physalaemus pustulosus* species group; Anura: Leptodactylidae). **Mol. Phylogenet. Evol. 39**: 392-402, 2006

Rosa, C., Aguiar-Jr., O., Giaretta, A.A., Recco-Pimentel, S.M. Karyotypic variation in the genus *Megaelosia* (Anura, Hylodinae) with the first description of a B-chromosome in a leptodactylid frog. **Copeia 2003**: 166-174, 2003.

Ruvinsky, I., Maxson, L.M. Phylogenetic relationships among bufonoid frogs (Anura: Neobatrachia) inferred from mitochondrial DNA sequences. **Mol. Phylogenet. Evol. 5**: 533-547, 1996.

Ryuzaki, M.; Hanada, H.; Okumoto, H.; Takizawa, N.; Nishioka, M. Evidence for heteromorphic sex chromosomes in males of *Rana tagoi* and *Rana sakuraii* in Nishitama district of Tokyo (Anura: Ranidae). **Chrom. Res. 7**: 31-42, 1999.

Schempp, W.; Schmid, M. Chromosome banding in Amphibia. VI. BrdU-replication patterns in Anura and demonstration of XX/XY sex chromosomes in *Rana esculenta*. **Chromosoma 83**: 697-710, 1981.

Schmid, M. Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. **Chromosoma 66**: 361-388, 1978.

Schmid, M. Chromosome banding in Amphibia. V. Highly differentiated ZW/ZZ sex chromosomes and exceptional genome size in *Pyxicephalus adspersus* (Anura, Ranidae). **Chromosoma 80**, 69-96, 1980.

Schmid, M.; Haaf, T.; Geile, B.; Sims, S. Chromosome banding in Amphibia. VIII. An unusual XY/XX-sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). **Chromosoma 88**: 69-82, 1983.

Schmid, M.; Steinlein, C.; Friedl, R.; Almeida, C.G.; Haaf, T.; Hillis, D.M.; Duellman, W.E. Chromosome banding in Amphibia. XV. Two types of Y chromosomes and heterochromatin hypervariability in *Gastrotheca pseutes* (Anura, Hylidae). **Chromosoma 99**: 413-423, 1990.

Schmid, M., Nanda, I., Steinlein, C., Kausch, K., Haaf, T., Epplen, J. Sex-determining mechanisms and sex chromosomes in Amphibia. In **Amphibian Cytogenetics and Evolution** (D.M. Green and S.K. Sessions, eds.). Academic Press, San Diego pp. 393-430, 1991.

Schmid, M., Steinlein, C., Feichtinger, W. Chromosome banding in AmphibiaXVII. First demonstration of multiple sex chromosomes in amphibians: *Eleutherodactylus maussi* (Anura, Leptodactylidae). **Chromosoma, 101**: 284-292, 1992.

Schmid, M.; Ohta, S.; Steinlein, C.; Guttenbach, M. Chromosome banding in Amphibia. XIX. Primitive ZW/ZZ sex chromosomes in *Buergeria buergeria* (Anura, Rhacophoridae). **Cytogenet. Cell. Genet. 62**: 238:246, 1993.

Schmid, M., Feichtinger, W., Steinlein, C., Rupprecht, A., Haaf, T., Kaiser, H. Chromosome banding in Amphibia XXIII. Giant W sex chromosomes and extremely small genomes in *Eleutherodactylus euphronides* and *Eleutherodactylus shrevei* (Anura, Leptodactylidae). **Cytogenet. Genome Res. 97**: 81-94, 2002.

Schmid, M., Feichtinger, W., Steinlein, C., Visbal Garcia, R., Badillo, A.F. Chromosome banding in Amphibia XXVIII. Homomorphic XY sex chromosomes and a derived Y-autosome translocation in *Eleutherodactylus riveroi* (Anura, Leptodactylidae) **Cytogenetic and Genome Research, 101**: 62-73, 2003.

Schweizer, D. Reverse fluorescent chromosome banding with chromomycin and DAPI. **Chromosoma 58**: 307-324, 1976.

Schweizer, D. Simultaneous fluorescent staining of R bands and specific heterochromatic regions (DA-DAPI bands) in human chromosomes. **Cytogenet. Cell Genet. 27**: 190-193, 1980.

Scott, E. A phylogeny of ranid frogs (Anura : Ranoidea : Ranidae), based on a simultaneous analysis of morphological and molecular data. **Cladistics 21**: 507-574, 2005.

Silva, A. P. Z., Haddad, C.F.B., Galassi, G.G., Kasahara, S. Multiple nucleolus organizer regions in *Leptodactylus mystacinus* (Amphibia, Anura) and comments on its systematic position in the *L. fuscus* group based on cytogenetic and molecular analyses. **Genetica 127**: 35-44, 2006.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P. Evolution, Weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. **Entomol. Soc. Amer. 87**: 651-671, 1994.

Singh, L.; Purdom, I.F.; Jones, K.W. Satellite DNA and evolution of sex chromosomes. **Chromosoma 59**: 43-62, 1976.

Springer, M.S., DeBry R.W., Douady, C., Amrine H.M., Madsen, O., Jong, W.W., Stanhope, M.J. Mitochondrial versus nuclear gene sequences in deep-level mammalian phylogeny reconstruction. Molecular Biology and Evolution, 18: 132-143, 2001.

Sumida, M. Mitochondrial DNA differentiation in the japanese brown frog *Rana japonica* as revealed by restriction endonuclease analysis. **Genes Genet. Syst. 72**: 79-90, 1997.

Veloso, A., Celis-Diez, J.L., Guerrero, P.C., Méndez, M.A., Iturra, P., Simonetti, J.A. Description of a new Euspsophus species (Amphibia, Leptodactylidae) from the remnants of Maulino forest, central Chile. **Herpetol. J. 15**: 159-165, 2005.

Wiley, J.E. Replication banding and FISH analysis reveal the origin of the *Hyla femoralis* karyotype and XY/XX sex chromosomes. **Cytogenetic and genome Research, 101**: 80-83, 2003.

Zoldos, V., Papes, D., Cerbath, M., Panaud, O., Besendorfer, V., Siljak-Yakovlev, S. Molecular-cytogenetic studies of ribosomal genes and heterochromatin reveal conserved genome organization among 11 *Quercus* species. **Theor. Appl. Genet. 99**: 969-977, 1999.

Manuscrito a ser submetido à publicação (revista: Molecular Phylogenetics and Evolution)

Cytogenetic and molecular analyses in Amazonian populations of the frog *Engystomops petersi* (Anura; Leiuperidae)

Targueta, C.P.[1], Rivera, M.[2], Souza, M.B.[3], Recco-Pimentel, S.M.[1], Lourenço, L.B[1].

[1]Departamento de Biologia Celular, IB, Universidade Estadual de Campinas - UNICAMP, 6109, 13083-863, Campinas, SP, Brasil

[2]Citogenética de Anfíbios, Pontifícia Universidad Católica Del Ecuador, Ecuador

[3]Universidade Federal do Acre – UFAC, Rodovia BR 364, n° 6637 (Km 04), Distrito Industrial, 500, 69915-900, Rio Branco, AC, Brasil

Keywords: *Engystomop*s; Anura; Amphibia; cytogenetics; phylogenetics

Abstract

The frog *Engystomops petersi* is widely distributed in the Amazon basin. Genetic divergence and speciation mechanisms of *E. petersi* populations have been inferred mainly from mitochondria DNA (mtDNA) and microsatellite polymorphisms, male advertisement call and sexual selection. In spite of the significant progress resulting from these studies, many cytogenetic, taxonomic and evolutionary aspects of *E. petersi* populations remain unclear. In the present study, cytogenetic analysis and nucleotide divergence in mtDNA and in the rhodopsin nuclear gene of *E. petersi* populations are described in an attempt to contribute to the understanding of this anuran. High cytogenetic variation distinguished karyotypic patterns, which included two populations with no prezygotic isolation. The Puyo and Acre populations showed heteromorphic sexual chromosomes (XX/XY), which were not identified in the other Equatorial populations. Since all the Equatorial populations were placed in a clade separated from the Acre populations, we hypothesize that the sex chromosomes X and Y is a plesiomorphic condition in this group. The results from mtDNA found here were very similar to those found in previous studies. In the rhodopsine nucleotide sequences, 6 SNPs (Single Nucleotide Polymorphism) were identified and various specimens were heterozygous for these nucleotide sites. The karyotypic and molecular data presented herein, in conjunction with previously reported data, corroborate the hypothesis that *Engystomops petersi* is a complex of distinct species and suggested hybridization and introgression events between populations. Moreover, tha data indicated that karyotypic evolution patterns may have played a substantial role in the *Engystomops* speciation.

Introduction

The *Engystomops petersi* is an Amazonian frog considered particularly interesting for speciation studies. The geographical distribution of *E. petersi* extends from the Andean foothills of Ecuador, Peru, and Bolivia to the Colombian, Brazilian, and French Guianan Amazon basin. The wide geographical distribution of this mainland frog in a region of several rivers and Andean foothills allowed Funk *et al.* (2007) to test some rivers and elevation gradient as barriers to gene flow. In that study, Funk *et al.* (2007) provided some support for the barrier hypothesis for the Madre de Dios River, but little evidence for the elevational gradient hypothesis of barrier. Interestingly, the authors found three distinct and well supported clades. The Ecuador and northern Peru populations composed one clade, referred as 'northwestern clade'. The second clade, referred as 'southwestern clade', comprised the populations of Acre, southern Peru and Bolivia. A population sampled in the state of Pará, Brazil, composed the third clade. The authors discussed the existence of a complex of species being confused and put together in the same taxon (*E. petersi*). In addition, Funk *et al.* (2007) suggested that *E. petersi* (named *Physalaemus petersi* by those authors) encompass the northwestern clade while the southwestern Amazon clade would be *E. freibergi* (named *Physalaemus freibergi* by them) corroborating the proposal of Ron *et al*. (2006).

Based on analyses of male advertisement call, Ron *et al.* (2006), Boul *et al.* (2004, 2007) and Guerra & Ron (2008) proposed that sexual selection has played an important role in divergence and speciation of the Amazonian *Engystomops*. Distinct calls were identified among populations of diverse geographic Amazon region. For instance, an interesting variation was found between La Selva and Yasuní, two populations in opposite sides of the Napo River, in Ecuador (Boul & Ryan, 2004; Ron *et al*., 2006; Boul *et al.*, 2007; Guerra &

Ron, 2008). The females from La Selva and Yasuní show preference for calls from the same population besides calls from foreign populations in crossing experiments made in laboratory according to Boul *et al.* (2007). Also females from Puyo, other equatorian locality, did not recognize the call from La Selva, although both populations had simple calls. However, matting preference drived by call selection was not observed when experiments were conducted with females from Puyo, which did not discriminate between the local call and the call from Yasuní (Guerra & Ron, 2008).

Since sexual selection did not explain all the genetic variation observed, Guerra & Ron (2008) suggested that additional factors are involved in the prezygotic isolation of *Engystomops* populations. The authors consider that selection against hybridization (reinforcement) can be involved in *Engystomops* speciation, favoring genetic divergence in mate recognition traits and/or mate preferences and leading to reproductive isolation.

Cytogenetic analyses in three *E. petersi* populations from the Acre State, in the Brazilian Amazon, revealed the sympatry of two distinct karyotypes (Lourenço *et al*., 1998, 1999). The studies revealed high variation in NOR and C-band sites among specimens identified as cytotype I. Additionally, heteromorphic sex chromosomes, a rare condition in anurans, were identified in this cytotype.

In spite of the recent substantial progress that resulted from recent investigations, many cytogenetic, taxonomic and evolutionary aspects of *E. petersi* populations remain unclear. To contribute to the understanding of this anuran, in the present work we described cytogenetically the population of *Engystomops petersi* from Puyo, Ecuador, a site in the region of Napo-Pastaza, considered to be the type-locality of this species (Cannatella & Duellman, 1984). We also analyzed the populations from Yasuní and La Selva, two populations from Ecuador that show prezygotic isolation (Guerra & Ron, 2008), and

another population from the state of Acre, in Brazil. All the specimens analyzed cytogenetically were included in a phylogenetic study using mtDNA sequences. In addition, the nucleotide divergence in the rhodopsin nuclear gene was analyzed for the same specimens.

Materials and methods

Specimen sampling for cytogenetic analysis

The cytogenetic analyses comprised six males and five females from Puyo ('Puyo' specimens), at the Provincia of Pastaza, Ecuador, a site within the region described as the type-locality of this species; five males ('Yasuní' specimens) from the Estación Científica Yasuní, Província of Orellana, Ecuador; one male ('Yasuní km 26' specimen) sampled about 26 km distant from the Estación Científica Yasuní; one juvenile ('La Selva' specimen) from La Selva, Provincia del Orellana, Ecuador; four males and four females from the Parque Zoobotânico da Universidade Federal do Acre, Brazil, here referred as 'UFAC' specimens. Specimens sampled in Ecuador were deposited in Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ), Quito, Ecuador. The Brazilian specimens were sampled under a permit issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA/10678-1) and Voucher specimens were deposited in the Museu de Zoologia Prof. Adão José Cardoso (ZUEC), Universidade Estadual de Campinas (UNICAMP), SP, Brazil.

Chromosome preparation

Chromosomes were obtained from metaphasic cells from intestine and testes of animals previously treated with colchicine, according to Schmid (1978) and Schmid *et al.* (1979) with few modifications. Prior to the intestine and testes removal, the animals were deeply anesthetized. Cell suspensions were dripped in clean plates and stored at -20°C. Chromosomes were stained with Giemsa 10%, silver-stained by the Ag-NOR method (Howell & Black, 1980) and submitted to C-banding (King, 1980). As for heterochromatin characterization, the karyotypes were sequentially stained with the fluorochromes 4'-6-diamidino-2-fenilidone (DAPI) and mytramycin (MM). Several plates were firstly submitted to C-banding, without Giemsa treatment, and then stained with fluorochromes. The NORs were also detected by *in situ* hybridization with the rDNA probe HM123 (Meunier-Rotival *et al*., 1979), according to the technique described by Viegas-Péquignot (1992). Metaphases were photographed under an Olympus microscope and analyzed using Image Pro-Plus version 4 (Media Cybernetics). Chromosomes were ordered and classified according to Green and Sessions (1991).

Molecular analysis

The sequences used in the molecular analysis were obtained from specimens collected at the same localities as the ones cytogenetically analyzed or from GenBank, as shown in Figure 1 and Appendix 1. Specimens collected at Rio Tejo, Acre, Brazil, population previously studied by Lourenço *et al.* (1998) were also included in the molecular analysis.

Genomic DNA was extracted from liver or muscle tissues stored at -70°C in the tissue bank at the Department of Cell Biology-Unicamp, Campinas-SP, Brazil, using the

TNES method. Tissue samples were immersed in TNES buffer solution (250 mM Tris pH 7.5, 2 M NaCl , 100 mM EDTA , 2.5% SDS. The solution was then supplemented with proteinase K (100 µg/ml) and the samples were incubated for 5 hours at 55°C. NaCl was added for protein precipitation. DNA was precipitated with isopropyl alcohol, washed with ethanol (70%), resuspended in TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and stored at -20°C.

The sequences of the 12S, tRNA-val, 16S mitochondrial genes were PCR amplified using the primers MVZ 59(L), MVZ 50(H), 12L13, Titus I (H), Hedges16L2a, Hedges16H10, 16Sar-L and 16Sbr-H (for primer sequences, see Goebel *et al.*, 1999). The primers Rhod1A and Rhod1C (Bossuyt and Milinkovitch, 2000) were used for PCR amplification of the rhodopsin nuclear gene. The DNA segment from each specimen was bi-directionally sequenced.

The PCR amplification products were purified with GFX PCR and Gel Band DNA Purification kit (GE Healthcare) and directly used as templates for sequencing in an automatic DNA sequencer ABI/Prism (Applied Biosystems, Inc.) using the BigDye Terminator kit (Applyed Biosystems), as recommended by the manufacturer. DNA sequences were edited using Bioedit version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using ClustalW.

Phylogenetic relationships were inferred from mtDNA sequences by the Maximum Parsimony (MP) (Camin & Sokal, 1965) and Maximum Likelihood (ML) methods, using PAUP* 4.0β10 (Swofford, 2000). Transitions and transversions were considered to have the same weight. The most appropriate evolution model for likelihood analyses was selected by Modeltest 3.7 (Posada and Crandall, 1998). All the constant and non-

informative characters were deleted in the ML analysis in order to make the analysis feasible. The phylogenetic trees were searched using heuristic algorithm, with 10 random addition-sequence replicates. Nodal support was assessed through nonparametric bootstrap analysis (Felsenstein, 1985), with heuristic search based on 1000 pseudoreplicates. Additional *Engystomops* species were used as outgroup in the parsimony analysis (Appendix 1).

Results

Cytogenetics of the Puyo population

In all analyzed individuals, the chromosome diploid number was 22, except for one female (QCAZ 34935) that was 2n=23. The karyotype of the 2n=22 karyotypes consisted of 7 pairs of metacentric chromosomes (pairs 1, 2, 5, 6, 8, 9 and 10), 3 submetacentric (pairs 3, 4 and 7) and one subtelocentric (pair 11), as shown in Figure 2. The 11^{th} pair was characterized as a sexual chromosome pair of the system XX/XY. The X chromosomes were homomorphic in three females analysed and all the males showed a chromosome Y. Two females showed a heteromorphic X pair, but the difference here was concerned the terminal C-band, which in one chromosome was larger than the other (insights in Figure 2A, B, E).

Secondary constrictions were observed in the terminal region of the long arm of pair 8 and in the pericentromeric region of the long arm of the sex chromosome X (Figure 2A). These regions were identified as NORs by the Ag-NOR method (Figure 2B) and *in situ* hybridization (Figures 2C and 2D). In two specimens, one female and one male, an additional NOR was observed in an interstitial region of the long arm of one homologue in pair 9 (Figure 2D).

Centromeric regions were identified by C-banding in all chromosomes, including the sexual pair (Figure 2E), as well as the terminal region of both arms of chromosomes 8 and 9, and of the long arm of chromosomes 10 and X. Frequently, the terminal regions were more bluish than the centromeric regions. In one specimen QCAZ 34937, one homologue of pair 10 also had a terminal C-band in the short arm. The terminal C-band in the long arm of chromosome 8 was apparently adjacent to the NOR (Figures 2B and 2C). In the NOR-bearing homologue 9, the terminal C-band in the long arm was adjacent to the NOR.

Chromosomes not submitted to C-banding were uniformly stained by DAPI, except for the NORs, which were negatively stained. After MM staining, the chromosomes were uniformly stained, but the terminal region of the short arm of chromosome 8 and of the long arm of chromosome 9 remained unstained (Figure 2F). The centromeric regions were not distinguishable by the fluorochrome staining. The previously C-banded plates showed a different result, since they had the centromeric and terminal C-bands of the chromosomes 8, 9, 10 and X brightly stained by DAPI. A heteromorphism in pair 10 for the presence of a terminal C-band in the short arm, was also observed upon DAPI staining of C-banded karyotypes (Figure 2G). Most likely, this heteromorphism justifies the differences found on the morphology of the homologues of this pair (Figures 2A and 2G).

The MM staining revealed pericentromeric regions in some chromosomes pairs, including the NOR-bearing X chromosome (Figure 2G). A large telomeric region, coincident with a DAPI positive region, was strongly stained with the MM fluorochrome in the X chromosome of the specimen QCAZ 34940. The same telomeric region was positive for DAPI but negative for MM in the specimen QCAZ 34939 (Figure 2G).

A pericentromeric region of the short arm of chromosome 1 was observed as a secondary constriction after Giemsa or fluorochrome staining without previous treatment (Figures 2A and 2F) and after silver staining (Figure 2B) or C-banding (Figure 2E). In C-banded metaphases stained with MM and specially with DAPI, this pericentromeric region seemed stained (Figure 2G), and that could represent some class of heterochromatin.

The extra chromosome found in all the 76 metaphases analyzed from the specimen QCAZ 34935 was tentatively identified to be a chromosome 8, since it had a NOR at the long arm and the terminal C-bands at both arms, but we do not discard the possibility of being a chromosome 9 (Figure 2H).

The analysis of meiotic cells did not show multivalent configurations (data not shown).

Cytogenetics of the Yasuní population

All specimens analyzed had the same diploid number (2n=22). The chromosomes were classified as metacentric (pairs 1, 2, 3, 4, 6, 8, 9), submetacentric (pair 10) and subtelocentric (pairs 5 and 11). The pair 11 was not heteromorphic in the five males analyzed, in contrast with the observed in Puyo karyotype (Figure 3A).

Giemsa staining discriminated the chromosome pairs 3 and 8, with secondary constrictions in their long arm (Figure 3A). The secondary constriction of the chromosome 3 was the only one recognized as NOR using the Ag-NOR technique (Figure 3B). This secondary constriction showed a weird feature, characterising an intraindividual variation. This secondary constriction was observed as a small constriction region in 39 of the 164 metaphases in the six specimens analyzed, but in 58 metaphases, it appeared as a large chromosome distention. In 67 metaphases, the distended region could only be seen in one

of the homologues (Figure 3A). All of those regions were also stained by Ag-NOR technique (Figure 3B).

The centromere in all chromosome pairs was detected after C-banding (Figures 3C and 3D). In addition, terminal C-bands were observed in both arms of the chromosomes 3 and 8, in the long arm of the chromosomes 4, 10 and 11, and in the short arm of chromosome 6 were also seen. A weakly stained C-band could be distinguished at the short arm of one homologue of the chromosome pair 10 (Figure 3C). In chromosome 3, there were two consecutive C-bands in the short arm, as well as terminal and a small interstitial C-band adjacent to the NOR in the long arm.

All chromosomes were homogeneously stained with DAPI, except for the NOR. Some of the terminal heterochromatic regions stained slightly brighter. The MM fluorochrome did not stain the terminal heterochromatic region of the chromosomes 3, 4, 8, 9 and 11 (Figure 3E). After C-banding, all centromeres and terminal C-bands of the chromosomes 3, 4, and 8 were evidenced by DAPI (Figure 3F). The MM fluorochrome did not evidence any of the telomeric regions, but some centromeres were weakly stained in metaphases previously submitted to C-banding (Figure 3F).

The presence of NOR and terminal C-bands in both arms of the chromosome pair 9 distinguished the male QCAZ 34948 of the Yasuní population (Figure 3D). These characteristics resembled the chromosome 3, but chromosome 9 is smaller and lacks the consecutive C-bands observed in both arms of pair 3. In chromosome 9, only one large block of C-band was observed in the terminal region of both arms. Another peculiar characteristic of this *E. petersi* specimen is a slight heteromorphism in the chromosome homologue morphology of the pairs 2, 3 and 6 (Figure 3D).

The analysis of meiotic cells did not show multivalent configurations (data not shown).

Karyotype of the specimen Yasuní-km 26

The karyotype of the Yasuní-km26 specimen (QCAZ 30826) was remarkably distinct from the other analyzed *E. petersi* specimens of this geographic region (Figure 4A). Interestingly, this karyotype was highly similar to the Puyo specimens, differing only in NOR distribution (Figures 4B and 4C). In the Yasuní-km26 specimen metaphases that were submitted to the Ag-NOR technique and *in situ* hybridization with the HM123 probe the NORs were observed at the pericentromeric region in the short arm of chromosome 4, long-arm of chromosome 6 and in a large extension of the long arm of the X chromosome. In several metaphases, there were three blocks of NORs in the pericentromeric X region, in association with heterocromatic regions (Figures 4D, 4E and 4F).

Karyotype of the juvenile La Selva specimen

This karyotype had a diploid complement of 22 chromosomes, with nine metacentric chromosome pairs (1, 3, 4, 6, 7, 8, 9, 10, 11), one pair of submetacentric (pair 5) and one pair of subtelocentric (pair 2) (Figure 5). A secondary constriction was observed in the short arm of the chromosome pair 6 (Figure 5A), which was revealed as NOR by the Ag-NOR method (Figure 5C). In the pair 6, a longer short arm characterized one of the homologues. This heteromorphism was not due to the NOR size, but was indeed a size difference related to a chromosome segment between the NOR and the telomere (Figure 5).

The C-banding revealed conspicuous centromeric heterochromatic regions in all chromosomes (Figure 5B). In addition, terminal C-bands were observed at the telomeres of

both arms of the chromosomes 6, 7, 10 and 11. Telomeric bands could also be seen in the long arm of the chromosome 3, and in the short arms of chromosomes 8 and 9. Two additional C-bands were observed in interstitial regions of the long arm of chromosome 5 and of the short arm of chromosome 8.

After the C-banding treatment, the central area of all centromeric heterochromatin was strongly stained with DAPI, and the pericentromeric regions were brightly stained with MM. Terminal heterochromatic regions were simultaneously stained with both fluorochromes. The interstitial C-band of the chromosome 5 was stained by DAPI and was adjacent to the pericentromeric MM-positive band in the long arm of this chromosome. The NOR was not stained with MM or DAPI, but the regions adjacent to the NOR were strongly evidenced by these fluorochromes (Figures 5C-G).

Cytogenetics of the UFAC-Acre population

In all UFAC-Acre specimens the karyotype was 2n=22 and consisted of 6 metacentric chromosome pairs (1, 2, 5, 6, 7, 9), 3 submetacentric pairs (3, 10, 11) and 2 subtelocentric pairs (4 and 8) (Figure 6A). Because the chromosome pair 8 was heteromorphic in all males and homomorphic in the females, it was classified as a sexual chromosome pair (Figure 6). Another heteromorphic pair, which was observed in four specimens, was present in both male and female specimens and so it was considered non-related to sex determination (Figures 6 and 7).

NOR sites, in a total of five, were individually detected in the regions pericentromeric of the long arm of the chromosome 3, distal of the long arm of chromosome 7, distal of the long arm of chromosome 9, interstitial in the long arm of chromosome 11 (Figures 6B and 7). The only apparently fixed NOR was observed in the

chromosome 7. The remaining NOR sites were not always present, thus related to the NOR polymorphism detected in this population (Figures 6B and 7). In the UFAC-Acre specimens, a maximum of eight NOR-bearing chromosomes was observed (Figure 7C).

All the centromeres were C-banded and terminal C-bands were present in 4p, 7p, 7q, 9p, 9q, and Xq, while interstitial C-bands were seen in 11q and Xq. The terminal C-bands in the chromosomes 7 and 9, and the interstitial C-band in 11q were adjacent to NOR sites. The interstitial C-band in 11q was occasionally absent, similarly to its adjacent NOR, which implied in a morphological chromosome difference (Figure 7). The sex chromosome pair of the female ZUEC 14435 was heteromorphic according to its C-band pattern (Figure 7A).

All the centromeres were MM positive in the plates not treated with C-banding and also in those previously C-banded (Figures 6D and 6E). Only the interstitial region of the X chromosome was stained with DAPI before C-banding (Figure 6D), neither the NOR and the terminal regions were stained by this fluorochrome before C-banding. In contrast, all the non-centromeric heterochromatic regions in the X chromosome and in the chromosomes 7, 9 and 11 were strongly stained with DAPI in C-banded metaphases (Figure 6E). The NORs in pairs 7 and 9 could be detected by MM in the C-banded metaphases (Figure 6E).

Chromosome Breaks

Chromosome breaks were observed in some of the metaphases. In the Puyo population, there were three metaphases with breaks. Many chromosome breaks were observed in the specimen QCAZ 34935 and, apparently, there were chromosomes with more than one centromere as well (Figure 8A). The specimen QCAZ 34937 of the Puyo

population showed a break in the short arm of chromosome 10 (Figure 8B). Chromosome breaks were present in two metaphases of the Yasuní specimens QCAZ 34944 and QCAZ 34947. In the Yasuní QCAZ 34947, the break apparently occurred in the chromosome 3 (Figure 8C). The UFAC-Acre population showed four metaphases with breaks in the specimens ZUEC 14455, 14432 and 14434. As for the ZUEC 14455 specimen, the break probably occurred in the X chromosome (Figure 8D). Although these abnormal chromosomes were present in quite low frequencies, it is conceivable that breaks indicate fragile DNA regions involved in the karyotypic differentiation of these anurans.

Molecular Analysis

The ML analysis used a GTR+G+I model of sequence evolution. The search for the best trees comprised 106 OTUs (Operational Taxonomic Units), without outgroups, consisted of 258 nucleotides each. Of 2418 nucleotides in the MP analysis, 571 were informative. A hundred of MPT (most parsimony trees) was generated, with 1474 steps. The strict consensus of these MPT is shown in Figure 9. The ML tree (not shown) was similar to the ML tree reported by Funk *et al.* (2007).

The Amazonian *Engystomops* were clustered in three major clades of the MP trees (Figure 9). The first clade was composed by the *Engystomops* specimens from Pará, which was supported by a high value of bootstrap (100%). The second major clade (southwestern clade) was weakly supported by bootstrap analysis and comprised all the Brazilian populations, the specimens from North bank of Madre De Dios River and South bank of Madre de Dios River, in Peru, and the population from Bolivia. The remaining populations were grouped in a third clade (northwestern clade), supported by 92% bootstrap value.

Inside the southwestern clade, the Brazilian UFAC population, not sampled by Funk *et al.* (2007), was clustered with a high bootstrap support (100%) together with the population from Rondônia and with the population from North bank of Madre De Dios River (Peru).

Sub-groups were also observed inside the northwestern clade. The Yasuní and Tiputini populations (Ecuador) remained together in a same sub-group with 100% bootstrap value. The populations from Puyo, Jatun Sacha, Shell, Cando and Napo (Ecuador) were grouped together with highly supported value (100%). Except for the Cando specimens, the other specimens were not grouped according to their sampling locality. Loreto (Peru) and Sucumbios (Ecuador) populations were paraphyletic in relation to the populations of the northwestern clade (Figure 9).

The relationships among the populations from Puyo, La Selva and Estación Cientifica Yasuní are still unclear. The first ones were closely related in the MP analysis, but with a low bootstrap support. In contrast, the La Selva and Estación Cientifica Yasuní populations showed high phylogenetic proximity in the ML analysis. In both MP and ML analyses, the specimen QCAZ 30826 from the *E. petersi* 'Yasuní km 26' population was grouped together with the specimens from Puyo, regardless of their geographical distance.

The variation in the rhodopsin gene was low among the haplotypes (Appendix 2). Of 312 nucleotides analyzed, only six were variable. The Single Nucleotide Polymorphism (SNP) identified as a C-T transition in the nucleotide site (nt) 17 clearly discriminated the specimens of the three populations from Ecuador (Yasuní, Yasuní-26km and Puyo) from the two Brazilian populations UFAC-Acre and Rio Tejo-Acre. The nt14 was not polymorphic in the Ecuador specimens and was equal to a few of the specimens from Brazil. Intrapopulational polymorphism was found in five nucleotide sites within the

Brazilian populations and four of the Ecuador populations. The typical sites of Puyo specimens were also found in the specimen QCAZ 30826, Yasuní-26km specimen. It was also interesting to note the presence of heterozygotes in the Puyo and Yasuní populations, and specially in the UFAC-Acre population. In all the variable nucleotide sites, only two different states (nucleotide types) were observed.

Discussion

Preliminar taxonomic considerations

The phylogenetic relationships between the *Engystomops* populations inferred in our ML analysis were very similar to the ML inferences reported by Funk *et al.* (2007), but differed in minor arrangements from our MP analysis. The principal differences referred to the relatedness of the Yasuní, La Selva and Puyo populations. La Selva and Yasuní were closely related in the ML analysis done in the present work and by Funk *et al.* (2007). Nevertheless, such arrangement was not maintained in the MP analyses described herein, such that the La Selva population was grouped in the same clade of the Puyo population, although supported by low bootstrap value. The other inconsistecies between MP and ML analysis did not affect the relevant groups in the Amazonian *Engystomops*.

In the MP tree and the ML tree, the UFAC-Acre population, which had not been previously analyzed, was grouped together with the specimens from Guajará-Mirim (Rondônia, Brazil) and from Amazonian Cusco, in the north bank of the Madre De Dios River (Peru), within a highly supported clade in the southwestern clade of the Amazonian *Engystomops*. Therefore, according to the proposal of *E. freibergi* revalidation to allocate the southwestern clade (Ron *et al.*, 2006; Funk *et al.*, 2007), the UFAC-Acre specimens

would be *E. freibergi* representatives. However, the bootstrap support was low for the southwestern clade in the MP analysis reported here.

The karyotypes found in Acre state of Brazil

The UFAC-Acre karyotypes shed new light on the previously described Rio Tejo-Acre karyotypic data (Lourenço *et al*., 1998, 1999). In the Rio Tejo-Acre karyotype referred to as type I, possibly the chromosome 8 is actually the X chromosome, as these chromosomes were very similar in both morphology and C-banding pattern. This equivocated chromosome pairwise could be due to the high polymorphism in the analyzed sample, which comprised heteromorphic pairs. By increasing the Acre sample size and including several females, the present work has contributed to the understanding of chromosome variation in *E. petersi*. At least three of the six C-banded karyotypes (type I) described by Lourenço et al. (1999) could be reorganized according to the chromosome classification of the karyotypes from the UFAC-Acre population.

However some of the karyotypes I previously described really differ from those from UFAC. In the C-banded karyotype of the single specimen from the Tejo River Mouth (in the REAJ) and of one of the specimens from the Humaitá Reserve (ZUEC 9620) previously analyzed (Lourenço *et al.*, 1999), the chromosome now classified as 7 (identified as chromosome 6 by Lourenço *et al*., 1999) had no terminal heterochromatic bands, in contrast with the chromosome 7 of the karyotypes from UFAC population. Another difference observed between the karyotype I previously described and those presented herein was noted in pair 10 of the former. This chromosome was very similar to that chromosome 10 found in the Puyo karyotype, but differed from all the chromosomes in the UFAC-Acre karyotype. Despite different, the chromosome 10 from the previously

described karyotype I reseamble the UFAC-Acre chromosome 9, differing from that only by the absence of a terminal C-band in the short arm. These real differences represent some interpopulational cytogenetic divergence between the acrean populations.

The interpopulational variations in the *Engystomops* from Ecuador

High cytogenetic variation was observed among the specimens from Puyo, Yasuní and La Selva populations. Interestingly, each of karyotypic group was supported by high bootstrap value in the MP analyses. The karyotype of the juvenile La Selva specimen was the most distinct among the studied populations. In this specimen from La Selva there was no chromosome similar to the X chromosome of Puyo population. High amounts of heterochromatin specially in the centromeric regions and in the terminal regions of some chromosomes characterized the La Selva specimen. The centromeric heterochromatin was clearly heterogeneous with a DAPI-positive band near the centromere plus MM-positive pericentromeric bands. In addition, the NOR site in the chromosome pair 6 was exclusive to the La Selva specimen.

Fluorochrome staining has been widely used in cytogenetic analyses and it may discriminate different heterochromatin classes in karyotypes from different species (Odierna *et al*., 2001; Kasahara *et al.,* 2003; Silva *et al.,* 2006). Herein, it allowed us to clearly discriminate the centromeric heterochromatin in the *Engystomops* karyotypes. Besides La Selva karyotype, only the UFAC karyotype had MM positive heterochromatin associated to the centromeres. The other populations had karyotypes with AT-rich centromeric heterochromatin. Although helpful in discriminating the karyotypes, the presence of CG-rich heterochromatin associated to the centromeres was not phylogenetically informative, since La Selva and UFAC population are inside different

clades. The presence of DAPI or MM positive heterochromatin may be a result of selective amplification of different classes of repetitive sequences and it can vary between species of the same genus. Such an interespecific variation was already seen for *Mantella* species by Odierna *et al*. (2001), that also concluded for the limited phylogenetic relevance of the heterochromatin classification in that genus.

The Puyo and Yasuní populations have no prezygotic isolation (Guerra and Ron, 2008). Even though, karyotype homologies between these two populations were not clear. The most similar chromosome pairs between Puyo and Yasuní were the 8 and 10, specially regarding terminal C-bands, but the pair 8 was NOR-bearing only in the Puyo karyotypes.

Interestingly, the karyotype of the Yasuní-26km specimen (QCAZ 30826) differed from the geographically related Yasuní specimens. The Yasuní-26km karyotype was similar to the Puyo karyotype and was included in the Puyo clade in the MP and ML analyses. It suggested that this specimen is more related to Puyo specimens and this finding is a clear evidence of the consistency of the karyotypic patterns proposed here.

Since the hybrid offspring of specimens with very distinct karyotypes may have unbalanced genome, we hypothesize that a selective pressure against hybrids of diverse karyotypic groups could have played a role in the differentiation of *Engystomops*. Hence, karyological traits could be an important factor involved in the arisement of prezygotic isolation independent of sexual selection driven by divergent calls. In spite of that, the karyological traits would have been involved in the reinforcement phenomenon, previoulsy proposed by Guerra & Ron (2008) to be an important process involved in *Engystomops* evolution.

The hybridization hypothesis

Guerra and Ron (2008) found that females from Puyo recognize the Yasuní calls and consider them as much attractive as the local calls, and conclude that a prezygotic isolation via mate choice is unlikely between these two populations. Even though, these two populations were clearly distinguished in the cytogenetic analysis. Thus, it is conceivable that rare events of hybridization and introgression could have occurred between the Puyo and Yasuní populations. This hypothesis could explain the diverse karyotype of the Yasuní QCAZ 34948 specimen, which contains heteromorphic chromosome pairs and the chromosome pair 9 with terminal C-bands not observed in the remaining Yasuní specimens. In addition, in the Yasuní QCAZ 34948 specimen, the NOR was in the chromosome pair 9, whereas in the other specimens it was in the pair 3.

Most likely, the high polymorphism detected in the analyzed Brazilian Acre populations, specially the Tejo River Mouth (in the REAJ), is a result of hybridization and introgression events. In addition, the great difficult in pairwising the chromosomes of the karyotypes of some specimens (like ZUEC 9620 and ZUEC 9625) described by Lourenço *et al*. (1998, 1999), even after the correct identification of the X chromosome proposed here, could be explained by these events. This hypothesis is corroborated by the presence of multivalent meiotic configurations in the Acrean specimens from REAJ and Humaitá Reserve (Lourenço *et al.*, 2000).

The heterozygous nucleotide sites in the gene rhodopsin, a nuclear gene considered highly conserved among *Engystomops* populations, support above proposed hybridization hypothesis. However, these heterozygotes could as yet be explained by ancestral polymorphisms, which might have occurred before population divergence and persisted in these populations. The rhodopsin nucleotide polymorphism, although relatively low as is

expected for this conserved gene, was substantial in distinguishing the diverse populations. Further analysis increasing specimen numbers could verify if the nt17 rhodopsin SNP is suitable to be a diagnostic marker capable of discriminating the geographically distant Amazonian *Engystomops* populations from Ecuador and Brazil.

Sex chromosome divergence

Heteromorphic sexual chromosomes X and Y were observed in the specimens from Puyo and Acre locations, but not in Yasuní and La Selva. The Yasuní male karyotypes contained homomorphic chromosomes in the pair 11, which were quite similar to X chromosome in the Puyo karyotypes. In the phylogenetic inferences reported herein and by Funk *et al.* (2007), the Puyo, Yasuní and La Selva Ecuadorian populations were closer to each other than to the Brazilian populations from Acre. If so, then the heteromorphic sex chromosomes could be a plesiomorphic condition in this Amazonian anuran group.

The sex chromosomes from Puyo and Acre were similar, but with a few different characteristics. The X chromosome of the Brazilian Acre specimens contained interstitial heterochromatic segments not observed in the Puyo karyotypes. As for the Y chromosome, there was an additional variation in some of the Brazilian males from Acre consisting of a terminal NOR in the long arm (Lourenço *et al.*, 1998).

In the sex chromosome differentiation hypotheses, the heterochromatinization of the Y and W chromosomes is considered relevant. Apparently, heterochromatinization has happened in diverse anuran species, such as *Gastrotheca riobambae* (Schmid *et al.*, 1983), *Pyxecephalus adspersus* (Schmid e Bachmann, 1980), *Leiopelma hamiltoni* (Green, 1988), *Crinia bilingua* (Mahony, 1991), *Hoplobatrachus tigerinus* (= *Rana tigrina*) (Chakrabarti *et al.*, 1983), *Pseudis tocantins* (Busin *et al.*, 2008), *Pristimantis euphronides* (=

Eleutherodactylus euphronides), *Pristimantis shrevei* (= *Eleutherodactylus shrevei*) (Schmid *et al.*, 2002) and *Proceratophrys boiei* (Ananias *et al.*, 2007). Nevertheless, in the Puyo and Acre *Engystomops*, a large amount of heterochromatin was present in the X chromosome and was absent in the Y chromosome. Only the centromeric region was C-banded in the chromosome Y. In *Eupsophus migueli* (Iturra & Veloso, 1989), the Y chromosome did not show any heterochromatic region, whereas the X chromosome seemed similar to the *Engystomops* from Puyo and Acre. In some *Engystomops* species, this morphological differentiation of sex chromosomes may have involved similar heterochromatinization of the X chromosome in a homomorphic ancestral pair. However, further studies are needed towards a better understanding of the evolutionary pathway of the *Engystomops* sex chromosomes.

Conclusions

The cytogenetic analysis revealed great similarity within each population and allowed the recognition of diverse karyological groups in the Amazonian *Engystomops*. The data suggested that karyological variation might be substantial in the speciation mechanisms of this anuran group.

The intrapopulational polymorphisms, the presence of multivalent meiotic configurations previously described (Lourenço *et al*., 2000), and the heterozygous nucleotide sites in the rhodopsin gene suggested that hybridization and introgression might have been relevant in the evolution of the Amazonian *Engystomops*.

Acknowledgements

The authors gratefully acknowledge Dr. Santiago Ron for valuable discussions. This work was supported by Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo).

References

Ananias, F., Modesto, A.D.S., Mendes, S.C., Napoli, M.F. Unusual primitive heteromorphic ZZ/ZW sex chromosomes in *Proceratophrys boiei* (Anura, Cycloramphidae, Alsodinae), with description of C-band interpopulational polymorphism. **Hereditas, 144**: 206-212, 2007.

Bossuyt, F., Milinkovitch, M.C. Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. **Proc. Natl. Acad. Sci. USA 97**: 6585-6590, 2000.

Boul, K.E., Ryan, M.J. Population variation of complex advertisement calls in *Physalaemus petersi* and comparative laryngeal morphology. **Copeia 2004**: 624-631, 2004.

Boul, K.E., Funk, W.C., Darst, C.R., Cannatella, D.C., Ryan, M.J. Sexual selection drives speciation in an Amazonian frog. **Proc. R. Soc. B: Biol. Sci. 274**: 399-406, 2007.

Busin, C.S., Andrade, G.V., Bertoldo, J., Del Grande, M.L., Uetanabaro, M., Recco-Pimentel, S.M. Cytogenetic analysis of four species of *Pseudis* (Anura, Hylidae), with the description of ZZ/ZW sex chromosomes in *P.tocantins*. **Genetica, 133**:119-127, 2008.

Camin, J. H., Sokal, R. R. A method for deducing branching sequences in phylogeny. **Evolution 19**: 311-326, 1965.

Cannatella, D.C.; Duellman, W. Leptodactylid frogs of the *Physalaemus pustulosus* group. **Copeia 1984**: 902-921, 1984.

Chakrabarti, S., Banerjee, S.N., Neogi, L.N., Roy-Choudhuri, S. C-band positive W chromosome in the female Indian frog**. Experientia, 39**: 321-322, 1983.

Felsenstein, J. Confidence lmits on phylogeny: an approach using bootstrap. **Evolution 39**: 783-791, 1985.

Funk, W.C., Caldwell, J.P., Peden, C.E., Padial, J.M., De la Riva, I., Cannatella, D.C., Tests of Biogeografic hypotheses for diversification in the Amazonian Forest frog, *Physalaemus petersi*. **Molecular Phylogenetics and Evolution 44**: 825-837, 2007.

Goebel, A.M., Donnelly, J.M., Atz, M.E. PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome b in bufonids and other frogs, and an overview of PCR primers which have amplied DNA in amphibians successfully. **Mol. Phylogenet. Evol. 11**: 163-199, 1999.

Green, D.M. Heteromorphic sex chromosomes in the rare and primitive frog *Leiopelma hamiltoni* from New Zealand. **J. Hered. 79**: 165-169, 1988.

Green, D.M., Sessions, S.K. Nomenclature for chromosomes. In: **Amphibian cytogenetics and evolution** (eds DM Green and SK Sessions) Academic Press, San Diego, p. 431-432, 1991.

Guerra, M.A. and Ron, S.R. Mate choice and courtship signal differentiation promotes speciation in an Amazonian frog. **Behavioral Ecology** 1-8, 2008.

Howell, W.M., Black, D.A. Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. **Experientia 36**: 1014-1015, 1980.

Iturra, P.; Veloso, A. Further evidence for early sex chromosome differentiation of anuran species. **Genetica 78**: 25-31, 1989.

Kasahara, S., Zampieri Silva, A.P., Gruber, S.L., Haddad, C.F.B. Comparative cytogenetic analysis an four tree frog species (Anura, Hylidae, Hylinae) from Brazil. **Cytogenet Genome Res 103**: 155-162, 2003.

King, M. C-banding studies on Australian hylid frogs: secondary constriction structure and the concept of euchromatin transformation. **Chromosoma 80**: 191-217, 1980.

Lourenço, L.B., Recco-Pimentel, S.M., Cardoso, A.J. Polymorphism of the nucleolus organizer regions (NORs) in *Physalaemus petersi* (Amphibia, Anura, Leptodactylidae) detected by silver-staining and fluorescence *in situ* hibridization. **Chrom**. **Res**.**6**: 621-628, 1998.

Lourenço, L.B., Recco-Pimentel, S.M., Cardoso, A.J. Two karyotypes and heteromorphic sex chromosomes in *Physalaemus petersi* (Anura, Leptodactylidae). **Can. J. Zool. 77**: 624-631, 1999.

Lourenço, L.B., Recco-Pimentel, S.M., Cardoso, A.J. A second case of multivalent meiotic configurations in diploid specimes of Anura. **Genetics and Molecular Biology 23**, 1: 131-133, 2000.

Mahony, M.J. Heteromorphic sex chromosomes in the Australian frog *Crinia bilingua* (Anura: Myobatrachidae). **Genome 34**: 334-337, 1991.

Meunier-Rotival, M., Cortadas, J., Macaya, G., Bernardi, G. Isolation and organization of calf ribosomal DNA. **Nucl. Acids Res**. **6:** 2109-2123, 1979.

Odierna, G., Vences, M., Aprea, G., Lötters, S., Andreone, F. Chromosome data for Malagasy Poison frogs (Amphibia: Ranidae: Mantella) and their bearing on taxonomy and phylogeny. **Zoological Science 18**: 505-514, 2001.

Posada, D. and Crandall, K.A. ModelTest: testing the modelo f DNA substitution. **Bioinformatics 14**: 817 – 818, 1998.

Ron, S.R., Santos, J.C., Cannatella, D.C. Phylogeny of the túngara frog genus *Engystomops* (=*Physalaemus pustulosus* species group; Anura: Leptodactylidae). **Mol. Phylogenet. Evol. 39**: 392-402, 2006

Schmid, M. Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. **Chromosoma 66**: 361-388, 1978.

Schmid, M.; Olert, J.; Klett, C. Chromosome banding in Amphibia. III. Sex chromosomes in *Triturus*. **Chromosoma 71**: 29-55, 1979.

Schmid, M. Chromosome banding in Amphibia. V. Highly differentiated ZW/ZZ sex chromosomes and exceptional genome size in *Pyxicephalus adspersus* (Anura, Ranidae). **Chromosoma 80**, 69-96, 1980.

Schmid, M.; Haaf, T.; Geile, B.; Sims, S. Chromosome banding in Amphibia. VIII. An unusual XY/XX-sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). **Chromosoma 88**: 69-82, 1983.

Schmid, M., Feichtinger, W., Steinlein, C., Rupprecht, A., Haaf, T., Kaiser, H. Chromosome banding in Amphibia XXIII. Giant W sex chromosomes and extremely small genomes in *Eleutherodactylus euphronides* and *Eleutherodactylus shrevei* (Anura, Leptodactylidae). **Cytogenet. Genome Res. 97**: 81-94, 2002.

Silva, A. P. Z., Haddad, C.F.B., Galassi, G.G., Kasahara, S. Multiple nucleolus organizer regions in *Leptodactylus mystacinus* (Amphibia, Anura) and comments on its systematic position in the *L. fuscus* group based on cytogenetic and molecular analyses. **Genetica 127**: 35-44, 2006.

Swofford, D. L. Phylogenetic analysis using parsimony, version 4.0b4a (Ilinois Natural History Survey, Champaign, 2000.

Viegas-Péquignot, E. "In situ" hybridization to chromosomes with biotinylated probes. In: **"In situ" hybridization: a practical approach** (D. Willernson, ed.). Oxford/New York/Tokyo: IRL Press , 1992, p. 137-158

Legends

Figure 1. A. Partial South America map in small scale displaying the sampling locations (yellow stars) in which *Engystomops* populations were surveyed. Geographical distributions of the *Engystomops* northwestern and southwestern clades are indicated in rectangles and shown in B and C large scales, respectively.

Figure 2. Karyotypes of male specimens of *Engystomops petersi* from Puyo (**A**) stained with Giemsa, (**B**) submitted to the Ag-NOR technique, (**E**) C-banded, (**F**) DAPI-stained (top) and MM-stained (bottom), (**G**) DAPI-stained (top) and MM-stained (bottom) after C-banding technique. The insets in A, B and E show the homomorphic and the heteromorphic pair XX from the females ZUEC 34939 (A;B), ZUEC 34935 (E) and ZUEC 34937, respectively. **C**. NOR-bearing chromosomes of a female hybridized with the rDNA probe HM 123. Note that some heterochromatic regions clearly evidenced by Ag-NOR in B are not detected in C. **D**. NOR-bearing chromosomes of the male QCAZ 34940 after the Ag-NOR technique (left) and hybridized with the rDNA probe HM 123 (right). Note the additional NOR in one homologue 9. **H**. Giemsa-stained karyotype from the Puyo female QCAZ 34935 with 2n=23. The arrow points the trissomic set. In the insets, the chromosomes 8 after the Ag-NOR technique (left) and C-banding (right). Bar = 2 µm.

Figure 3. Karyotypes of the *E. petersi* Yasuní specimens QCAZ 34944, QCAZ 34947 and QCAZ 34946 submitted to (**A**) Giemsa staining, (**B**) the Ag-NOR technique and (**C**) C-banding, respectively. The arrowheads in C indicate the two heterochromatic bands flanking the NOR. The insets in A and B show the chromosome pair 3 homomorphic (left) and heteromorphic (right) for NOR distension. **D**. C-banded karyotype from the specimen QCAZ 34948. The inset shows the NOR-bearing chromosome 9 after the Ag-NOR technique. **E**. DAPI-stained (top) and MM-stained (bottom) karyotypes. F. DAPI-stained (top) and MM-stained (bottom) after C-banding technique. Bar = 2 µm.

Figure 4. Cytogenetics of the Yasuní specimen QCAZ 30826 **A**. Karyotype submitted to C-banding. B. Karyotype after Ag-NOR staining. The NORs were pointed by arrows. **C**. Mitotic metaphase hybridized with the rDNA probe HM 123 and stained with propidium iodide. Note that some heterochromatic regions seen in A are clearly detected by the Ag-NOR method. **D**. X chromosome hybridized with the HM123 probe and stained with DAPI (left). In the right, only the DAPI stained. **E**. X chromosome after C-banding. **F**. X chromosome after the Ag-NOR technique. Note the presence of three NOR blocks in B and F, separated by heterochromatic regions (**E**).

Figure 5. Cytogenetics of the La Selva specimen **A**. Giemsa-stained karyotype. **B**. C-banded karyotype **C-E**. The 6th pair after Ag-NOR, DAPI and MM staining, respectively. Note the heterochromatic blocks flanking the NOR. **F-G.** The same metaphase stained with DAPI and MM, respectively, after C-banding. Note the different regions stained by these fluorochromes. The arrow in **F** points an interstitial heterochromatic band in the chromosome 5. The arrowheads in **F** and **G** indicate the NOR. Bar = 2 µm.

Figure 6. Giemsa-stained (**A**), silver stained (**B**) and C-banded (**C**) karyotypes from the female ZUEC 14432 from UFAC population. The inset in A shows the heteromorphic pair from a male. **D-E**. DAPI-stained (top) and MM-stained (bottom) karyotypes from the female ZUEC 14432, after C-banding technique (**E**) or not (**D**). Bar = 2 µm.

Figure 7. NOR-bearing chromosome pairs from seven different UFAC specimens (**A-G**) stained by Ag-NOR (top) and hybridized with the rDNA probe HM 123 (middle). In the bottom, the C-banded pairs 11 and sexual of the seven specimens. Bars = 2 µm.

Figure 8. Mitotic metaphases with chromosome breaks. **A-B**. Puyo specimens QCAZ 34935 and QCAZ 34937 . **C**. Specimen QCAZ 34947 from Estación Cientifica Yasuní. **D**. UFAC specimen ZUEC 14455 . Arrowheads indicate the chromosome breaks. The arrows in **A** indicate chromosomes that probably have 2 centromeres. Bars = 2 µm.

Figure 9. Strict consensus cladogram of *Engystomops* inferred by MP analysis using sequences of the mitochondrial genes 12S rDNA, val-tRNA and 16S rDNA. Numbers above the branches are bootstrap values. Branches without numbers had bootstrap <50. In the parentheses, the specimens numbers as indicated in Appendix 1. Available data of vocal calls (Boul *et al*., 2007) are indicated for the cytogenetically studied populations.

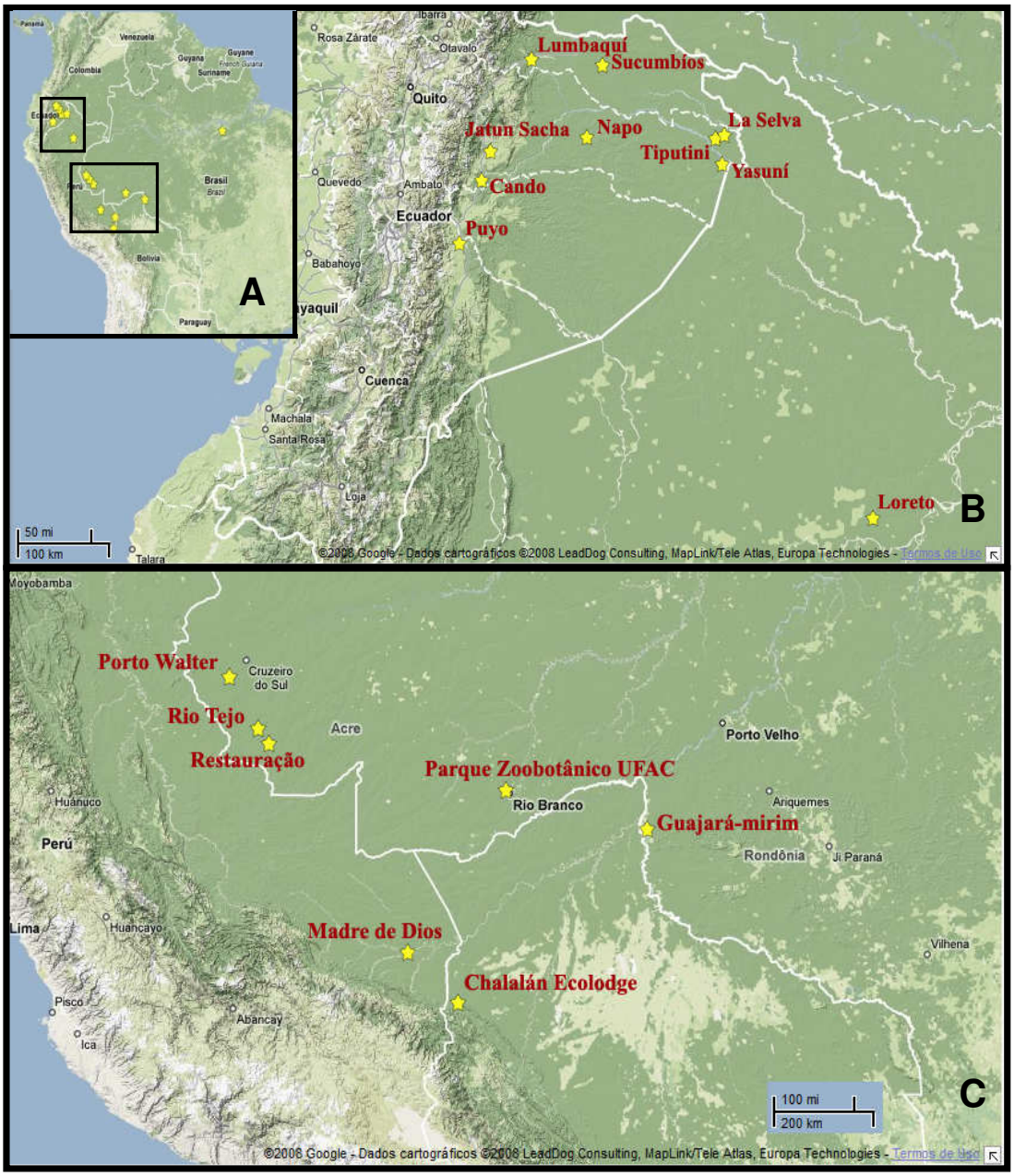

A
B
C
Lumbaquí
Sucumbíos
Jatun Sacha
Napo
La Selva
Tiputini
Yasuní
Cando
Puyo
Loreto
Quito
Cuenca
Ecuador
Porto Walter
Cruzeiro do Sul
Rio Tejo
Restauração
Acre
Parque Zoobotânico UFAC
Rio Branco
Porto Velho
Guajará-mirim
Rondônia
Ji Paraná
Madre de Dios
Chalalán Ecolodge
Perú
Lima
Huancayo
Pisco
Ica
Abancay
Vilhena
Ariquemes
Huánuco
Moyobamba
50 mi
100 km
100 mi
200 km
©2008 Google - Dados cartográficos ©2008 LeadDog Consulting, MapLink/Tele Atlas, Europa Technologies - Termos de Uso

Figure 1

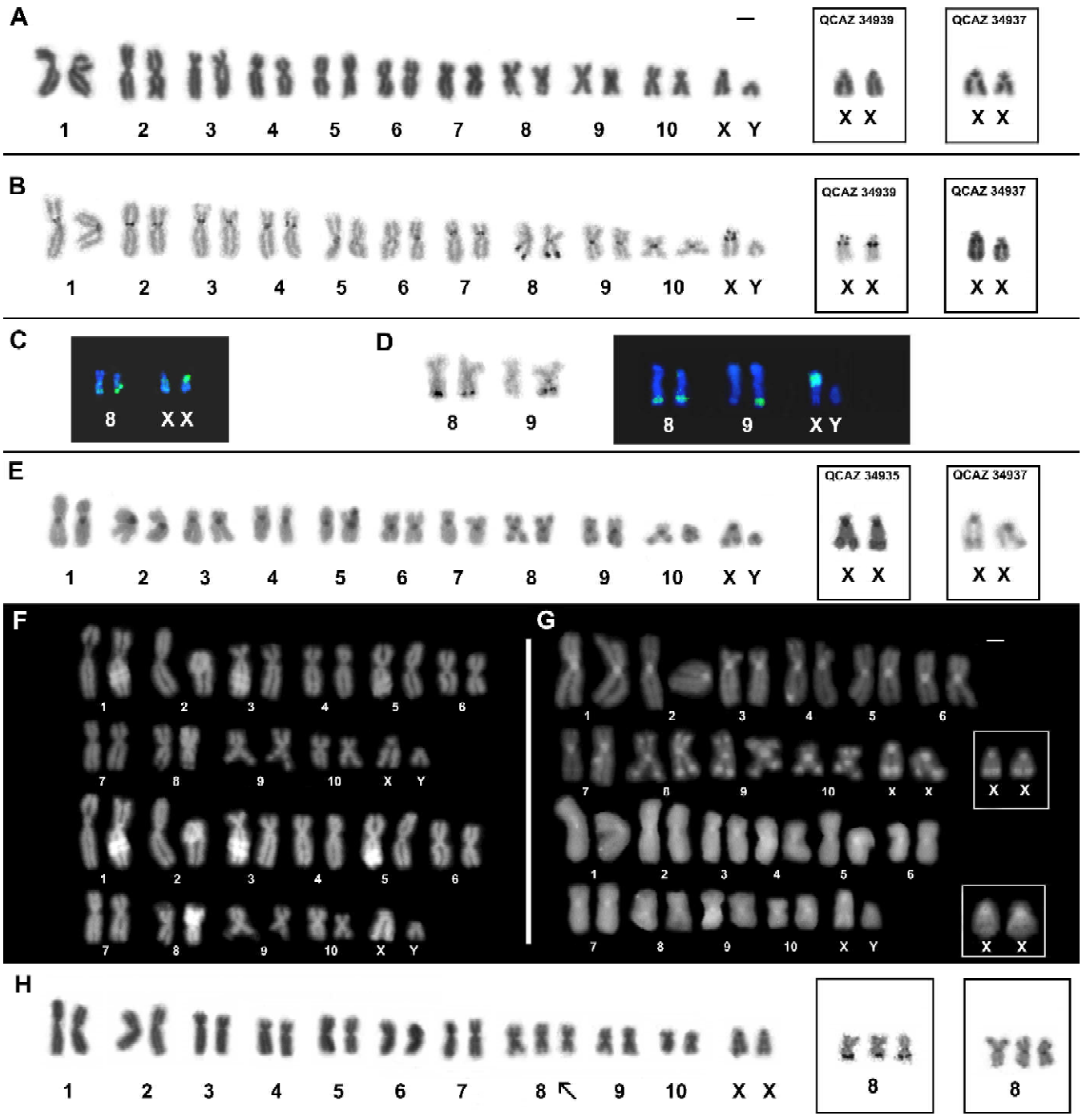

A
1 2 3 4 5 6 7 8 9 10 X Y
QCAZ 34939
X X
QCAZ 34937
X X
B
1 2 3 4 5 6 7 8 9 10 X Y
QCAZ 34939
X X
QCAZ 34937
X X
C
8 X X
D
8 9
8 9 X Y
E
1 2 3 4 5 6 7 8 9 10 X Y
QCAZ 34935
X X
QCAZ 34937
X X
F
1 2 3 4 5 6
7 8 9 10 X Y
1 2 3 4 5 6
7 8 9 10 X Y
G
1 2 3 4 5 6
7 8 9 10 X X
X X
1 2 3 4 5 6
7 8 9 10 X Y
X X
H
1 2 3 4 5 6 7 8 9 10 X X
8
8

Figure 2


A
1 2 3 4 5 6 7 8 9 10 11
3 3
B
1 2 3 4 5 6 7 8 9 10 11
3 3
C
1 2 3 4 5 6 7 8 9 10 11
D
1 2 3 4 5 6 7 8 9 10 11
9
E
1 2 3 4 5 6
7 8 9 10 11
1 2 3 4 5 6
7 8 9 10 11
F
1 2 3 4 5 6
7 8 9 10 11
1 2 3 4 5 6
7 8 9 10 11

Figure 3

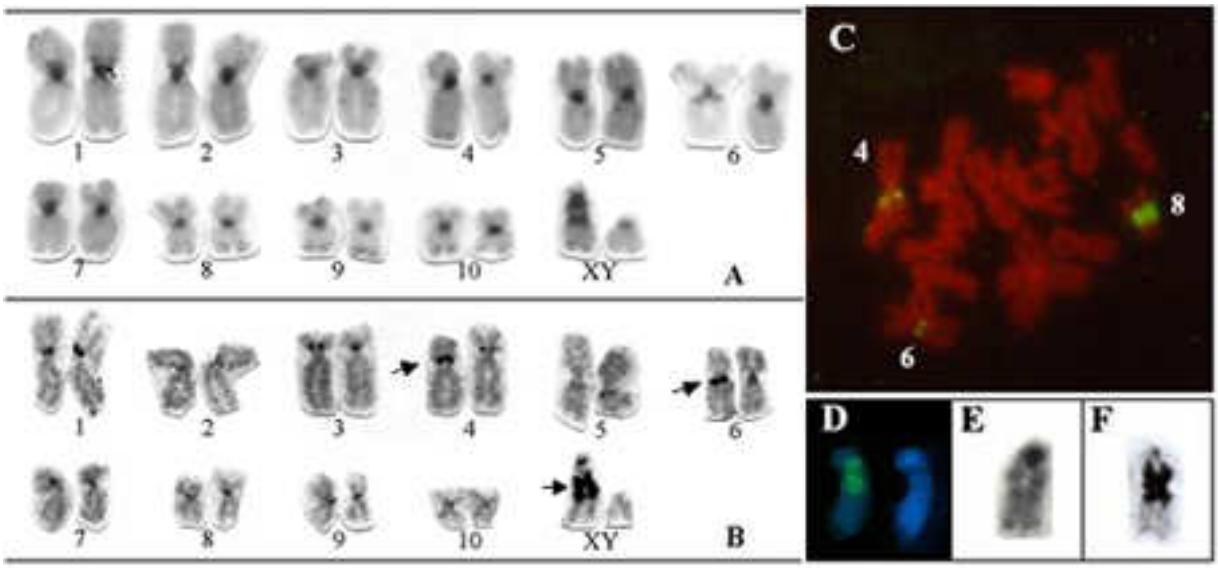


Figure 4

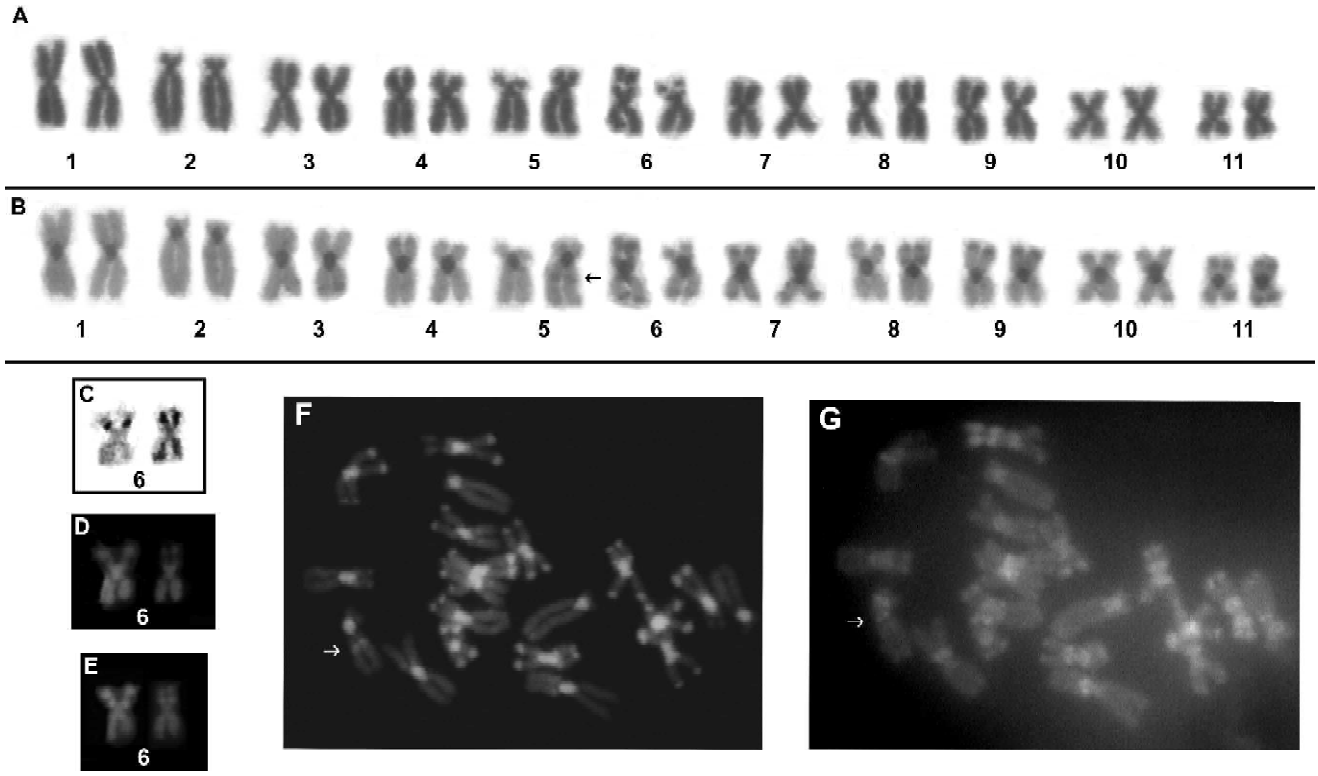


Figure 5

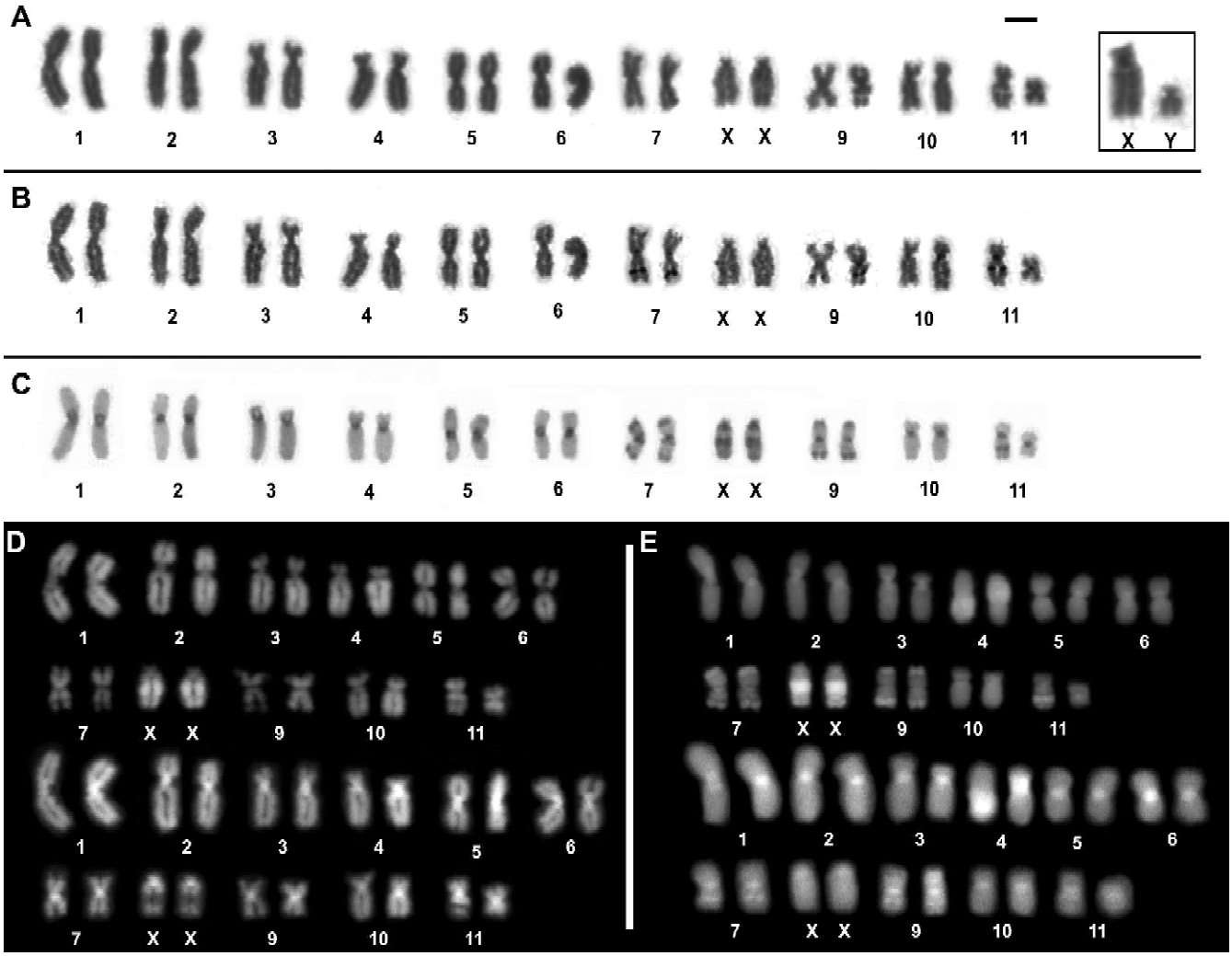

A
1 2 3 4 5 6 7 X X 9 10 11 X Y
B
1 2 3 4 5 6 7 X X 9 10 11
C
1 2 3 4 5 6 7 X X 9 10 11
D
1 2 3 4 5 6
7 X X 9 10 11
1 2 3 4 5 6
7 X X 9 10 11
E
1 2 3 4 5 6
7 X X 9 10 11
1 2 3 4 5 6
7 X X 9 10 11

Figure 6

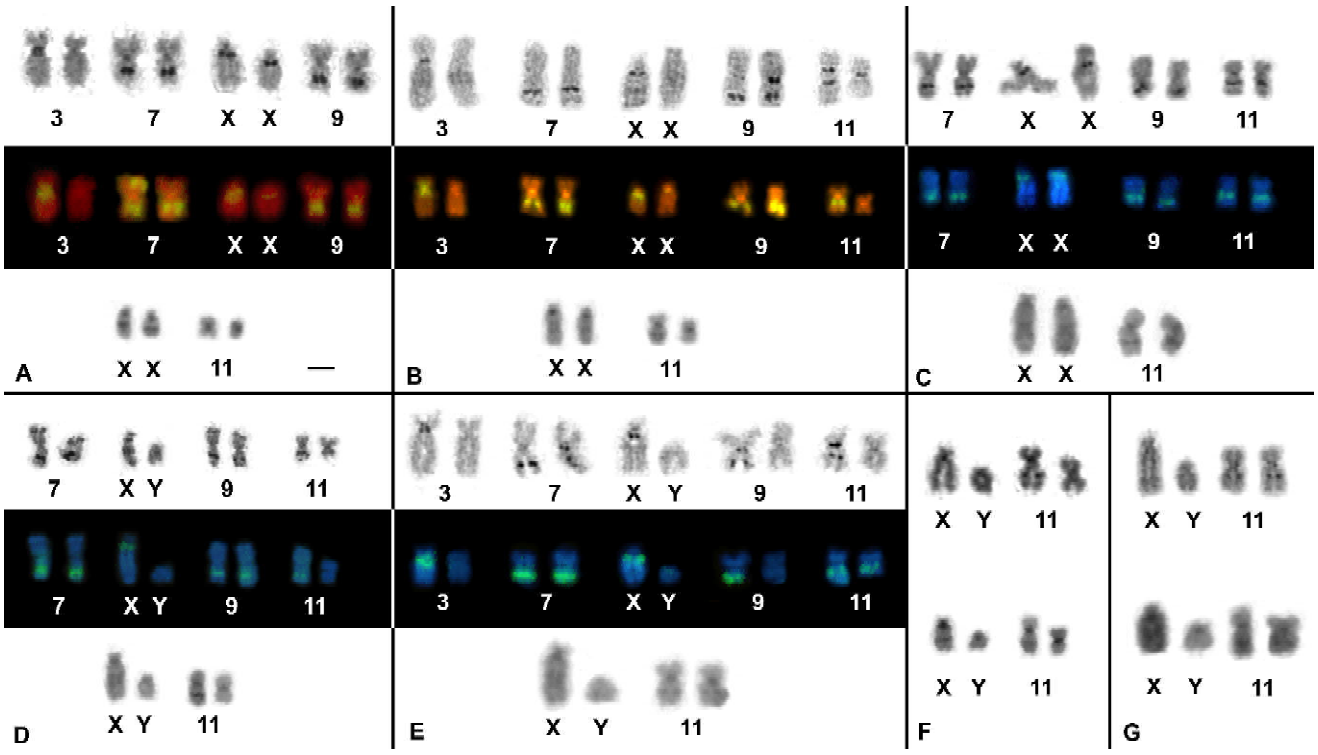

3 7 X X 9
3 7 X X 9 11
7 X X 9 11
3 7 X X 9
3 7 X X 9 11
7 X X 9 11
A X X 11 —
B X X 11
C X X 11
7 X Y 9 11
3 7 X Y 9 11
X Y 11
X Y 11
7 X Y 9 11
3 7 X Y 9 11
X Y 11
X Y 11
D X Y 11
E X Y 11
F
G

Figure 7

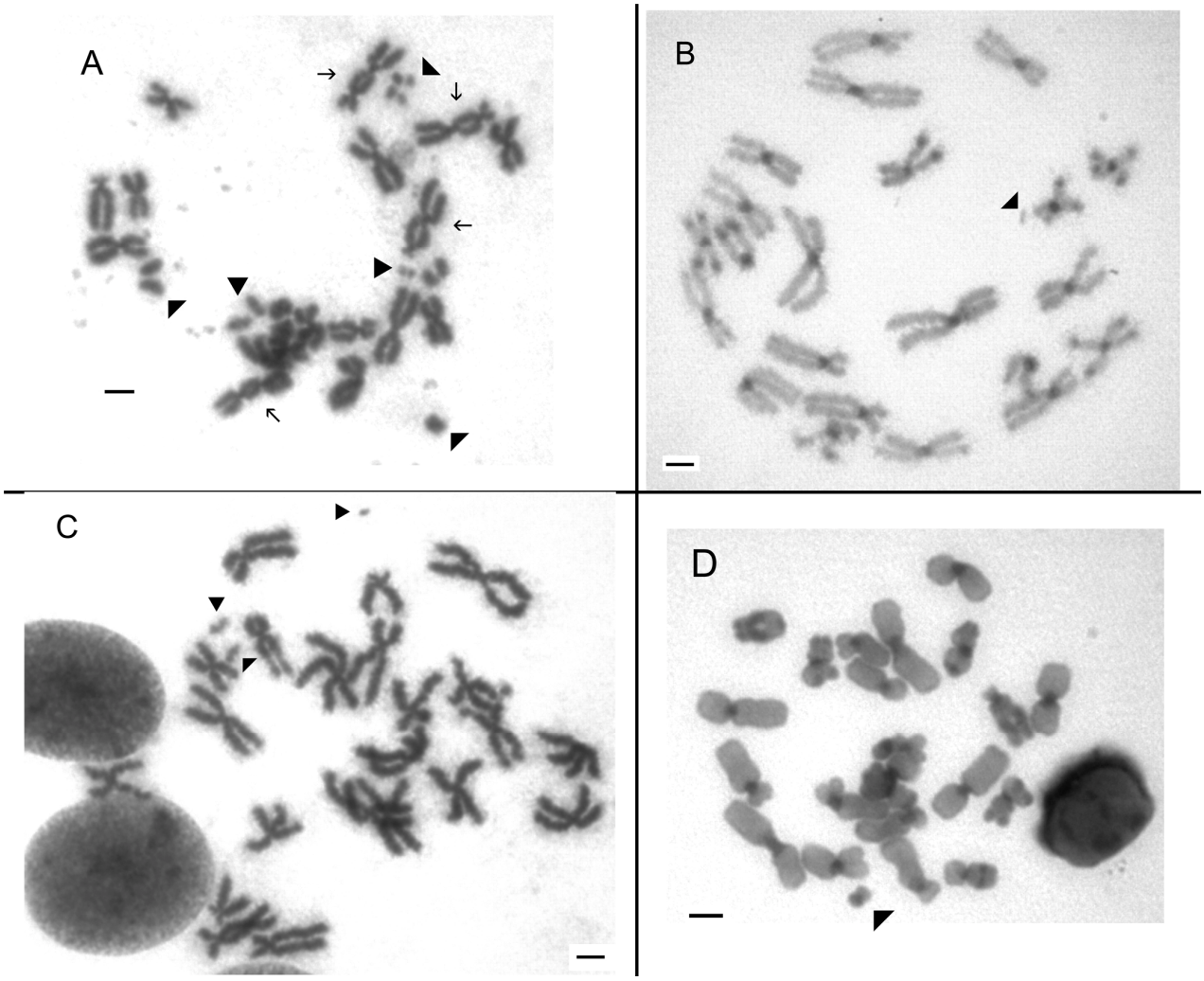


Figure 8

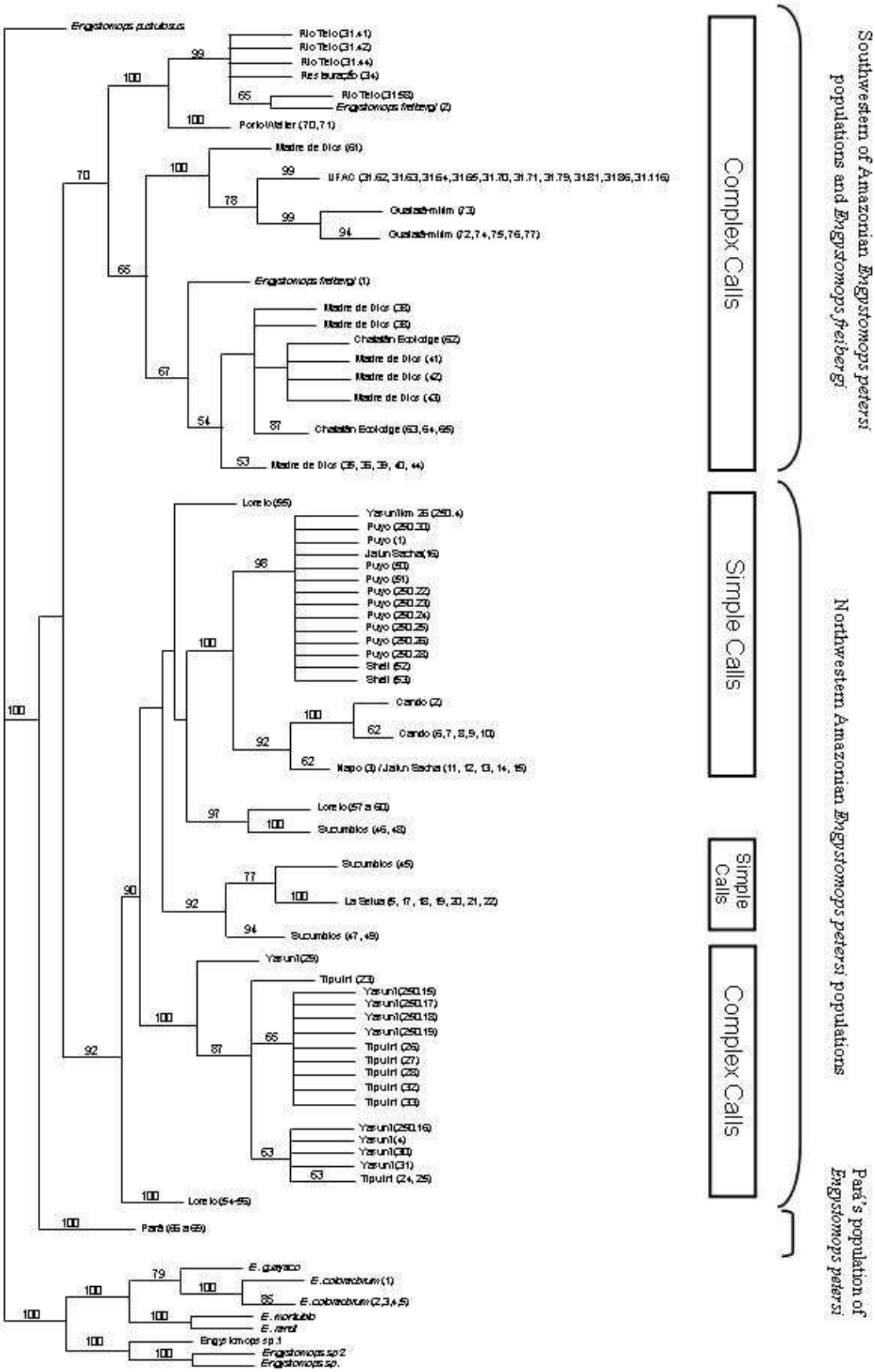

Engystomops pustulosus
Rio Telo (31.41)
Rio Telo (31.42)
Rio Telo (31.44)
Restauração (34)
Rio Telo (3158)
Engystomops freibergi (2)
Porto Velho (70, 71)
Madre de Dios (61)
UFAC (31.62, 31.63, 3164, 3165, 31.70, 31.71, 31.79, 3181, 3186, 31.116)
Guatuso-milim (73)
Guatuso-milim (72, 74, 75, 76, 77)
Engystomops freibergi (1)
Madre de Dios (38)
Madre de Dios (39)
Chalalán Ecologe (62)
Madre de Dios (41)
Madre de Dios (42)
Madre de Dios (43)
Chalalán Ecologe (63, 64, 65)
Madre de Dios (35, 36, 39, 40, 44)
Loreto (55)
Yasuni km 25 (250.4)
Puyo (250.30)
Puyo (1)
Jatun Sacha (16)
Puyo (50)
Puyo (51)
Puyo (250.22)
Puyo (250.23)
Puyo (250.24)
Puyo (250.25)
Puyo (250.26)
Puyo (250.28)
Shell (52)
Shell (53)
Cando (2)
Cando (6, 7, 8, 9, 10)
Napo (3) / Jatun Sacha (11, 12, 13, 14, 15)
Loreto (57 a 60)
Sucumbios (46, 48)
Sucumbios (45)
La Selva (5, 17, 18, 19, 20, 21, 22)
Sucumbios (47, 49)
Yasuni (29)
Tipuirí (23)
Yasuni (250.15)
Yasuni (250.17)
Yasuni (250.18)
Yasuni (250.19)
Tipuirí (26)
Tipuirí (27)
Tipuirí (28)
Tipuirí (32)
Tipuirí (33)
Yasuni (250.16)
Yasuni (4)
Yasuni (30)
Yasuni (31)
Tipuirí (24, 25)
Loreto (54+56)
Pará (66 a 69)
E. guayaco
E. cobanchrum (1)
E. cobanchrum (2,3,4,5)
E. montubio
E. ronsi
Engystomops sp.1
Engystomops sp.2
Engystomops sp.
Complex Calls
Simple Calls
Simple Calls
Complex Calls
Southwestern Amazonian Engystomops petersi populations and Engystomops freibergi
Northwestern Amazonian Engystomops petersi populations
Pará's population of Engystomops petersi

Figure 9

Appendix 1. Specimens used for the phylogenetic analysis, their geographic procedence, and DNA sequences used.12S/16S: 12S rDNA, val-tRNA and 16S rDNA mitochondrial sequences; Rhod: nuclear gene of rhodopsin

Species	Locality	Molecular Reference	GenBank accession number	Voucher number	Cytogenetic Reference	Genes	Symbol
Engystomops petersi	Brazil: Rondônia: Parque Estadual Guajará-mirim	Funk *et al*., 2007	EF470285	LSUMZ 17523	-	12S/16S	77
Engystomops petersi	Brazil: Rondônia: Parque Estadual Guajará-mirim	Funk *et al*., 2007	EF470284	LSUMZ 17489	-	12S/16S	76
Engystomops petersi	Brazil: Rondônia: Parque Estadual Guajará-mirim	Funk *et al*., 2007	EF470283	LSUMZ 17467	-	12S/16S	75
Engystomops petersi	Brazil: Rondônia: Parque Estadual Guajará-mirim	Funk *et al*., 2007	EF470282	LSUMZ 17459	-	12S/16S	74
Engystomops petersi	Brazil: Rondônia: Parque Estadual Guajará-mirim	Funk *et al*., 2007	EF470281	LSUMZ 17427	-	12S/16S	73
Engystomops petersi	Brazil: Rondônia: Parque Estadual Guajará-mirim	Funk *et al*., 2007	EF470280	LSUMZ 17422	-	12S/16S	72
Engystomops petersi	Brazil: Acre: Porto Walter	Funk *et al*., 2007	EF470279	LSUMZ 13687	-	12S/16S	71
Engystomops petersi	Brazil: Acre: Porto Walter	Funk *et al*., 2007	EF470278	LSUMZ 13649	-	12S/16S	70
Engystomops petersi	Brazil: Pará: Agropecuária Treviso	Funk *et al*., 2007	EF470277	LSMUZ 18731	-	12S/16S	69
Engystomops petersi	Brazil: Pará: Agropecuária Treviso	Funk *et al*., 2007	EF470276	LSMUZ 18730	-	12S/16S	68
Engystomops petersi	Brazil: Pará: Agropecuária Treviso	Funk *et al*., 2007	EF470275	LSMUZ 18729	-	12S/16S	67
Engystomops petersi	Brazil: Pará: Agropecuária Treviso	Funk *et al*., 2007	EF470274	LSMUZ 18728	-	12S/16S	66
Engystomops petersi	Bolivia: La Paz: Chalalán Ecolodge	Funk *et al*., 2007	EF470273	MNCN/ADN 2846	-	12S/16S	65
Engystomops petersi	Bolivia: La Paz: Chalalán Ecolodge	Funk *et al*., 2007	EF470272	MNCN/ADN 2845	-	12S/16S	64
Engystomops petersi	Bolivia: La Paz: Chalalán Ecolodge	Funk *et al*., 2007	EF470271	MNCN/ADN 2823	-	12S/16S	63
Engystomops petersi	Peru: Madre de Dios: Trail between Madre de Dios and Lago Sandoval	Funk *et al*., 2007	EF470270	KUNHM 215133	-	12S/16S	62
Engystomops petersi	Peru: Madre de Dios: Cusco Amazónico	Funk *et al*., 2007	EF470269	KUNHM 215534	-	12S/16S	61
Engystomops petersi	Peru: Loreto: Amazon Conservancy for Tropical Studies	Funk *et al*., 2007	EF470268	MUSM 21564	-	12S/16S	60
Engystomops petersi	Peru: Loreto: Amazon Conservancy for Tropical Studies	Funk *et al*., 2007	EF470267	MUSM 21562	-	12S/16S	59
Engystomops petersi	Peru: Loreto: Amazon Conservancy for Tropical Studies	Funk *et al*., 2007	EF470266	MUSM 21556	-	12S/16S	58
Engystomops petersi	Peru: Loreto: Amazon Conservancy for Tropical Studies	Funk *et al*., 2007	EF470265	MUSM 21546	-	12S/16S	57
Engystomops petersi	Peru: Loreto: San Jacinto	Funk *et al*., 2007	EF470264	KUNHM 222071	-	12S/16S	56
Engystomops petersi	Peru: Loreto: San Jacinto	Funk *et al*., 2007	EF470263	KUNHM 222070	-	12S/16S	55
Engystomops petersi	Peru: Loreto: San Jacinto	Funk *et al*., 2007	EF470262	KUNHM 222069	-	12S/16S	54
Engystomops petersi	Ecuador: Pastaza: Shell	Funk *et al*., 2007	EF470261	QCAZ 25039	-	12S/16S	53
Engystomops petersi	Ecuador: Pastaza: Shell	Funk *et al*., 2007	EF470260	QCAZ 25038	-	12S/16S	52
Engystomops petersi	Ecuador: Pastaza: Puyo	Funk *et al*., 2007	EF470259	QCAZ 28857	-	12S/16S	51
Engystomops petersi	Ecuador: Pastaza: Puyo	Funk *et al*., 2007	EF470258	QCAZ 26211	-	12S/16S	50
Engystomops petersi	Ecuador: Sucumbíos: Puerto Bolivar	Funk *et al*., 2007	EF470257	QCAZ 28178	-	12S/16S	49
Engystomops petersi	Ecuador: Sucumbíos: Puerto Bolivar	Funk *et al*., 2007	EF470256	QCAZ 28172	-	12S/16S	48
Engystomops petersi	Ecuador: Sucumbíos: Puerto Bolivar	Funk *et al*., 2007	EF470255	QCAZ 28169	-	12S/16S	47
Engystomops petersi	Ecuador: Sucumbíos: Puerto Bolivar	Funk *et al*., 2007	EF470254	QCAZ 27813	-	12S/16S	46
Engystomops petersi	Ecuador: Sucumbíos: Lumbaquí	Funk *et al*., 2007	EF470253	QCAZ 25790	-	12S/16S	45
Engystomops petersi	Peru: Madre de Dios: south side of Tambopata River across from Tambopata Research Center	Funk *et al*., 2007	EF011554	MUSM 19382	-	12S/16S	44
Engystomops petersi	Peru: Madre de Dios: south side of Tambopata River across from Tambopata Research Center	Funk *et al*., 2007	EF011553	MUSM 19380	-	12S/16S	43
Engystomops petersi	Peru: Madre de Dios: south side of Tambopata River across from Tambopata Research Center	Funk *et al*.,2007	EF011552	MUSM 19381	-	12S/16S	42
Engystomops petersi	Peru: Madre de Dios: south side of Tambopata River across from	Funk *et al*., 2007	EF011551	MUSM 19348	-	12S/16S	41

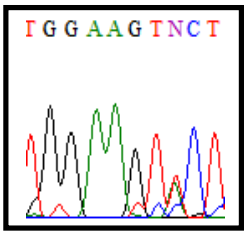
	Tambopata Research Center						
Engystomops petersi	Peru: Madre de Dios: Tambopata Research Center	Funk *et al*., 2007	EF011550	MUSM 19363	-	12S/16S	40
Engystomops petersi	Peru: Madre de Dios: Tambopata Research Center	Funk *et al*., 2007	EF011549	MUSM 19404	-	12S/16S	39
Engystomops petersi	Peru: Madre de Dios: Tambopata Research Center	Funk *et al*., 2007	EF011548	MUSM 19403	-	12S/16S	38
Engystomops petersi	Peru: Madre de Dios: Tambopata Research Center	Funk *et al*., 2007	EF011547	MUSM 19368	-	12S/16S	37
Engystomops petersi	Peru: Madre de Dios: Explorer's Inn	Funk *et al*., 2007	EF011546	USNM 343260	-	12S/16S	36
Engystomops petersi	Peru: Madre de Dios: Explorer's Inn	Funk *et al*.,2007	EF011545	USNM 343264	-	12S/16S	35
Engystomops petersi	Brazil: Acre: Restauração	Funk *et al*., 2007	EF011544	ZUEC 9523	-	12S/16S	34
Engystomops petersi	Ecuador: Orellana: Estación Científica Yasuní	Funk *et al*., 2007	EF011543	QCAZ 15136	-	12S/16S	33
Engystomops petersi	Ecuador: Orellana: Estación Científica Yasuní	Funk *et al*., 2007	EF011542	QCAZ 15138	-	12S/16S	32
Engystomops petersi	Ecuador: Yasuní	Boul *et al*., 2006	EF011541	DCC 3682	-	12S/16S	31
Engystomops petersi	Ecuador: Yasuní	Boul *et al*., 2006	EF011540	DCC 3685	-	12S/16S	30
Engystomops petersi	Ecuador: Yasuní	Boul *et al*., 2006	EF011539	QCAZ 11863	-	12S/16S	29
Engystomops petersi	Ecuador: Tiputini	Boul *et al*., 2006	EF011538	QCAZ 28612	-	12S/16S	28
Engystomops petersi	Ecuador: Tiputini	Boul *et al*., 2006	EF011537	QCAZ 28607	-	12S/16S	27
Engystomops petersi	Ecuador: Tiputini	Boul *et al*., 2006	EF011536	QCAZ 28608	-	12S/16S	26
Engystomops petersi	Ecuador: Tiputini	Boul *et al*., 2006	EF011535	QCAZ 28610	-	12S/16S	25
Engystomops petersi	Ecuador: Tiputini	Boul *et al*., 2006	EF011534	QCAZ 28611	-	12S/16S	24
Engystomops petersi	Ecuador: Tiputini	Boul *et al*., 2006	EF011533	QCAZ 28620	-	12S/16S	23
Engystomops petersi	Ecuador: La Selva	Boul *et al*., 2006	EF011532	DCC 3705	-	12S/16S	22
Engystomops petersi	Ecuador: La Selva	Boul *et al*., 2006	EF011531	QCAZ 24029	-	12S/16S	21
Engystomops petersi	Ecuador: La Selva	Boul *et al*., 2006	EF011530	QCAZ 23975	-	12S/16S	20
Engystomops petersi	Ecuador: La Selva	Boul *et al*., 2006	EF011529	QCAZ 28576	-	12S/16S	19
Engystomops petersi	Ecuador: La Selva	Boul *et al*., 2006	EF011528	QCAZ 28577	-	12S/16S	18
Engystomops petersi	Ecuador: La Selva	Boul *et al*., 2006	EF011527	QCAZ 28578	-	12S/16S	17
Engystomops petersi	Ecuador: Jatun Sacha	Boul *et al*., 2006	EF011526	MJR 001	-	12S/16S	16
Engystomops petersi	Ecuador: Jatun Sacha	Boul *et al*., 2006	EF011525	MJR 008	-	12S/16S	15
Engystomops petersi	Ecuador: Jatun Sacha	Boul *et al*., 2006	EF011524	MJR 006	-	12S/16S	14
Engystomops petersi	Ecuador: Jatun Sacha	Boul *et al*., 2006	EF011523	MJR 005	-	12S/16S	13
Engystomops petersi	Ecuador: Jatun Sacha	Boul *et al*., 2006	EF011522	MJR 004	-	12S/16S	12
Engystomops petersi	Ecuador: Jatun Sacha	Boul *et al*., 2006	EF011521	QCAZ 24045	-	12S/16S	11
Engystomops petersi	Ecuador: Cando	Boul *et al*., 2006	EF011520	DCC 3712	-	12S/16S	10
Engystomops petersi	Ecuador: Cando	Boul *et al*., 2006	EF011519	DCC 3711	-	12S/16S	9
Engystomops petersi	Ecuador: Cando	Boul *et al*., 2006	EF011518	DCC 3710	-	12S/16S	8
Engystomops petersi	Ecuador: Cando	Boul *et al*., 2006	EF011517	DCC 3701	-	12S/16S	7
Engystomops petersi	Ecuador: Cando	Boul *et al*., 2006	EF011516	DCC 3699	-	12S/16S	6
Engystomops petersi	Ecquador: Sucumbíos: La Selva	Ron *et al*., 2006	DQ337234	QCAZ 23976	-	12S/16S	5
Engystomops petersi	Ecuador: Yasuní	Boul *et al*., 2006	DQ337233	QCAZ 12128	-	12S/16S	4
Engystomops petersi	Ecuador: Napo: Napo-Galeras, Ishquinambi	Ron *et al*., 2006	DQ337232	QCAZ 14723	-	12S/16S	3
Engystomops petersi	Ecuador: Cando	Boul *et al*., 2006	DQ337231	QCAZ 11965	-	12S/16S	2
Engystomops petersi	Ecuador: Pastaza: El Puyo	Ron *et al*., 2006	DQ337230	QCAZ 26210	-	12S/16S	1
Engystomops petersi	Ecuador: 26 Km Estação Científica Yasuní	This work	-	QCAZ 30826	This work	Rhod/12S/16S	250.04
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34926	-	Rhod	250.07
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34927	-	Rhod	250.08
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34929	-	Rhod	250.09
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34924	-	Rhod	250.10
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34928	-	Rhod	250.11
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34932	-	Rhod	250.13
Engystomops petersi	Ecuador: Puyo	-	-	QCAZ 34933	This work	-	250.20

Engystomops petersi	Ecuador: Puyo	-	-	QCAZ 34934	This work	-	250.21
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34935	This work	Rhod/12S/16S	250.22
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34936	This work	Rhod/12S/16S	250.23
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34937	This work	Rhod/12S/16S	250.24
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34938	This work	Rhod/12S/16S	250.25
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34940	This work	Rhod/12S/16S	250.26
Engystomops petersi	Equador: Puyo	-	-	QCAZ 34939	This work	-	250.27
Engystomops petersi	Equador: Puyo	-	-	QCAZ 34941	This work	Rhod	250.28
Engystomops petersi	Equador: Puyo	-	-	QCAZ 34942	This work	-	250.29
Engystomops petersi	Ecuador: Puyo	This work	-	QCAZ 34943	This work	Rhod/12S/16S	250.30
Engystomops petersi	Ecuador: Estação Científica Yasuní, Província de Orellana	This work	-	QCAZ 34944	This work	Rhod/12S/16S	250.15
Engystomops petersi	Ecuador: Estação Científica Yasuní, Província de Orellana	This work	-	QCAZ 34945	This work	Rhod	250.16
Engystomops petersi	Ecuador: Estação Científica Yasuní, Província de Orellana	This work	-	QCAZ 34946	This work	Rhod/12S/16S	250.17
Engystomops petersi	Ecuador: Estação Científica Yasuní, Província de Orellana	This work	-	QCAZ 34947	This work	Rhod/12S/16S	250.18
Engystomops petersi	Ecuador: Estação Científica Yasuní, Província de Orellana	This work	-	QCAZ 34948	This work	Rhod	250.19
Engystomops petersi	Ecuador: La Selva	-	-	-	This work	-	La Selva
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9620	Lourenço *et al.*, 1998	Rhod/12S/16S	31.41
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9621	-	Rhod/12S/16S	31.42
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9651	-	Rhod/12S/16S	31.44
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9652	Lourenço *et al.*, 1998	Rhod	31.45
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9624	Lourenço *et al.*, 1998	Rhod	31.46
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9625	Lourenço *et al.*, 1998	Rhod	31.47
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9639	Lourenço *et al.*, 1998	Rhod	31.49
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9641	Lourenço *et al.*, 1998	Rhod	31.51
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9642	Lourenço *et al.*, 1998	Rhod	31.52
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9643	Lourenço *et al.*, 1998	Rhod	31.53
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9644	-	Rhod	31.54
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9645	Lourenço *et al.*, 1998	Rhod	31.56
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9646	-	Rhod	31.57
Engystomops petersi	Brazil: Acre: Rio Tejo	This work	-	ZUEC 9647	Lourenço *et al.*, 1998	Rhod/12S/16S	31.58
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	-	-	Rhod/16S	31.62
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	-	-	Rhod/12S/16S	31.63
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	-	-	Rhod	31.64
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	-	-	Rhod/12S/16S	31.65
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	-	-	Rhod	31.66
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	-	-	Rhod/12S/16S	31.70
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	-	-	Rhod/12S/16S	31.71
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	ZUEC 14432	This work	Rhod/16S	31.79

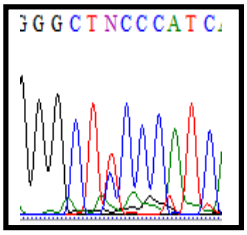
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	ZUEC 14434	This work	Rhod/12S/16S	31.81
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	ZUEC 14435	This work	Rhod	31.82
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	ZUEC 14436	This work	Rhod	31.83
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	-	-	ZUEC 14437	This work	-	31.84
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	ZUEC 14439	This work	Rhod/16S	31.86
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	-	-	ZUEC 14440	This work	-	31.87
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	ZUEC 14445	-	Rhod	31.92
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	-	-	ZUEC 14455	This work	-	31.102
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	-	-	ZUEC 14458	This work	-	31.105
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	ZUEC 14463	-	Rhod	31.110
Engystomops petersi	Brazil: Acre: Parque Zoobotânico da UFAC	This work	-	ZUEC 14469	-	Rhod/16S	31.116
Physalaemus coloradorum	Ecuador: Pichincha 5Km NW La Florida	Ron *et al*., 2005	AY834182	QCAZ19418	-	12S/16S	Color3
Physalaemus freibergi	Peru: Madre de Dios: Tambopata, Explorer's Inn	Cannatella *et al*., 1998	AF058962	-	-	12S/16S	Freib1
Engystomops cf. freibergi	Brazil: Acre: Reserva Extrativista do Alto Juruá, Rio Tejo	Ron *et al*., 2006	DQ337229	ZUEC 9511	-	12S/16S	Freib2
Engystomops guayaco	Ecuador: Guayas: 11 km N Cerro Masvale	Ron *et al*., 2005	AY834175	QCAZ23533	-	12S/16S	Guay4
Engystomops guayaco	Ecuador: Guayas: 11 km N Cerro Masvale	Ron *et al*., 2006	DQ337219	QCAZ 23533	-	12S/16S	Guay6
Engystomops montubio	Ecuador: Manabí Estero Ancho, 52 km W El Carmen	Ron *et al*., 2005	AY834177	QCAZ23190	-	12S/16S	Mont1
Engystomops pustulosus	Panama: Panama: Gamboa	Cannatella *et al*, 1998	AF058960	-	-	12S/16S	Pust1
Engystomops randi	Ecuador: Guayas: Cerro Masvale	Ron *et al*., 2005	AY834179	QCAZ19559	-	12S/16S	Rand1
Engystomops sp. B (E. caicai)	Peru: Lambayeque: Olmos, 8,5 km N Motupe	Ron *et al*., 2006	DQ337216	MR 726	-	12S/16S	Sp1
Engystomops sp. D DCC-2006	Ecuador: El Oro: Puyango	Ron *et al*., 2006	DQ337217	QCAZ 26981	-	12S/16S	Sp2
Engystomops sp. D DCC-2006	Ecuador: Loja: Zapotillo	Ron *et al*., 2006	DQ337218	QCAZ 26959	-	12S/16S	Sp3

Appendix 2. SNPs sites of the rhodopsin gene. M refers to cytosine or adenine; Y refers to thymine or cytosine; W refers to thymine or adenine. In the side, electropherograms from rhodopsin sequences of different specimens from UFAC showing double peaks at the SNP site.

Specimens	SNPs sites					
	14	17	23	91	137	311
31.41 Rio Tejo, Acre	C	T	C	T	T	C
31.42 Rio Tejo, Acre	.	.	.	.	Y	T
31.44 Rio Tejo, Acre	.	.	.	.	Y	Y
31.45 Rio Tejo, Acre	M	.	.	W	Y	Y
31.46 Rio Tejo, Acre	.	.	.	.	Y	T
31.47 Rio Tejo, Acre	.	.	.	.	C	T
31.49 Rio Tejo, Acre	.	.	Y	.	.	N
31.51 Rio Tejo, Acre	.	.	.	.	Y	T
31.52 Rio Tejo, Acre	.	.	.	.	Y	T
31.53 Rio Tejo, Acre	.	.	.	.	.	T
31.54 Rio Tejo, Acre	.	.	.	.	.	T
31.56 Rio Tejo, Acre	.	.	Y	.	Y	T
31.57 Rio Tejo, Acre	M	.	.	.	.	Y
31.58 Rio Tejo, Acre	A	.	.	.	.	.
31.62 UFAC, Acre	.	.	.	A	.	T
31.63 UFAC, Acre	.	.	.	W	.	T
31.64 UFAC, Acre	A	.	.	W	.	Y
31.65 UFAC, Acre	.	.	.	W	.	Y
31.66 UFAC, Acre	.	.	.	W	.	Y
31.70 UFAC, Acre	.	.	.	W	.	T
31.71 UFAC, Acre	.	.	.	W	.	T
31.79 UFAC, Acre	.	.	.	.	.	T
31.81 UFAC, Acre	.	.	.	.	.	T
31.82 UFAC, Acre	.	.	.	W	.	T
31.83 UFAC, Acre	A	.	.	.	.	T
31.86 UFAC, Acre	.	.	.	W	.	T
31.92 UFAC, Acre	A	.	.	W	.	T
31.110 UFAC, Acre	.	.	.	W	.	T
31.116 UFAC, Acre	.	.	.	.	.	T
250.04 26Km Yasuní, Ecuador	A	C	.	A	.	.
250.15 Yasuní, Ecuador	A	C	.	.	.	.
250.16 Yasuní, Ecuador	A	C	.	.	.	.
250.17 Yasuní, Ecuador	A	C	.	.	.	.
250.18 Yasuní, Ecuador	A	C	.	W	.	.
250.19 Yasuní, Ecuador	A	C	.	.	.	.
250.07 Puyo, Ecuador	A	C	.	A	.	T
250.08 Puyo, Ecuador	A	C	.	A	.	T
250.09 Puyo, Ecuador	A	C	.	A	.	T
250.10 Puyo, Ecuador	A	C	.	A	.	T
250.11 Puyo, Ecuador	A	C	.	A	.	Y
250.13 Puyo, Ecuador	A	C	.	A	.	Y
250.22 Puyo, Ecuador	A	C	.	A	.	T
250.23 Puyo, Ecuador	A	C	.	A	.	.
250.24 Puyo, Ecuador	A	C	.	A	.	T
250.25 Puyo, Ecuador	A	C	.	A	.	Y
250.26 Puyo, Ecuador	A	C	.	A	.	T
250.28 Puyo, Ecuador	A	C	.	A	.	T
250.30 Puyo, Ecuador	A	C	.	A	.	Y



A



B

Considerações Finais

- Cada população de *Engystomops* aqui analisada possuiu um padrão cariotípico diferente, inclusive as populações de Puyo e Yasuní, que não apresentam isolamento pré-zigótico. Esse fato permitiu sugerir que a diferenciação citogenética possa exercer algum papel no isolamento de populações e, conseqüentemente, nos processos de especiação desses anuros.
- A análise citogenética da população de UFAC (Acre, Brasil) permitiu uma nova interpretação dos dados cariotípicos antes descritos para outras populações do Acre e uma re-descrição daqueles cariótipos foi apresentada.
- As populações de Puyo e do Acre possuíram um par de cromossomos sexuais heteromórficos do sistema XX/XY, uma característica rara em anuros. As populações de Yasuní e de La Selva não apresentaram essa característica. A análise comparativa desses dados com as inferências filogenéticas permitiu sugerir que a presença de cromossomos sexuais heteromórficos seja uma condição plesiomórfica para esse grupo de *Engystomops* amazônicos.
- A presença de heterozigotos para nucleotídeos simples no gene da rodopsina pode ser interpretada como resultante de uma possível ocorrência de eventos de hibridação e introgressão entre as populações. Esses eventos podem favorecer a grande variação cariotípica encontrada inter e intra-populacionais e sua ocorrência é corroborada pela identificação de indivíduos com cariótipos em que o reconhecimento inequívoco de alguns pares cromossômicos não é possível e pela ocorrência de configurações meióticas multivalentes em alguns espécimes.

- Os resultados da filogenia utilizando DNA mitocondrial corroboraram o trabalho de Funk *et al.* (2007). Três grandes clados foram reconhecidos dentre os *Engystomops* da Amazônia: o clado com espécimes do Pará, o clado referente às populações do Equador e norte do Peru (denominado de clado noroeste), e o clado que inclui as populações do Acre, sul do Peru e Bolívia (denominado de clado sudoeste). A população da UFAC, não incluída nas análises de Funk *et al.* (2008), pertence a esse último clado.
- Os resultados moleculares e citogenéticos obtidos corroboram a presença de um complexo de espécies dentre os *Engystomops* amazônicos, mas sugerem a possibilidade de ocorrência de híbridos e introgressão entre espécies crípticas dessa região.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Mestrado** intitulada: "Estudo Citogenético e das relações filogenéticas de *Engystomops petersi* e *Engystomops* sp.(Anura, Leiuperidae)":

( ) não se enquadra no Artigo 1º, § 3º da Informação CCPG 01/2008, referente a bioética e biossegurança.

(X) está inserido no Projeto CIBio (Protocolo nº 2005/03), intitulado Citogenética e biologia molecular de anfíbios anuros

( ) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo nº _____).

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (?) (Protocolo nº _____).

Cintia Pelegrineti Targueta de Azevedo Brito

Aluno(a): Cíntia Pelegrineti Targueta de Azevedo Brito

Luciana Bolsoni Lourenço

Orientador(a): Luciana Bolsoni Lourenço Morandini

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Helena C F Oliveira

Nome:
Função:

Profa. Dra. HELENA COUTINHO F. DE OLIVEIRA
Presidente
Comissão Interna de Biossegurança
CIBio/IB - UNICAMP