

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

DÉBORA SILVA RODRIGUES

“ESTUDO DA ORGANIZAÇÃO DO GENE RIBOSSOMAL 5S
EM POPULAÇÕES DE *Engystomops* DA AMAZÔNIA
(ANURA, LEIUPERIDAE)”

Este exemplar corresponde à redação final
da tese defendida pelo(a) candidato (a)
Débora Silva Rodrigues
Luciana Bolsoni Lourenço
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, 2012

FICHA CATALOGRÁFICA ELABORADA POR
ROBERTA CRISTINA DAL' EVEDOVE TARTAROTTI – CRB8/7430
BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP

R618e	<p>Rodrigues, Débora Silva, 1986- Estudo da organização do gene ribossomal 5S em populações de Engystomops da Amazônia (Anura, Leiuperidae) / Débora Silva Rodrigues. – Campinas, SP: [s.n.], 2012.</p> <p>Orientador: Luciana Bolsoni Lourenço Morandini. Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.</p> <p>1. RNA ribossômico 5S. 2. Gene NTS. 3. Engystomops. 4. Marcadores moleculares. 5. Anfíbio – Genética. I. Lourenço, Luciana Bolsoni, 1972-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.</p>
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Informações para Biblioteca Digital

Título em Inglês: The study of the organization of the 5S ribosomal gene in populations of Amazonian Engystomops (Anura, Leiuperidae)

Palavras-chave em Inglês:

5S Ribosomal RNA

NTS gene

Engystomops

Molecular markers

Amphibians - Genetics

Área de concentração: Biologia Celular

Titulação: Mestre em Biologia Celular e Estrutural

Banca examinadora:

Luciana Bolsoni Lourenço Morandini [Orientador]

Sanae Kasahara

Cesar Martins

Data da defesa: 16-02-2012

Programa de Pós Graduação: Biologia Celular e Estrutural

Campinas, 16 de fevereiro de 2012.

BANCA EXAMINADORA

Profa. Dra. Luciana Bolsoni Lourenço Morandini (Orientadora)

Luciana Bolsoni
Assinatura

Prof. Dr. Cesar Martins

Cesar
Assinatura

Profa. Dra. Sanae Kasahara

Sanae
Assinatura

Profa. Dra. Ana Cristina Prado Veiga Menoncello

Assinatura

Prof. Dr. Luis Felipe de Toledo Ramos Pereira

Assinatura

"Além da mente humana e como um impulso livre, cria-se a ciência. Esta se renova, assim como as gerações, frente a uma atividade que constitui o melhor jogo do *homo ludens*: a ciência é, no mais estrito e melhor dos sentidos, uma gloriosa diversão"

Jacques Barzun

Dedico este trabalho a todos os amantes de anfíbios, que se aventuraram a entender um pouquinho desse universo incrível que ainda tem muito a ser desvendado.

Agradecimentos

Agradeço primeiramente aos meus pais, Luiz Felipe e Neide, pelo exemplo de vida que levo comigo todos os dias. Sem a boa educação que proporcionaram a mim – pessoal e profissional – eu não seria a pessoa que sou hoje. Por isso, grande parte dessa conquista é mérito deles. Obrigada por tudo o que sacrificaram para que hoje pudéssemos estar colhendo esses belos frutos. Amo vocês!

Agradeço também à minha irmã Luiza, vovó América e Maria e tia Ligia, pelo carinho e alegria que trazem à minha vida. Cada uma, de sua forma, contribuiu muito durante essa jornada, pela presença de sempre, a preocupação diária e o simples interesse. Pequenas coisas essenciais que fazem toda a diferença no dia a dia. Obrigada!

Alguém que não poderia deixar de citar aqui, e agradecer sinceramente, é aquele que foi o meu companheiro durante muito tempo, que procurou sempre estar presente em minha vida e de uma forma ou de outra acreditou e me incentivou a ser quem eu queria ser. Por um tempo seguimos caminhos próprios, mas agora os caminhos se encontraram de novo, e eu agradeço muito a Deus por ter você novamente ao meu lado. Obrigada, Leandro, por acreditar em mim, estar comigo e me fazer muito feliz.

Às minhas amigas, em especial Jaque, Cíntia, Camis, Jú, Lívia, Mi e Bruna, que hora longe, hora perto, sempre torceram por mim e acreditaram na minha capacidade. Sou feliz por ter vocês comigo nessa vida. Vocês tornam tudo mais simples e alegre. Obrigada pelos conselhos e pelo aprendizado, seja ele pessoal ou profissional, mas sempre muito valiosos. Adoro vocês, minhas irmãs!

A um amigo em especial, Thiago, que esteve ao meu lado durante grande parte dessa jornada de Mestrado, me dando conselhos, me apoiando nos meus momentos de loucura, me ensinando muito sobre tantas coisas que engrandeceram minha visão acadêmica, amparando minhas conquistas, enfim, sendo um grande companheiro. Obrigada, pra sempre.

À minha orientadora, Profa. Luciana, que foi minha maior professora desde que nos conhecemos, em 2008, na minha Iniciação Científica. Obrigada por acreditar no meu potencial e me aceitar como sua aluna. Por ser presente, me ajudar nos momentos que

necessito e estimular meu crescimento técnico e intelectual. Obrigada também pelas oportunidades que me ofereceu nesse último ano, acreditando na minha capacidade. Fico muito feliz em saber que atendo suas expectativas. É um retorno muito importante que me faz ter a certeza de que estou no lugar certo na minha profissão.

À Profa. Shirlei, que antes de todos, foi a primeira pessoa que me aceitou em seu laboratório. Obrigada por abrir as portas e me proporcionar essa oportunidade.

Em especial, à Profa. Ana Cristina (Aninha), pelas conversas e conselhos sempre construtivos. Espero que nossa amizade continue a crescer. Tenho muito ainda o que aprender contigo!

À Miryan Rivera, colaboradora do trabalho que será originado desta dissertação, pelo apoio e disposição prestados, pela revisão e discussão dos resultados gerados, e pelo coleguismo de sempre.

Aos amigos de laboratório, Cíntia, Daniel, Juliana, Lívia, Stenio, Kaleb, Reinaldo, Maurício, Júlio, Maria, Lenita, Gisele, Ane, Saulo, Ariane, Amanda, Caroline e Rafael. Deixo aqui meu sincero agradecimento pelo companheirismo e pelo aprendizado que todos vocês me proporcionam no ambiente de trabalho, que sem dúvidas muito auxiliou no desenvolvimento desse trabalho.

À técnica Klélia, que não está mais em nosso laboratório, mas foi quem me recebeu e me auxiliou em meus primeiros contatos com os procedimentos técnicos na bancada. Obrigada pela disposição e paciência.

À Alessandra, atual técnica de nosso laboratório, pelo auxílio na manutenção do nosso ambiente de trabalho.

Aos colegas do departamento, em especial Juliana Godoy, Giane, Rafaela e Marina, pela amizade conquistada nesses anos de convivência e pela contribuição profissional que cada uma me proporcionou.

Ao Prof. Fábio Costa, por disponibilizar o microscópio de fluorescência de seu laboratório para a obtenção de algumas imagens dessa dissertação. Em especial ao técnico, Nelson, pelo auxílio prestado no manuseio do equipamento e pela sua disponibilidade sempre que necessária.

Ao Prof. Hernandes F. Carvalho, por disponibilizar o seu termociclador para a obtenção de alguns resultados desse trabalho também. Em especial à técnica Fabiana, pelo auxílio prestado.

A todos que estagiaram no nosso laboratório por curtos períodos ou que simplesmente foram aprender alguma técnica em que eu pude auxiliar. Agradeço a oportunidade e a paciência de aprenderem comigo. Esses pequenos momentos de ensino e aprendizado mútuo são sempre experiências interessantes.

Aos mestres Ricardo Catalano, Edmilson Gonçalves e Miralva Aparecida da Pontifícia Universidade Católica de Campinas, que foram os “cupidos” responsáveis pela minha paixão na Graduação. E também pelo incentivo para cursar o Programa de Pós-graduação em Biologia Celular e Estrutural da UNICAMP.

Ao Programa de Pós-graduação em Biologia Celular e Estrutural pela oportunidade de realizar o Mestrado.

Aos professores do Departamento de Biologia Estrutural e Funcional pela contribuição que tiveram na minha formação durante as disciplinas que cursei. E também, aos professores que participaram da disciplina BS 111, que também tiveram importante participação na minha formação durante o PED.

Aos amigos e colegas de profissão que foram feitos pelo caminho, pela sua contribuição, pequena ou não, mas igualmente importante para o desenvolvimento desse trabalho.

Agradeço à CNPq, CAPES/PROEX e CAPES/PROAP, e FAPESP pelo auxílio financeiro.

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Resumo

O gênero *Engystomops* apresenta ampla distribuição geográfica e constitui um interessante grupo de anuros para estudos cariotípicos. As populações de *Engystomops* encontradas na Amazônia têm sua identificação taxonômica ainda controversa. Análises genéticas e citogenéticas apoiam hipóteses que sugerem a existência de um complexo de espécies crípticas e especiação incipiente. Muitas vezes a variação citogenética observada entre diferentes populações estudadas dificultou o reconhecimento de homeologias cromossômicas entre os cariotípos. Uma caracterização cromossômica mais detalhada poderia auxiliar no possível reconhecimento de homeologias cromossômicas e, dessa forma, contribuir para o estudo dos processos envolvidos na divergência desses anuros. Já que o gene do DNAr 5S tem sido importante marcador genético e citogenético para estudos evolutivos e para a identificação e comparação de espécies em diversos grupos, no presente trabalho o DNAr 5S de *Engystomops freibergi* e de exemplares de *Engystomops petersi* de duas localidades Equatorianas (Puyo e Yasuní) foi estudado. Em todos os casos, dois tipos de DNAr 5S, facilmente diferenciados pelo tamanho e composição da sequência do seu espaçador não transcrito, foram isolados. A provável região promotora do gene do RNAr 5S (ICR) foi localizada nos dois tipos de sequências de DNAr 5S e a presença de possíveis sequências regulatórias adicionais foi discutida. No cariotípico de *E. freibergi*, sonda contendo a unidade repetitiva do DNAr 5S tipo I hibridou na região pericentromérica do braço curto dos cromossomos do par 3, e o DNAr 5S tipo II foi mapeado na região distal do braço longo dos cromossomos do par 6. A sonda formada somente pela região de NTS do DNAr 5S tipo I claramente detectou a região pericentromérica de 3p nos cariotípicos de *E. freibergi* e *E. petersi* (Puyo) e de 5p no cariotípico de *E. petersi* (Yasuní), porém nenhum sinal distal ou intersticial foi observado. A sonda formada pela região de NTS do DNAr 5S tipo II detectou apenas a região distal de 6q nos três cariotípicos estudados, corroborando a distribuição diferencial dos dois tipos de DNAr 5S nesses cariotípicos. Tais sítios de DNAr 5S constituem novos marcadores cromossômicos, os quais permitem sugerir a homeologia entre o cromossomo 6 dos cariotípicos de *E. freibergi* e de *E. petersi*, e entre o cromossomo 5 do cariotípico de *E. petersi* de Yasuní e o cromossomo 3 dos cariotípicos de *E. freibergi* e de *E.*

petersi de Puyo. Já que os dois tipos de DNAr 5S encontrados em *Engystomops* são relacionados àqueles de *Physalaemus* tanto quanto à composição nucleotídica quanto à localização cromossômica, é ainda possível inferir que a origem desses dois tipos de sequências tenha antecedido a divergência evolutiva desses gêneros.

Abstract

The *Engystomops* genus is widely distributed geographically and constitutes an interesting group for karyotypic studies. The taxonomic identifications of Amazonian populations of *Engystomops* is still controversial. Genetic and cytogenetic analyses suggest the existence of a complex of cryptic species and incipient speciation. The cytogenetic variations found among some populations prevent the recognition of chromosomal homeologies between the described karyotypes. A more detailed chromosomal characterization could help in the recognition of chromosome homeologies and, therefore, could contribute to the study of the processes involved in the divergence of these anurans. Since the 5S rDNA gene has been an important genetic and cytogenetic marker for evolutionary studies and even for the identification and comparison of species in diverse groups, in the present work the 5S rDNA of *Engystomops freibergi* and exemplars of *Engystomops petersi* from two Ecuadorian locations (Puyo and Yasuní) was studied. In all cases, two types of 5S rDNA, easily differed by size and composition of their non-transcribed spacer, were isolated. A putative promoting region of the 5S rRNA gene (ICR) was recognized in the two types of 5S rDNA sequences and the presence of possible additional regulatory sequences was discussed. In the *E. freibergi* karyotypes, the entire type I 5S rDNA repeating unit hybridized to the pericentromeric region of 3p, whereas the entire type II 5S rDNA repeating unit mapped to the distal region of 6q, suggesting a differential localization of these sequences. The type I NTS probe clearly detected the 3p pericentromeric region in the karyotypes of *E. freibergi* and *E. petersi* from Puyo and the 5p pericentromeric region in the karyotype of *E. petersi* from Yasuní, but no distal or interstitial signals were observed. Interestingly, this probe also detected many centromeric regions in the three karyotypes, suggesting the presence of a satellite DNA family derived from 5S rDNA. The type II NTS probe detected only distal 6q regions in the three karyotypes, corroborating the differential distribution of the two types of 5S rDNA. Because the 5S rDNA types found in *Engystomops* are related to those of *Physalaemus* with respect to their nucleotide sequences and chromosomal locations, their origin likely preceded the evolutionary divergence of these genera. In addition, our data indicated homeology between chromosome 5 in *E.*

petersi from Yasuní and chromosomes 3 in *E. freibergi* and *E. petersi* from Puyo. In addition, the chromosomal location of the type II 5S rDNA corroborates the hypothesis that the chromosomes 6 of *E. petersi* and *E. freibergi* are homeologous despite the great differences observed between the karyotypes of the Yasuní specimens and the others.

Introdução

A Classe Amphibia está dividida em três Ordens: Anura (sapos, rãs e pererecas), Gymnophiona (cobras-cegas) e Urodea (salamandras). A Ordem Anura abriga aproximadamente 90% da Classe Amphibia com 5966 espécies descritas, agrupadas em 49 famílias (Frost, 2011). Os anuros habitam todos os continentes, com exceção dos polos, da maioria das ilhas oceânicas e regiões desérticas. Cerca de um terço das espécies atualmente descritas se encontram na América do Sul, principalmente na Amazônia e nas florestas tropicais úmidas (Frost, 2011).

Os anuros apresentam características morfológicas bastante conservadas, o que torna difícil a utilização destes caracteres como ferramentas exclusivas para análises filogenéticas (Hillis, 1991). Portanto, existe uma necessidade cada vez maior de se aliarem novas ferramentas aos estudos morfológicos, em busca de novos caracteres que possam auxiliar em tais análises. Interessantes e diversos fenômenos cromossômicos têm sido descritos através de estudos citogenéticos em anuros, com a utilização das regiões organizadoras de nucléolo (NORs) (Quinderé *et al.*, 2009; Nascimento *et al.*, 2010; Cardozo *et al.*, 2011), bandas heterocromáticas (Schmid *et al.*, 2002, Veiga-Menoncello *et al.*, 2003; Busin *et al.*, 2006, Cuevas *et al.*, 2008), e utilização de fluorocromos (Kaiser *et al.*, 2003; Campos *et al.*, 2009; Targueta *et al.*, 2010). Os sítios de genes ribossomais 5S de anuros, no entanto, ainda têm sido pouco estudados e podem vir a representar importante ferramenta para estudos dessa natureza.

O gene ribosomal 5S como marcador cromossômico

Os RNA ribossomais são moléculas que constituem a estrutura básica das subunidades ribossomais maior e menor e são transcritos por duas famílias multigênicas distintas, sendo elas a família do DNA 45S, responsável pela síntese de RNA 18S, 5.8S e 28S, e a família do DNA 5S, responsável pela transcrição do RNA 5S (ver revisões de Long & Dawid, 1980; Drouin & de Sá, 1995; e Martins & Wasko, 2004).

Os genes ribossomais 5S são compostos por múltiplas cópias organizadas *in tandem* de regiões conservadas de 120 pb intercaladas por espaçadores não transcritos variáveis (NTS), os quais podem diferir tanto no comprimento quanto na sua composição nucleotídica (ver revisões de Gerbi, 1985; e Martins & Wasko, 2004).

Segmentos presentes na região transcrita dos genes ribossomais 5S têm importante função no controle transcracional desses genes. Tais segmentos são denominados box A, elemento intermediário (IE) e box C e juntos são conhecidos por região de controle interna (ICR: **I**nternal **C**ontrol **R**egion) do gene ribossomal 5S (Pieler *et al.*, 1987). Essas regiões são promotores ativos da transcrição do gene ribossomal 5S, sendo a box A uma sequência geral para a associação da RNA polimerase III, enquanto o elemento intermediário e a box C são sítios de interação com o fator de transcrição TFIIIA (Pieler *et al.*, 1987). Outra característica tipicamente encontrada nos genes 5S é a região terminadora poli-T, inicialmente reportada por Korn & Brown (1978).

Apesar da grande variação observada para o NTS do DNAr 5S, muitos estudos demonstraram que elementos conservados estão presentes no espaçador não transcrito e têm um importante papel na regulação da expressão do gene ribossomal 5S, tais como sequências TATA-like (para referências ver Martins & Wasko, 2004), sequências de oligonucleotídeos (Korn & Brown, 1978) e, em mamíferos, uma região denominada box D (Hallenberg & Frederiksen, 2001; Nederby-Nielsen *et al.*, 1993).

O DNAr 5S tem sido utilizado como marcador genético e citogenético para estudos evolutivos e na identificação e comparação de espécies, híbridos e identificação de linhagens (Vitelli *et al.*, 1982; Suzuki *et al.*, 1994; Martins & Galetti, 1999; Fernández-Tajes & Méndez, 2007; Ferreira *et al.*, 2007). A região de 120 pb é altamente conservada mesmo dentre espécies não-relacionadas, tornando possível o isolamento das unidades repetitivas do DNAr 5S de algumas espécies baseado nas sequências disponíveis para espécies não necessariamente relacionadas. Além da região conservada, o NTS também pode ser isolado para ser utilizado como marcador genético, uma vez que o NTS está flanqueado pelas cópias do RNAr 5S no arranjo do DNAr 5S.

Uma característica interessante do NTS de vertebrados é a presença de duas classes de sequências, um assunto extensamente estudado em peixes (revisão de Martins & Wasko,

2004; Pasolini *et al.*, 2006; Pinhal *et al.*, 2009). Em várias espécies de peixes (Martins & Galleti, 2001b) e também em alguns outros organismos, como plantas (Falistocco *et al.*, 2007), mamíferos (Hallenberg *et al.*, 1994, Frederiksen *et al.*, 1997) e nos anuros *Xenopus laevis* e *Xenopus borealis* (Brown & Sugimoto, 1973; Brownlee *et al.*, 1974; Carroll & Brown, 1976a, 1976b; Jacq *et al.*, 1977; Fedoroff & Brown, 1978; Miller *et al.*, 1978; Korn & Brown, 1978; Peterson *et al.* 1980) e *Physalaemus cuvieri* (Vitorazzi *et al.*, 2011), distintas classes de DNAr 5S foram descritas, diferenciadas principalmente pelo comprimento e composição do NTS.

Em espécies de peixe do gênero *Leporinus*, a análise conjunta da organização molecular das classes de DNAr 5S com sua localização cromossômica mostrou que classes distintas de DNAr 5S ocupavam diferentes sítios cromossômicos (Martins & Galleti, 2001a). Nesse estudo, os autores também comprovaram uma maior similaridade entre as unidades repetitivas que ocupavam sítios cromossômicos homeólogos em diferentes espécies do que entre aquelas encontradas em diferentes sítios da mesma espécie. Esse interessante fenômeno tem importantes implicações na análise evolutiva das espécies, pois permite o uso dessas diferentes classes de DNAr 5S para distinções de espécies relacionadas (exemplo em Pendas *et al.*, 1994) e também para inferências filogenéticas (Ferreira *et al.*, 2007).

Em anuros, a localização cromossônica de genes ribossomais 5S é conhecida para *Xenopus laevis*, *Xenopus borealis*, *Xenopus muelleri* (Harper *et al.*, 1983; Schmid *et al.*, 1987), para espécies das famílias Ascaphidae, Discoglossidae e Ranidae (Vitelli *et al.*, 1982), para *Physalaemus ephippifer* (Nascimento *et al.*, 2010) e *Physalaemus cuvieri* (Vitorazzi *et al.*, 2011). Em *X. laevis* e *X. muelleri*, as sequências de DNAr 5S estão distribuídas nas regiões teloméricas do braço longo da maioria dos cromossomos (Pardue 1973; Pardue *et al.*, 1975). Em quatro espécies de *Rana* (*Rana esculenta*, *Rana catesbeiana*, *Rana graeca*, *Rana temporaria*), o DNAr 5S foi localizado em um ou dois pares cromossômicos nos telômeros e próximo aos centrômeros (Vitelli *et al.*, 1982). É interessante notar que em Anura as sequências de DNAr 5S estão mais dispersas pelo genoma do que as sequências de DNAr 18S+28S. Enquanto em *Ascaphus truei*, *Alytes obstetricans*, *Bombina variegata* e *Discoglossus pictus*, por exemplo, as NORs estão

restritas a um único par cromossômico, as sequências de DNAr 5S estão localizadas em um a 6 pares cromossômicos. Curiosamente, o único sítio cromossômico de *D. pictus* em que os genes de RNAr 5S e os genes de RNAr 18S+28S estão co-localizados está no braço curto do cromossomo 7. Em *A. obstetricans*, um dos 4 sítios de DNAr 5S também está localizado próximo à NOR nos telômeros do par 10. Já no grupo *Physalaemus*, as NORs e o DNAr 5S ocupam diferentes sítios (Nascimento *et al.*, 2010). Um caso interessante neste grupo foi o descrito por Vitorazzi *et al.* (2011), ao estudarem uma sequência de DNA satélite de *P. cuvieri*. Os autores concluíram que a sequência satélite isolada era derivada do DNAr 5S e a mapearam na região centromérica de vários pares cromossômicos, enquanto os sítios de DNAr 5S ocorriam na região pericentromérica do cromossomo 3 (DNAr 5S do tipo I) e no braço longo do cromossomo 5 (DNAr 5S do tipo II). Dessa forma, o estudo dos genes ribossomais 5S pode representar uma valiosa ferramenta na caracterização cariotípica de anuros e, consequentemente, auxiliar no reconhecimento de homeologias interespécificas e também no estudo de variações intraespecíficas.

***Engystomops* da Amazônia – o objeto de estudo**

O gênero *Engystomops* ocorre do sul do México ao norte da América do Sul e é atualmente composto por oito espécies (para referências, ver Frost, 2011). Dentre elas, *Engystomops petersi* e *Engystomops freibergi* têm distribuição amazônica, estando *E. freibergi* distribuída ao sul dos rios Maranhão e Amazonas, enquanto *E. petersi* ocorre ao norte desses rios (Funk *et al.*, 2008). Muita variação genética (Funk *et al.*, 2007; Targueta *et al.*, 2010) e citogenética (Targueta *et al.*, 2010) foi observada dentre as populações amazônicas, indicando a possibilidade de que o grupo *Engystomops* seja composto por um complexo de espécies no qual haja a ocorrência de espécies crípticas. Além de *E. petersi* e *E. freibergi*, outras espécies crípticas parecem ocorrer na Amazônia, constituindo um complexo de espécies muito interessante para estudos sobre mecanismos de especiação.

A ampla distribuição geográfica desses anuros em uma região com muitos rios e elevações andinas permitiu que Funk *et al.* (2007) testassem a atuação de rios e do relevo

como barreiras ao fluxo gênico. Algum suporte foi obtido para a hipótese de barreira em relação ao rio Madre de Dios, mas poucas evidências corroboraram a hipótese de barreira relativa ao gradiente elevacional.

Baseados na análise do canto, Ron *et al.* (2006), Boul *et al.* (2004, 2007) e Guerra & Ron (2008) propuseram que mecanismos de seleção sexuais têm exercido importante papel na divergência e na especiação dos *Engystomops* da Amazônia. Diferentes tipos de canto permitem a identificação de populações de diferentes regiões da Amazônia. Uma interessante variação foi observada, por exemplo, entre espécimes de La Selva e Yasuní, duas populações que ocorrem em lados opostos do rio Napo no Equador (Boul & Ryan, 2004; Ron *et al.*, 2006; Boul *et al.*, 2007; Guerra & Ron, 2008). Em experimentos de cruzamento conduzidos no laboratório, as fêmeas de La Selva e Yasuní preferem cantos de machos da mesma população a cantos de machos de outras populações (Boul *et al.*, 2007). Também fêmeas de Puyo, outra localidade do Equador, não reconhecem o canto de machos de La Selva, embora os machos de ambas as populações tenham o canto classificado como simples. Entretanto, nenhuma preferência de cruzamento resultante da seleção de canto foi observada em experimentos realizados com fêmeas de Puyo, pois essas fêmeas não discriminaram entre cantos de machos locais e cantos de machos de Yasuní (Guerra & Ron, 2008).

Já que a seleção sexual não pode explicar todas as variações genéticas observadas, Guerra & Ron (2008) sugeriram também que fatores adicionais estejam envolvidos no isolamento de populações de *Engystomops*. Esses autores consideram que seleção contra hibridação (“reinforcement”) possa também exercer importante papel na especiação de *Engystomops*, favorecendo a divergência genética em características ligadas ao reconhecimento para acasalamento e levando ao isolamento reprodutivo.

A análise citogenética aliada a inferências filogenéticas baseadas em sequências de DNA de espécimes de *Engystomops* coletados nas proximidades da localidade-tipo de *E. petersi*, em duas outras localidades do Equador e em uma do Acre, no Brasil, levou Targueta *et al.* (2010) a sugerirem que as acentuadas variações cariotípicas interpopulacionais observadas possam estar relacionadas ao isolamento de algumas populações, sendo tais variações um dos prováveis fatores de “reinforcement”. Ainda

segundo esses autores, a ocorrência de anéis meióticos multivalentes descritos previamente para uma população do Brasil (Lourenço *et al.*, 2000) e a presença de heterozigotos em relação ao gene nuclear rodopsina, considerado bastante conservado evolutivamente, podem ser interpretados como indícios de eventual ocorrência de hibridação e introgessão entre os *Engystomops* amazônicos.

A variação citogenética descrita por Targueta *et al.* (2010), além de possibilitar a inferência da hipótese discutida no parágrafo anterior, mostrou também que as diferenças encontradas entre alguns dos cariótipos em questão são tão acentuadas que impedem o reconhecimento de homeologias cromossômicas. Isso pode ser notado especialmente quando o cariótipo de espécimes de Yasuní é comparado com os demais analisados por Targueta *et al.* (2010). Fica claro, portanto, que novos marcadores cromossômicos são necessários para uma melhor caracterização desses cariótipos e, consequentemente, para uma melhor inferência das homeologias cromossômicas e dos rearranjos responsáveis pelas diferenças hoje notadas.

Outro interessante dado citogenético relativo ao gênero *Engystomops* consiste na ocorrência de cromossomos sexuais heteromórficos em uma das populações de *E. petersi* analisada (população de Puyo, no Equador), enquanto em outras duas tal heteromorfismo não foi observado. Cromossomos sexuais heteromórficos semelhantes aos observados na população de Puyo de *E. petersi* foram também observados nas populações de *E. freibergi* do Acre (Targueta *et al.*, 2010). Segundo Funk *et al.* (2007) as populações de Puyo, Yasuní e La Selva estão mais próximas umas das outras do que as populações brasileiras do Acre. Dessa forma a presença de cromossomos sexuais seria uma condição plesiomórfica nos *Engystomops* (Targueta *et al.*, 2010). Porém, Targueta *et al.* (2010) ainda destacaram que, apesar da morfologia ser parecida, os cromossomos sexuais de Pyuo e Acre apresentam algumas diferenças, como no caso do cromossomo X dos espécimes brasileiros do Acre, em que é possível observar segmentos de heterocromatina intersticial que não é observada no cariótipo de Puyo, ou ainda a presença em alguns indivíduos do Acre de NOR terminal no braço longo do cromossomo Y. Dessa forma, levando-se em consideração essas diferenças e as análises filogenéticas, os autores sugerem ainda que a divergência desses cromossomos sexuais nas duas populações possa ter ocorrido mais de uma vez e de forma

independente. Pouco se sabe sobre os eventos que levaram a essa divergência dos cromossomos sexuais em *Engystomops* e um melhor entendimento das homeologias cromossômicas entre os cariótipos já descritos poderia auxiliar também na análise dessa questão.

Apesar dos problemas taxonômicos mencionados, todas as populações amazônicas de *Engystomops* são agrupadas em um mesmo clado, denominado Edentulus, que também inclui a espécie *E. pustulosus* (Ron *et al.*, 2006; Funk *et al.*, 2007; Targueta *et al.*, 2010). O clado Edentulus é grupo-irmão do clado Duovox, atualmente composto pelas espécies *Engystomops coloradorum*, *Engystomops guayaco*, *Engystomops montubio*, *Engystomops pustulatus*, *Engystomops puyango* e *Engystomops randi*, todas distribuídas em baixas altitudes a oeste da Cordilheira dos Andes, no Equador e no norte do Peru (Ron *et al.*, 2006; Ron *et al.*, 2010).

Dados publicados recentemente mostram que todas as espécies do clado Duovox apresentam $2n=20$ (Ron *et al.*, 2010, Targueta *et al.*, 2011). Considerando que todas as espécies já cariotipadas de *Physalaemus*, gênero considerado como grupo-irmão de *Engystomops*, apresentam $2n=22$, assim como as espécies do clado Edentulus (Morescalchi *et al.*, 1968; León, 1970; Targueta *et al.*, 2010), Targueta *et al* (2011) concluíram que a redução do número diploide de 22 para 20 representa uma sinapomorfia do clado Duovox.

Apesar dessa intrigante descoberta, com os dados disponíveis até o momento também não é possível inferir com segurança as homeologias cromossômicas entre os cariótipos com $2n=20$ e os demais já descritos para o gênero. Consequentemente, os possíveis rearranjos cromossômicos que levaram a essa variação cromossônica numérica em *Engystomops* ainda permanecem desconhecidos.

Justificativa

As interessantes variações cromossômicas já descritas para o gênero *Engystomops* fazem desse grupo monofilético um valioso modelo para estudos de evolução cromossônica. No entanto, muito acerca da evolução cromossônica nesse gênero ainda

não pode ser inferido, principalmente pela dificuldade no reconhecimento de homeologias cromossômicas entre os diferentes cariótipos encontrados. Considerando que os cariótipos mais bem estudados tiveram seus cromossomos caracterizados quanto ao tamanho, à morfologia e à localização de NORs e de bandas heterocromáticas DAPI-positivas e CMA-positivas (Targueta *et al.*, 2010; Targueta *et al.*, 2011), a localização de genes ribossomais 5S se apresenta como uma interessante estratégia de busca de novos marcadores cromossômicos no grupo.

Objetivos

Objetivos Gerais

Caracterizar as sequências de DNAr 5S de *E. freibergi* e *E. petersi* e mapeá-las nos cariótipos de diferentes populações dessas espécies, ampliando o conhecimento acerca dessa família gênica em anuros e fornecendo novos elementos para a análise da evolução cromossômica em *Engystomops*.

Objetivos Específicos

1. Isolar o gene ribossomal 5S de espécimes de *E. freibergi* provenientes do Acre (Brasil) e *E. petersi* provenientes de Yasuní e Puyo (Equador);
2. Analisar comparativamente as sequências isoladas das três populações em estudo;
3. Localizar *in situ* de sequências ribossomais 5S em cariótipos de espécies amazônicas de *Engystomops*.

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Molecular organization and chromosomal localization of 5S rDNA in Amazonian *Engystomops* (Anura, Leiuperidae)

Débora Silva Rodrigues^{1§}, Miryan Rivera², Luciana Bolsoni Lourenço¹

¹Department of Structural and Functional Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas, SP, 13083-863, Brazil

²Escuela de Ciencias Biológicas, Pontifícia Universidad Católica Del Ecuador, Quito, Ecuador

[§]Corresponding author

E-mail addresses:

DSR: ra05086095@gmail.com

MR: mriverai@puce.edu.ec

LBL: bolsoni@unicamp.br

Abstract

Background: For anurans, knowledge of 5S rDNA is scarce. For *Engystomops* species, chromosomal homeologies are difficult to recognize due to the high level of inter- and intraspecific cytogenetic variation. In an attempt to better compare the karyotypes of the Amazonian species *Engystomops freibergi* and *Engystomops petersi*, and to extend the knowledge of 5S rDNA organization in anurans, the 5S rDNA sequences of Amazonian *Engystomops* species were isolated, characterized, and mapped.

Results: Two types of 5S rDNA, which were readily differentiated by their NTS (non-transcribed spacer) sizes and compositions, were isolated from specimens of *E. freibergi* from Brazil and *E. petersi* from two Ecuadorian localities (Puyo and Yasuní). In the *E. freibergi* karyotypes, the entire type I 5S rDNA repeating unit hybridized to the pericentromeric region of 3p, whereas the entire type II 5S rDNA repeating unit mapped to the distal region of 6q, suggesting a differential localization of these sequences. The type I NTS probe clearly detected the 3p pericentromeric region in the karyotypes of *E. freibergi* and *E. petersi* from Puyo and the 5p pericentromeric region in the karyotype of *E. petersi* from Yasuní, but no distal or interstitial signals were observed. Interestingly, this probe also detected many centromeric regions in the three karyotypes, suggesting the presence of a satellite DNA family derived from 5S rDNA. The type II NTS probe detected only distal 6q regions in the three karyotypes, corroborating the differential distribution of the two types of 5S rDNA.

Conclusions: Because the 5S rDNA types found in *Engystomops* are related to those of *Physalaemus* with respect to their nucleotide sequences and chromosomal locations, their origin likely preceded the evolutionary divergence of these genera. In addition, our data indicated homeology between Chromosome 5 in *E. petersi* from Yasuní and Chromosomes 3 in *E. freibergi* and *E. petersi* from Puyo. In addition, the chromosomal location of the type II 5S rDNA corroborates the hypothesis that the Chromosomes 6 of *E. petersi* and *E. freibergi* are homeologous despite the great differences observed between the karyotypes of the Yasuní specimens and the others.

Key Words: 5S rDNA, non-transcribed spacer, chromosome, *Engystomops*

Background

Ribosomal RNAs (rRNA) are molecules that combine to form the basic structures of the small and large ribosomal subunits. rRNAs are transcribed by two distinct multigene families. The 45S rDNA family synthesizes the 18S, 5.8S and 28S rRNAs, and the 5S rDNA family transcribes the 5S rRNA (as reviewed in references [1-3]). The 5S ribosomal genes are found as conserved copies of 120-bp sequences arranged *in tandem* and interspersed with variable non-transcribed spacers (NTSs) that differ in length and nucleotide composition (see reviews in [3] and [4]). Within the conserved 120-bp sequence lies an internal control region (ICR) consisting of 3 characteristic regions: the A box, an intermediate element (IE), and the C box [5]. These regions act as promoters for transcription; the A box is a general binding sequence for RNA polymerase III, and the intermediate element and the C box are interaction sites for the transcription factor TFIIIA [5]. Another characteristic typically found in the presumed-functional 5S genes is a poly-T terminator region, as initially reported by Korn and Brown [6].

Despite the large variations observed for the NTSs found in 5S rDNA, several studies have reported that conserved elements are present in these regions and that they play an important role in the regulation of 5S rRNA gene expression, similar to TATA-like sequences [3], oligonucleotide sequences [6], and the D box found in mammals [7, 8]. It has also been reported that a C localized at position -1 of many previously described 5S rRNA gene sequences guarantees a correct and efficient transcription start [8].

The sequences of 5S rDNA have been used as genetic and cytogenetic markers in evolutionary studies and in the identification and comparison of species, hybrids and strains [9-13]. The 120-bp region is highly conserved even among unrelated species, making it possible to isolate the 5S rDNA repeats of several species based on the available sequence from another species that is not necessarily closely related. An interesting feature of the 5S rDNA NTS that is most likely widespread throughout all vertebrate groups is the presence of two differently sized sequences, a subject that has been extensively studied in fish (see reviews in [3,14,15]).

For anurans, knowledge combining the molecular organization of the 5S rDNA sequences and their chromosomal locations are known for *Xenopus laevis*, *X. borealis*, *X. muelleri* [20, 24], in families Ascaphidae, Discoglossidae e Ranidae [13, 24], *Physalaemus ephippifer* [22] and *Physalaemus cuvieri* [23]. In *Xenopus* species [21] and *P. cuvieri* [23], two types of 5S rDNA sequences were found and mapped to distinct chromosomal sites. In *Xenopus* species, the distinct types of 5S rDNA, named oocyte- and somatic types, show a differential pattern of gene expression [6, 16-20, 24-26]. In addition to these cases with combined molecular and chromosomal analyses, studies restricted to the nucleotide sequence of the transcribed region of the 5S rDNA have been reported for the anurans *Gastrotheca riobambae* [27], *Bufo americanus*, *Rana pipiens* and *Rana catesbeiana* [28].

Based on a genetic analysis of morphological data, the *Engystomops* genus was revalidated by Nascimento *et al.* [29] to allocate species previously grouped in the *Physalaemus pustulosus* group. Currently, nine species comprise the *Engystomops* genus (the source studies are cited in reference [30]), and some reproductive and genetic evidences suggest the occurrence of undescribed cryptic species among the Amazonian *Engystomops* [31-33]. The cytogenetic analyses of different populations of the Amazonian species *Engystomops petersi* and *Engystomops freibergi* revealed interesting inter- and intraspecific divergences, and the possible involvement of these cytogenetic variations in incipient speciation has been suggested [33-34]. Nevertheless, the lack of available cytogenetic markers prevents the proper identification of chromosomal homeologies in these populations, and consequently, the hypothesized rearrangements responsible for the karyotypic divergences remain unknown. In the present study, we isolated and characterized the 5S rDNA sequences of *E. freibergi* and two populations of *E. petersi* and mapped their chromosomal locations, extending the knowledge of 5S rDNA organization at the genomic and chromosomal levels in these amphibians. These data facilitate the identification of possible homeologous chromosomes among the *Engystomops*, serving as an important contribution for further studies on chromosomal divergence in anurans.

Results

5S ribosomal gene characterization and molecular analysis

PCR amplification of the segments containing the 5S rDNA from *E. freibergi* from Acre (ZUEC 9647), *Engystomops petersi* from Yasuní (QCAZ 34948), and *E. petersi* from Puyo (QCAZ 34937) using the primers 5S-A and 5S-B (Fig. 1) generated bands with fragments of approximately 750 and 200 bp in all experiments. The cloning of the *E. freibergi* sequences resulted in six recombinant colonies, three of which carried an insert of 201 bp, one with an insert of 764 bp, and two with inserts of 765 bp. All the cloned sequences contained a 118-bp region that corresponded to the coding sequence of the 5S ribosomal gene (Fig. 2a and b) and NTS regions of either 84 bp (5SACR 201-1 to 3) or approximately 647 bp (5SACR 764 and 5SACR765-1 to 2). A comparison of the 84-bp fragments revealed four base substitutions among the sequences. A comparison of the 647-bp fragments showed 3 base substitutions and a deletion-insertion at position 292 (Table I; Fig. 2a and b).

Three recombinant clones containing the 5S rDNA of *E. petersi* from Yasuní were recovered. The insert 5SYAS201 was shorter than the others (5SYAS766 and 5SYAS774) due to variations in their NTSs, which were 84 bp long in the insert 5SYAS201 and approximately 650 bp long in the others (Table I). When compared with the clone 5SYAS766, clone 5SYAS774 showed nine additional nucleotides in a microsatellite DNA region (indicated at positions 546 to 554 in Fig. 2b).

Nineteen recombinant clones containing the 5S rDNA of *E. petersi* from Puyo were obtained. Eleven had larger NTSs (649 bp in clone 5SPUY766 and 652 bp in clones 5SPUY769-1 to 10), whereas the remaining inserts had small NTSs of 84 bp (Fig. 2). The differences in the lengths of the larger NTS segments resulted from the variation of the number of repetitions of a microsatellite DNA sequence present in these regions (positions 552 to 554 in Fig. 2b). Thus, based on the NTSs, the sequences obtained can be classified into two types: type I 5S rDNA (with the small, 84-bp NTS) and type II 5S rDNA (with the large, ~650-bp NTS) (Table I). Interestingly, the presumed 5S rDNA coding region was

also characteristic for each of these types (see Table I), as could be inferred from the maximum likelihood analysis of this specific region, in which all the type I sequences were clustered together and apart from the type II sequences group (Fig. 3).

As expected, comparison of the 120 bp of the coding regions of the type I and type II 5S rDNA of *Engystomops* with those of the other anuran species and selected fish species available in GenBank revealed great similarity (Fig. 4). When the type I and type II sequences of *P. cuvieri* were excluded from the analysis, there was a higher similarity between the *Engystomops* type I 5S RNA gene and the remaining 5S sequences (88–90%) than between the latter and the *Engystomops* type II gene (82%). When the *Engystomops* and *P. cuvieri* 5S genes were compared, a higher similarity was observed among the sequences of the same type (Fig. 4).

A higher similarity was also observed between the *Engystomops* and the *P. cuvieri* type I NTS (97% -100%) than between the type II NTS of the *Engystomops* species (~650 bp) and that of *P. cuvieri* (~580 bp) (90% - 92%). Because the type I NTS sequences were the same in all the *Engystomops* analyzed, and the type II NTS sequences were quite similar (average pairwise similarity: 98%; Fig. 2), to better illustrate the comparison between the *Engystomops* and *P. cuvieri* NTSs, only the pairwise alignments between the sequences of single specimens of *E. petersi* from Puyo and of *P. cuvieri* are shown in Fig. 5. The three elements (A box, intermediate element and C box) of the 5S gene internal control region (ICR) were identified in the presumed coding regions of all the sequences isolated from the three species of *Engystomops* (Fig. 4). Interestingly, the control elements of the *Engystomops* type II sequences differed more from those available in GenBank than did the elements of the *Engystomops* type I sequences.

All sequences of the type I 5S rDNA contained the control element TATA-box in their NTS regions, which was located 25 bp upstream from the coding region. In addition, the type I sequences also had a TATA-like element located 13 nucleotides upstream from the TATA-box (Fig. 2a). In the NTS of the type II sequences, an element similar to the TATA-box motif was also detected, but it was very distant from the +1 position of the presumed coding region of the 5S gene, occupying positions -345 to -350 (Fig. 2b). Additionally, in the type II 5S rDNA sequences, were found two hexanucleotides (at

positions -53 to -48 and -21 to -16; Fig. 2b) resembling the regulatory hexanucleotides previously described for *Xenopus* [6].

Both the type I and II *Engystomops* 5S sequences showed a T-rich sequence containing 4 to 5 T-residues starting at position -119, which may correspond to the poly-T termination region of the 5S gene described in the literature [6, 35]. Interestingly, in addition to the poly-T region, the type I sequences showed a GAACAAA sequence very similar to the sequence GAAACAA, which is found downstream from the 5S rRNA gene in fish and has been suggested to act as a terminal region [3].

The consensus secondary structures for the presumed type I and type II 5S rRNA is shown in Figure 6. The secondary structure of all analyzed 5S rRNAs consists of five helices (I-V), two hairpin loops (C and E), two internal loops (B and D), and a hinge region (A), arranged into the three-helix junction.

Chromosome mapping of 5S rDNA

As expected, the karyotypes of the specimens of *E. freibergi* and *E. petersi* analyzed here were the same as those described previously by Targueta *et al.* [33]. Therefore, our study includes two of the three karyological groups recognized by Targueta *et al.* [33] among *E. petersi* specimens: the Puyo and Yasuní karyological groups. It is interesting to notice that the karyotypes of the specimens of *E. petersi* from Puyo are more similar to those of the specimens of *E. freibergi* than to the karyotype of *E. petersi* from Yasuní. To describe the mapping of the 5S rDNA sequences in these karyotypes, the previously proposed chromosome classification scheme [33] was used.

In the *E. freibergi* karyotype, a FISH probe containing the entire repeating unit of the type I 5S rDNA hybridized pericentromerically to the short arm of Chromosome 3 and also distally to the long arm of Chromosome 6 (Fig. 7a). When using the probe containing the entire repeating unit of the type II 5S rDNA, only the distal region of the long arm of Chromosome 6 was detected (Fig. 7b). Neither the site detected in Chromosome 3 nor the

site on Chromosome 6 coincided with any nucleolar organizer regions (NORs) reported by Targueta *et al.* [33] (Fig. 7).

Because these data suggested a differential localization of both types of 5S rDNA sequences in the *E. freibergi* karyotype, further analyses were performed using more specific probes that exclusively contained either the type I or type II NTS. In the karyotypes of *E. freibergi* and *E. petersi* from Puyo, which are morphologically very similar (for details, see [33]), the type I NTS probe detected the proximal region of the short arm of Chromosome 3 but did not detect a distal region of Chromosome 6. This probe also detected the centromeric region of several chromosomes (Fig. 8b). In contrast, the type II NTS probe detected only a distal site in the long arm of Chromosome 6 in the karyotypes of both *E. freibergi* (Fig. 8a) and *E. petersi* from Puyo (Fig. 8c). In the karyotype of *E. petersi* from Yasuní, the type I NTS probe detected the pericentromeric region of the short arm of Chromosome 5 and the centromeric regions of several chromosomes (Fig. 8d), whereas the type II NTS probe detected only a distal site on the long arm of Chromosome 6 (Fig. 8e).

Discussion

Molecular Organization of the 5S rDNA in *Engystomops*

This study verified the occurrence of two types of 5S rDNA in the genomes of the Amazonian species of *Engystomops*, a feature widely documented in many vertebrates, including fish [10-11; 36-42], the anurans *Xenopus laevis* and *Xenopus borealis* [6, 16-20, 43-44] chickens [45-47], and mammals [12, 48-49]. Similarly to the findings reported in these studies, the two types of 5S rDNA sequences found in *E. freibergi* and *E. petersi* varied slightly in their corresponding coding regions, with the main difference between them found in the NTS region, which varied in length (84 bp for the type I 5S rDNA and approximately 650 bp for the type II 5S rDNA) and nucleotide composition. These two types of 5S rDNA do not, however, appear to be related to the dual system observed in *Xenopus* (i.e., the oocyte and somatic types) [6, 13, 16-17, 19-21] because we found no

similarities between the NTSs of the oocyte- or somatic-type sequences to those in the present study.

Since Ohno's publication [50], the origin of genic variants has been attributed to events of sequence duplication followed by processes that result in the divergence of the duplicated sequences. This hypothesis has been corroborated by many studies (reviewed in references [51-55]) and may also explain the presence of two types of 5S rDNA. Gene duplication may result from unequal crossing over, retropositioning, or chromosomal (or genomic) duplication [51], and the outcomes of these events are quite different, including neofunctionalization, pseudogene origin or simple preservation of gene duplicates [50, 56-57].

Although our data for the Amazonian *Engystomops* do not allow us to elucidate the events involved in the origin of either type of the 5S rDNA, those events may have preceded the divergence of *Engystomops* and *Physalaemus*. Such an inference follows from the observation of higher nucleotide divergence between the sequences of the type I and type II 5S rDNAs of each species in these genera than among the sequences of the same type found in distinct species.

With respect to the functionality of the 5S rDNA sequences found in *Engystomops*, the analysis of the secondary structure of the presumed rRNAs shows that both types of sequences are consistent with the general eukaryotic 5S rRNA structure [58-59], suggesting that the type I and type II 5S rDNA sequences may have transcriptional potential. The functionality of the type I 5S rDNA sequences of *Engystomops* is corroborated by the recognition, in this type of sequences, of elements that are similar to those considered to be important for the transcriptional activity of 5S rRNA genes. Those type I 5S rDNA elements are: (i) sequences quite similar to the ICR elements; (ii) a T-rich region downstream from the presumed coding region; (iii) a TATA-box located 25 nucleotides upstream from the coding region; and (iv) the nucleotide C at position -1. In addition, in the type I 5S rDNA of *Engystomops*, the presence of a GAACAAA segment was noted, which is very similar to the sequence GAAACAA suggested to act as a terminal region of the 5S gene transcription in fish [3]. A region tentatively named the TATA-like region was also found in the *Engystomops* type I 5S rDNA, located 12 nucleotides upstream from the

TATA-box. Campo *et al.* [42] reported an additional TATA-like region in the NTS regions of the 5S rDNA sequences of the fishes *Merluccius merluccius*, *Merluccius senegalensis*, and *Merluccius capensis*, and suggested that this TATA-box may serve as a “backup”. The same hypothesis may be considered for the similar sequence found in the NTS of the type I 5S rDNA of *Engystomops*.

In contrast, some doubts remain about the transcriptional potential of the type II 5S rDNA sequences of *Engystomops*. In the type II repeats, the nucleotide at position -1 is a T and not a C; the ICR segment differed more from the ICRs of the other vertebrates used for comparison than the ICR of the type I repeats; and no TATA-box was found in the NTS. The only segment that resembles a TATA-like motif in the type II repeats of *Engystomops* was observed very distant from the region considered to be the coding region, approximately at position -420. However, a T-rich region downstream from the presumed coding region is also present in the *Engystomops* type II 5S rDNA.

It is also interesting to note that the results of previous experiments with *Xenopus* suggest that the oligonucleotides AGAACG and AAAAGT, located at positions -28 to -23 and -18 to -13, respectively, may be involved in the initiation of 5S rDNA transcription instead of a TATA-box [6]. In the type II 5S rDNA sequences of *Engystomops*, the hexanucleotides AGAACG and GCAAGT were found at positions -53 to -48 and -21 to -16, respectively. The similarity of these oligonucleotides to those described for *Xenopus*, despite the low similarity of the remaining NTS sequence, is an interesting issue to be considered in further analyses of the functionality of the *Engystomops* type II 5S rDNA.

Physical Mapping of the 5S rDNA in the *Engystomops* karyotypes X evolutionary diversification in 5S rDNA

The FISH assays suggest the existence of two sites of sequence accumulation of the 5S rDNA in the karyotypes of *E. freibergi* and *E. petersi*, one on 3p (*E. freibergi* and *E. petersi* from Puyo) or 5p (*E. petersi* from Yasuní) and another on 6q. The results of these

assays also suggest that the former site is exclusive to or preferentially constitutes type I sequences, whereas the latter, on 6q, is associated with type II sequences.

Ribosomal DNA repeating units are evolutionarily dynamic and appear to be able to spread throughout the genome, creating new rDNA loci [3, 60-62]. The presence of two distinct 5S rDNA sequence types organized in different chromosomal regions or even on different chromosomes has been described for several fish, *e.g.*, *Salmo solar* [36], *Oncorhynchus mykiss* [37], *Coregonus artedi*, *C. zenithicus* [63], and *Oreochromis niloticus* [40].

In anurans, the first chromosome mapping experiments for the 5S rRNA genes were conducted in *Xenopus laevis*. Using specific probes, Harper *et al.* [20] revealed a differential distribution of the two types of 5S rDNA in the *Xenopus* karyotypes, mapping the somatic-type 5S rDNA to the distal end of the long arm of Chromosome 9 in *Xenopus laevis* and *X. borealis*, and the oocyte-type to the distal ends of the majority of *Xenopus laevis* chromosomes. The authors also mapped a trace oocyte-type 5S rDNA in *X. laevis*, which is a minor class of the oocyte type, to the distal end of the long arm of Chromosome 13.

In addition to these data for the *Xenopus* karyotypes, only the 5S rDNA chromosomal sites were detected in the karyotypes of *Physalaemus ephippifer* [22] and *Physalaemus cuvieri* [23]. A probe containing the entire repeat of the type I 5S rDNA of *P. cuvieri* detected a pericentromeric region of the short arm of Chromosome 3 in both *Physalaemus* species karyotypes [22-23] whereas a probe with the entire repeat of the type II 5S rDNA of *P. cuvieri* preferentially detected a distal region of the long arm of Chromosome 6 [23]. Similarly to the above-mentioned cases, a differential localization of the two types of 5S rDNA found in the *Engystomops* species was observed in this study.

As mentioned above, taking into account the similarity of the sequences, probably the origin of the two types of 5S rDNA found in *Engystomops* and *Physalaemus* species preceded the divergence of these genera. Apparently, the origin of this dual-system involved translocation or transposition events that lead to the separation of two groups of 5S rDNA sequences, favoring the dominance of divergence forces over homogenization processes between these groups. On the other hand, the homogeneity of the 5S rDNA

repeats clustered in the same chromosomal site was maintained, what may be explained by concerted evolution. As a result of these processes, two distinct types of 5S rDNA sequences, occupying different chromosomal sites, have arisen. It is worth mentioning that purifying selection may also have been involved in this scenario. In addition to concerted evolution, purifying selection has been invoked to justify the homogenization in a gene family [64-65]. Since the comparison between both types of 5S rDNA sequences of *Engystomops* showed a higher variation between the presumed coding-regions than between their NTSs, it is likely that purifying selection has been acting over these coding-regions, avoiding high level of divergence.

Another intriguing finding of this study was the hybridization of the probe that corresponds to the type I NTS to the centromeric region of various chromosomes, sites which were not detected in this analysis by the probes that potentially contain the transcribed region of the 5S rDNA. A possible explanation for this result is that the sequences associated with the centromeric regions are segments of satellite DNA derived from the 5S rDNA, a phenomenon previously reported for the fish *Hoplias malabaricus* [66] and the frog *Physalaemus cuvieri* [23].

The 5S rDNA cluster has been reported to be linked to the major rDNA sequences [4, 36-37, 67-68, 70] but, in some cases, is localized to different chromosomes [22, 63, 69, 71-73]. The differential chromosomal localization of the 5S and 45S rDNAs is the prevalent condition, not only in the cited examples but also in other groups, including plants [74-77]. Similarly, the 5S rDNA clusters detected for *Engystomops* were not related to the major rDNA.

Several authors have previously discussed the prevalence of different chromosomal sites for the 45S and 5S rDNAs over their linkage in other organisms, and a probable explanation is intrinsically related to the repetitive nature of these sequences. Others have suggested that because *tandem* repeated sequences are frequently involved in events of unequal crossing-over and gene conversion, the separation of the two great families of ribosomal DNA at different chromosomal sites would avoid disruptive interference in its organization such as undesired rearrangements between the 45S and 5S arrays [3,11].

The 5S rDNA chromosomal sites unlinked to the NORs of the *Engystomops* species represent new cytogenetic markers to be considered for their karyotypic comparison. Based on classic cytogenetic techniques, CMA₃ and DAPI staining, and *in situ* localization of nucleolar rDNA, Targueta *et al.* [33] described the three *Engystomops* karyotypes of the present study and noted that recognizing chromosomal homeology was difficult, especially between the Yasuní karyotype and the other two. In addition, difficulties in differentiating the three very morphologically similar chromosome pairs (pairs 3, 6, and 8) in the Yasuní karyotype have been reported. In the present study, we were able to suggest a homeology between Chromosome 6 of the *E. petersi* karyotype from Yasuní and Chromosome 6 of *E. freibergi* and *E. petersi* from Puyo based on the mapping of the type II 5S rDNA sequences. Additionally, this chromosome site also constitutes a distinctive marker for Chromosome 6 in the karyotype of *E. petersi* from Yasuní, distinguishing it from Chromosome 8 and the NOR-bearing Chromosome 3. Finally, the mapping of the type I 5S rDNA sequence to Chromosome 5 of the specimens from Yasuní and Chromosome 3 of *E. freibergi* and of *E. petersi* from Puyo, which are similar chromosomes in size and morphology, may suggests that these chromosomes are homeologous.

In addition to the recognition of chromosomal homeologies among the *Engystomops* species, the 5S rDNA mapping performed here allows for a better cytogenetic comparison of *Engystomops* with its sister genus, *Physalaemus*. Chromosomes 3 of *P. cuvieri* [23] and *P. ephippifer* [22], bearing the type I 5S rDNA sequence, are morphologically similar to Chromosome 5 of *E. petersi* from Yasuní and Chromosome 3 of *E. freibergi* and *E. petersi* from Puyo. Therefore, the homeology of all these chromosomes can be strongly inferred. Similarly, we can deduce homeology among the metacentric Chromosome 6 of the *Engystomops* species and Chromosome 5 of *P. cuvieri*, which all carry the type II 5S rDNA sequences. Chromosome 5 or 6 of *P. ephippifer* likely also bears this type of sequence; however, the presence of this sequence has not been verified with a specific probe for type II 5S rDNA sequences [22]. These cytogenetic data suggest that the chromosomal sites of the 5S rDNA may be conserved in these leiuperid genera, which has not been observed for the NORs [22, 33, 79-84]. Therefore, the two 5S rDNA arrays appear to be independent units of evolution in *Engystomops* species, and further studies of their functionality and

their relation to a possible centromeric DNA satellite sequence are necessary to provide a better understanding of the evolution of these sequences.

Conclusions

In Amazonian *Engystomops*, two types of 5S rDNA were found and mapped to distinct chromosomal sites. Because these rDNA types are related to those found in *Physalaemus* with respect to their nucleotide sequences and chromosomal locations, their origin likely preceded the evolutionary divergence of these genera. In addition, our data revealed chromosomal homeologies among the three karyotypes of the Amazonian *Engystomops*, representing an important contribution for further studies of karyotype evolution in this genus.

Methods

Specimens

Specimens of *E. petersi* from Puyo (Provincia de Pastaza) and Estación Científica de Yasuní (Provincia de Olleana), located in the Ecuadorian Amazon, and specimens of *E. freibergi* from the Tejo estuary, state of Acre, Brazil were studied. The vouchers specimens are at the Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ), Quito, Ecuador or at the Museu de Zoologia “Prof. Adão José Cardoso” (ZUEC) at the Universidade Estadual de Campinas (UNICAMP), Campinas, state of São Paulo, Brazil.

Isolation and cloning of the 5S gene

Genomic DNA was isolated from liver and muscle samples stored at -80°C in the tissue collection deposited at the Laboratório de Estudos Cromossômicos em Anuros at IB-UNICAMP, Brazil. The genomic DNA isolation was performed as described [33], with a TNES solution (250 mM Tris-HCl pH 7.5, 2 M NaCl, 100 mM EDTA pH 8.0, and 2.5% SDS). After electrophoresis in 1% agarose, the DNA quality was evaluated, and its quantity was estimated. The entire repeating unit of the 5S rDNA (which includes the presumed

transcribed region and the NTS) was isolated by PCR using the primers 5S-A (5'-TACGCCGATCTCGTCCGATC-3') and 5S-B (5'-CAGGCTGGTATGCCGTAAAGC-3') [36]. The amplified fragments were purified using the GFX PCR and Gel Band DNA Purification kit (GE Healthcare - Little Chalfont, Buckinghamshire, UK) and cloned into the pGEM-T vector (pGEM-T easy Vector – Promega - Madison, WI, USA) according to the manufacturer's instructions. Recombinant vectors were used to transform competent *Escherichia coli* of the JM109 strain (Fermentas), and the cloned fragments were sequenced. Repeating units of two different sizes were obtained and named type I 5S rDNA and type II 5S rDNA. Based on their nucleotide sequences, specific primers were designed to exclusively isolate the presumed 120-bp transcribed region from the type I 5S rDNA (5S120T1-F: 5'-GCTTCGCTTACGGCCATACC-3'; 5S120T1-R: 5'-AGCTTACAGCACCTGGTATT-3') and the type II 5S rDNA (5S120T2-F: 5'-GTCTCTGCTTACGGCCATACC-3'; 5S120T2-R: 5'-AGTCTACAGCACCCGCGCTTC-3'). Specific primers were also designed to isolate the NTS region from the type I (5ST1-F: 5'-GCTGTAAGCTTTGTTTGAA-3'; 5ST1-R: 5'-GAAAGCTCAGGGCCTGTACAG-3') and type II (5ST2-F: 5'-GCTGTAGACCGTTATTTACCTT-3'; 5ST2-R: 5'-AGAGACAGGCCTCTCACTTGC-3') 5S rDNA repeats. The annealing sites of the primers used in this study are indicated in Figure 1. The resulting amplified fragments were all sequenced and analyzed.

DNA sequencing and analysis

The cloned fragments were amplified by PCR using the T7 and SP6 primers. The amplified products were purified using the GFX PCR and Gel Band DNA Purification kit (GE Healthcare - Little Chalfont, Buckinghamshire, UK) and used directly as templates in amplification reactions using the BigDye Terminator chemistry version 3.1 (Applied Biosystems - Austin, TX, USA) according to the manufacturer's recommendations. Each cloned fragment was bi-directionally sequenced in an automatic DNA sequencer. The sequences were edited using BioEdit version 7.0.1 [85] and aligned with ClustalW software. The fragments obtained were compared with other sequences available in the GenBank database [86] and in the 5S Ribosomal RNA Database [87]. All presumed coding

regions of the generated 5S rDNA sequences were assembled into a data matrix, and a Maximum Likelihood (ML) analysis was conducted using the PAUP* 4.0□10 software [88] with the evolution model K80, which was selected by Modeltest 3.7 [89] for this data set. Nodal support for the ML arrangement was assessed through a non-parametric bootstrap analysis [88], with a heuristic search based on 1,000 pseudoreplicates.

Chromosome preparations and Fluorescent *in situ* Hybridization (FISH):

Chromosome preparations were made from the intestine and testis cell suspensions obtained from the *E. freibergi* specimens ZUEC 14435, ZUEC 14439, ZUEC 14440, and ZUEC14458, and the *E. petersi* specimens QCAZ 34946, QCAZ 34947, QCAZ 34940, and QCAZ 34942. All of these cell suspensions were available at the collection of amphibian material deposited at the Laboratório de Estudos Cromossômicos em Anuros at IB-UNICAMP, Brazil, and were used previously by Targueta *et al.* [33] to describe the karyotypes of *E. freibergi* and *E. petersi*. The cell suspensions were spotted onto clean slides, and the chromosome preparations were hybridized with the 5S rDNA fragments isolated as described above and PCR-labeled with either biotin or digoxigenin. The hybridization was performed as described elsewhere [90]. The biotin-labeled probes were detected with an anti-biotin antibody (goat anti-biotin – Vector – Burlingame CA, USA), which is recognized by a FITC-conjugated secondary antibody (anti-goat IgG-FITC – Vector - Burlingame CA, USA). The digoxigenin-labeled probes were detected with an anti-digoxigenin antibody conjugated with rhodamine. The chromosomes were counterstained with DAPI (0.5 µg/mL).

List of abbreviations

rDNA: ribosomal DNA; NTS: non-transcribed spacer; DAPI: 4, 6-diamidino-2-phenylindole; 3p: short arm of Chromosome 3; 5p: short arm of Chromosome 5; 6q: long arm of Chromosome 6.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DSR acquired the data and drafted the manuscript. MR helped to collect the specimens and obtain the chromosome preparations and revised the manuscript. LBL designed and coordinated the study and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements and Funding

The authors gratefully acknowledge Ailín Blasco for helping in the field work and Shirlei M. Recco Pimentel for the constant encouragement and assistance in obtaining some tissue samples. This work was supported by the Brazilian agencies FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), and the Ecuadorian agency SENESCYT (Secretaría Nacional de Educación Superior, Ciencia, Tecnología y Innovación).

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Table 1. *Engystomops* type I and II 5S rDNA sequence data.

Identification name, specimen source, GenBank accession number and NTS size of all cloned 5S rDNA sequences of *Engystomops freibergi* and *Engystomops petersi*. ZUEC: Museu de Zoologia Prof. Adão José Cardoso, at the State University of Campinas, Brazil; QCAZ: Museo de Zoología de la Pontificia Universidad Católica del Ecuador.

Sequence name	Specimen source (Voucher number, species identification, and locality)	GenBank accession number	NTS size (bp)
5SACR201-1	ZUEC9651 – <i>E. freibergi</i> /Acre, Brazil	JF325868	84
5SACR201-2	ZUEC9651 – <i>E. freibergi</i> /Acre, Brazil	JF325869	84
5SACR201-3	ZUEC9651 – <i>E. freibergi</i> /Acre, Brazil	JF325870	84
5SYAS201	QCAZ34948 – <i>E. Petersi</i> /Yasuní, Ecuador	JF325859	84
5SPUY201-1	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325860	84
5SPUY201-2	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325861	84
5SPUY201-3	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325862	84
5SPUY201-4	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325863	84
5SPUY201-5	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325864	84
5SPUY201-6	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325865	84
5SPUY201-7	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325866	84
5SPUY201-8	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325867	84
5SACR764	ZUEC9647 – <i>E. freibergi</i> /Acre, Brazil	JF325843	647
5SACR765-1	ZUEC9647 – <i>E. freibergi</i> /Acre, Brazil	JF325844	648
5SACR765-2	ZUEC9647 – <i>E. freibergi</i> /Acre, Brazil	JF325845	648
5SYAS766	QCAZ34948 – <i>E. Petersi</i> /Yasuní, Ecuador	JF325846	649
5SYAS774	QCAZ34948 – <i>E. Petersi</i> /Yasuní, Ecuador	JF325847	658
5SPUY766	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325848	648
5SPUY769-1	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325849	651
5SPUY769-2	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325850	651
5SPUY769-3	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325851	651
5SPUY769-4	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325852	651
5SPUY769-5	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325853	651
5SPUY769-6	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325854	651
5SPUY769-7	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325855	651
5SPUY769-8	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador or	JF325856	651
5SPUY769-9	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325857	651
5SPUY769-10	QCAZ34937 – <i>E. petersi</i> /Puyo, Ecuador	JF325858	651

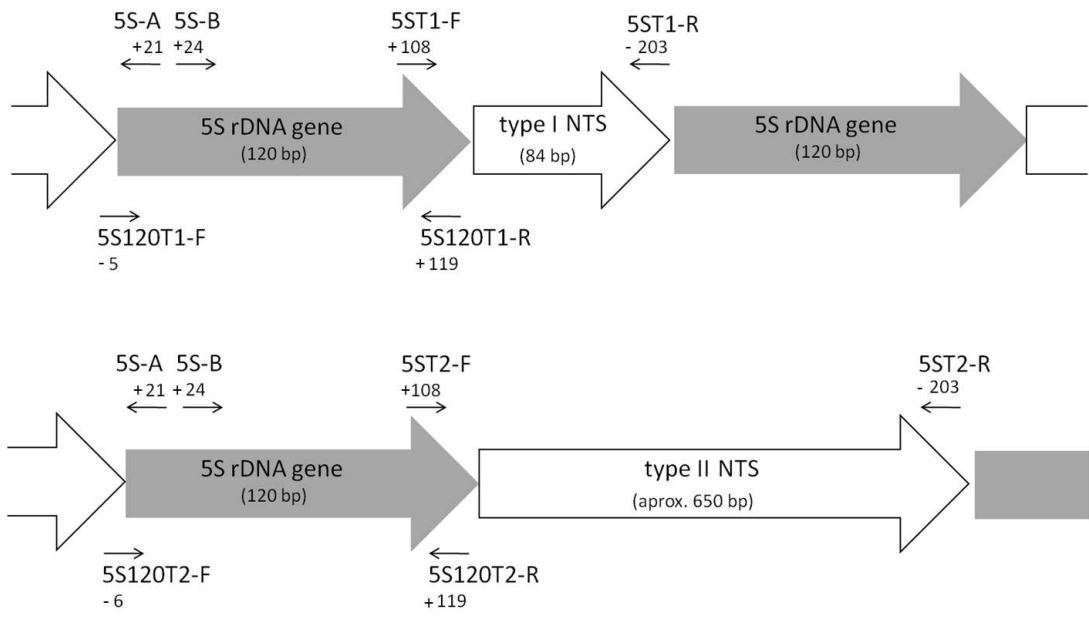


Figure 1: Annealing sites of the primers used for 5S rDNA analysis.

The primers 5S-A and 5S-B were described previously by Pendás et al. [36]. For the primers sequences, see Methods section.

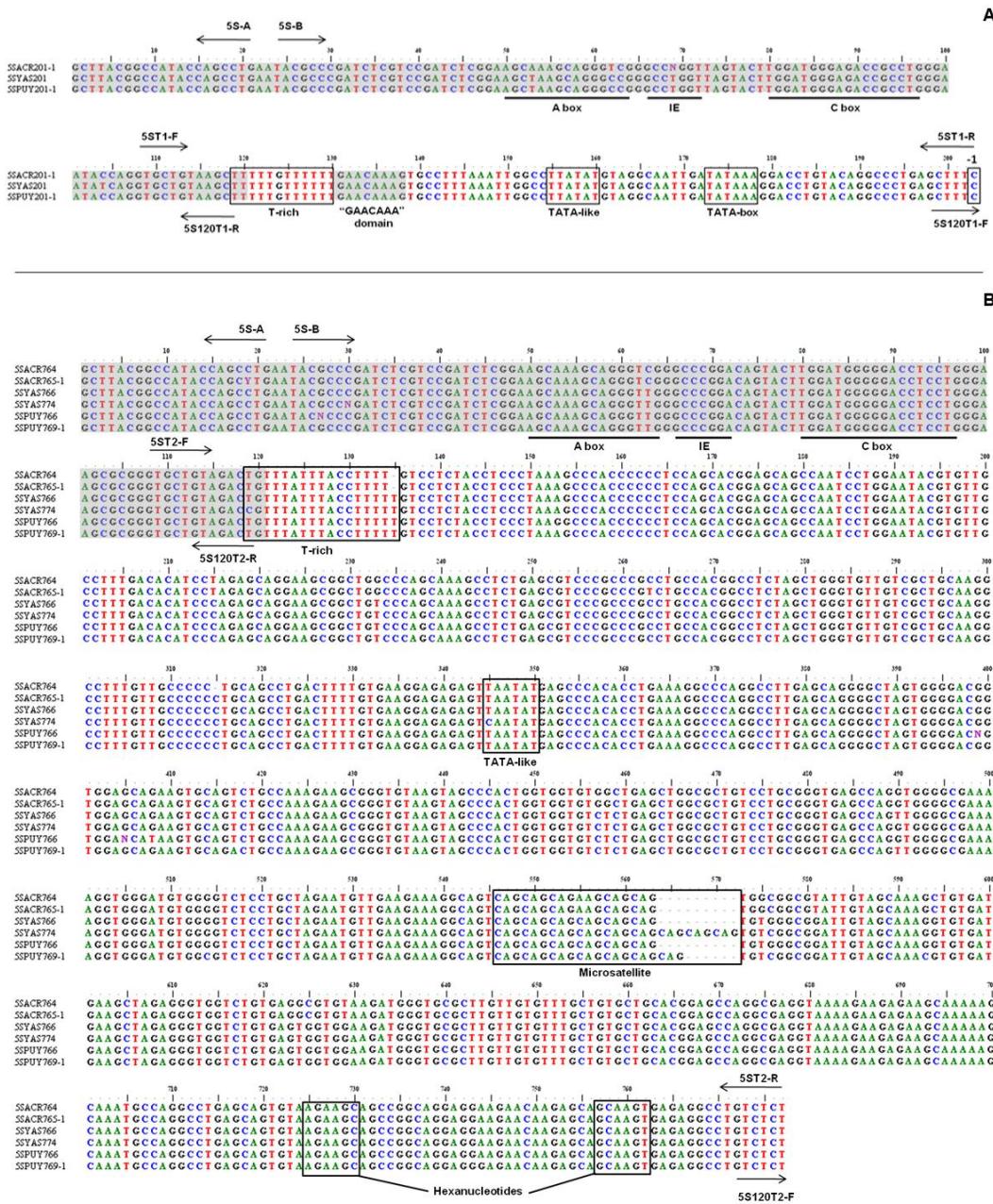


Figure 2. Aligned sequences of the cloned fragments of *Engystomops freibergi* (5SACR) and *Engystomops petersi* (5SYAS and 5SPUY).

A. Type I 5S rDNA sequences. B. Type II 5S rDNA sequences. The shaded areas indicate the presumed transcribing regions. The annealing sites of the primers used in this study are shown. Presumed regulatory elements are indicated (A box, IE, C box, poly-T, TATA box, TATA-like, hexanucleotides AGAACG and GCAAGT, and GAACAAA sequence). The arrow in A points to the C nucleotide at position -1 in all the type I sequences. A microsatellite observed in type II sequences is also indicated in B.

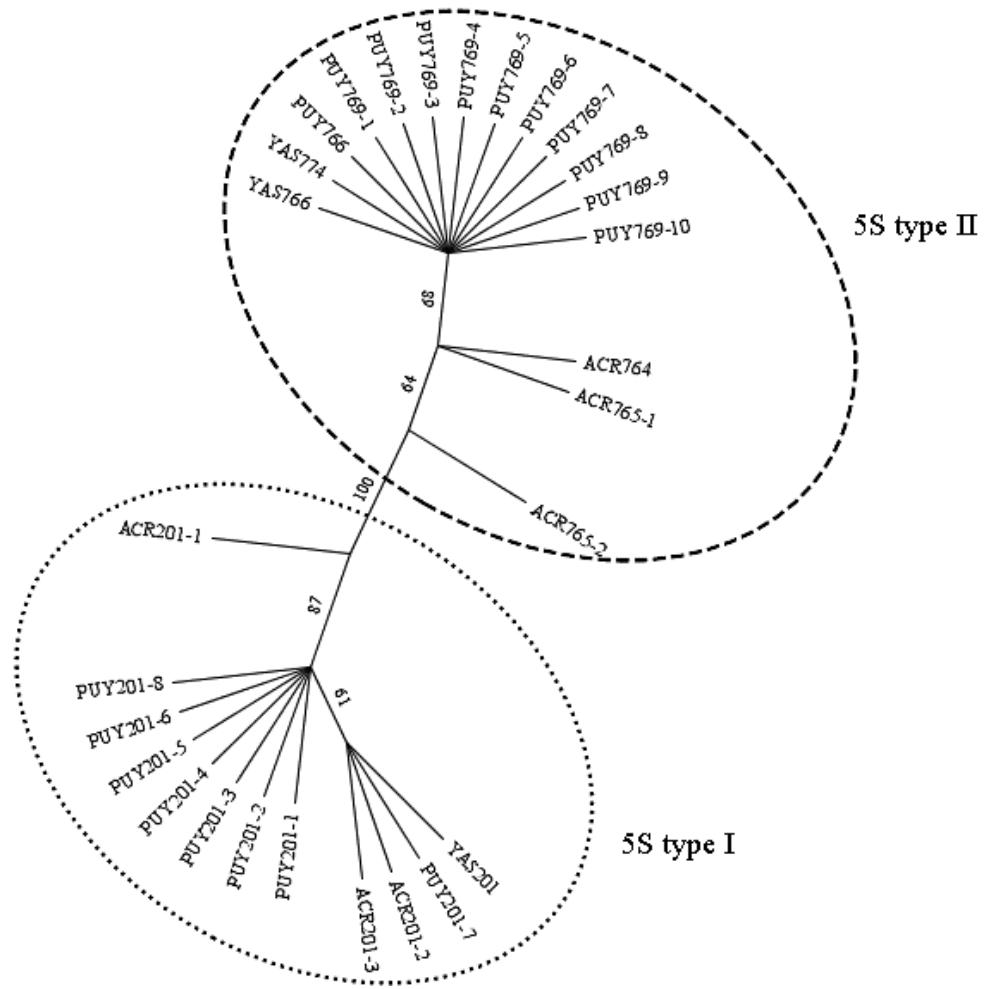


Figure 3. Maximum likelihood dendrogram inferred from the coding region of the 5S rDNA sequences of *Engystomops*.

The likelihood score was 265.2362. See Table 1 for a detailed description of the sequence symbols. The dotted circle groups the type I 5S rDNA and the dashed circle groups the type II 5S rDNA sequences. Numbers above branches are bootstrap values from 1000 pseudoreplicates. Bootstrap values under 0.5 were omitted.

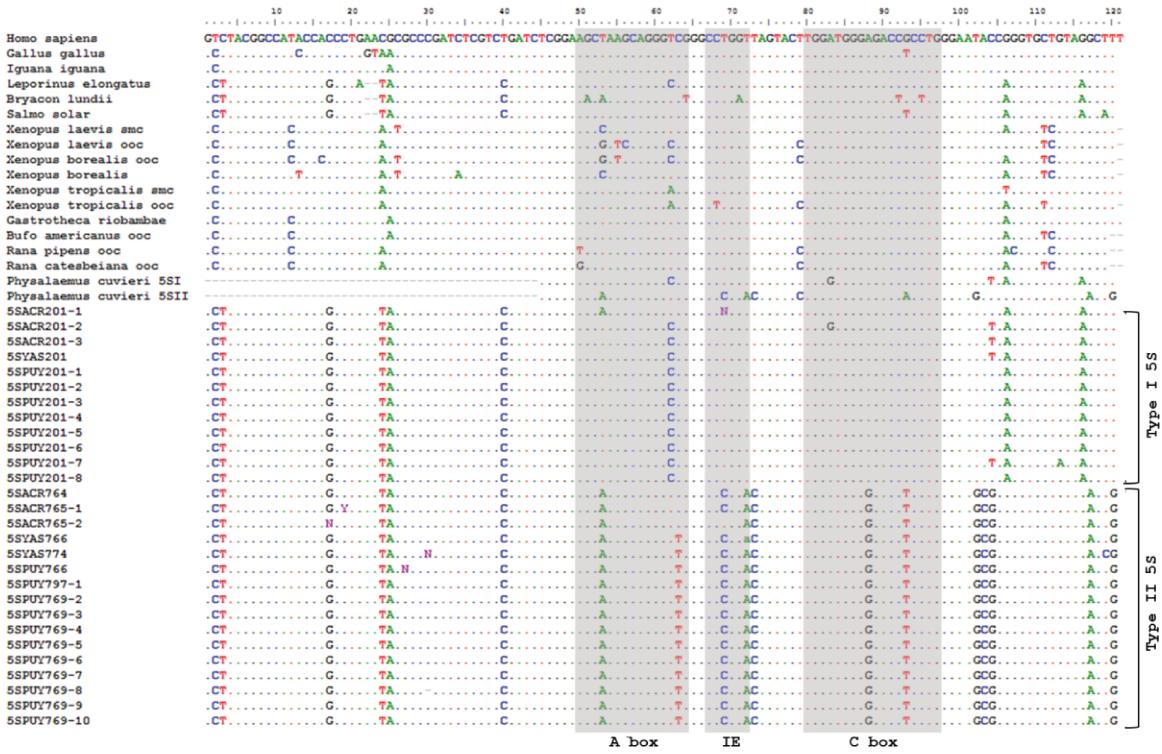


Figure 4. Comparison of the 5S rDNA sequences of *Engystomops* with 5S rDNA sequences available in the GenBank.

Alignment of the presumed coding regions of the type I and II 5S rDNA sequences of *Engystomops freibergi* (Acre) and *Engystomops petersi* (Yasuní and Puyo) with the 5S rDNA sequences of other vertebrates obtained from the GenBank (accession numbers: AF250511, AF284728, AF284742, AY271269, S73107, M24954, V00647, J01009, M35055, J01010, M30904, M35176, M63899, V01425, V01426, X12622, X12623, X12624, M74438, X58365, X58368, X58367, M10817, X01309, V00589). The internal control regions are in gray (A box = positions 50 to 64; intermediate element = positions 67 to 72; C box = position 80 to 97). Note that the control regions of the *Engystomops* type I 5S rDNA sequences are more similar to those found for the other vertebrate 5S rDNA sequences than are those of the *Engystomops* type II 5S rDNA. Ooc: oocyte-type. Smc: somatic-type.

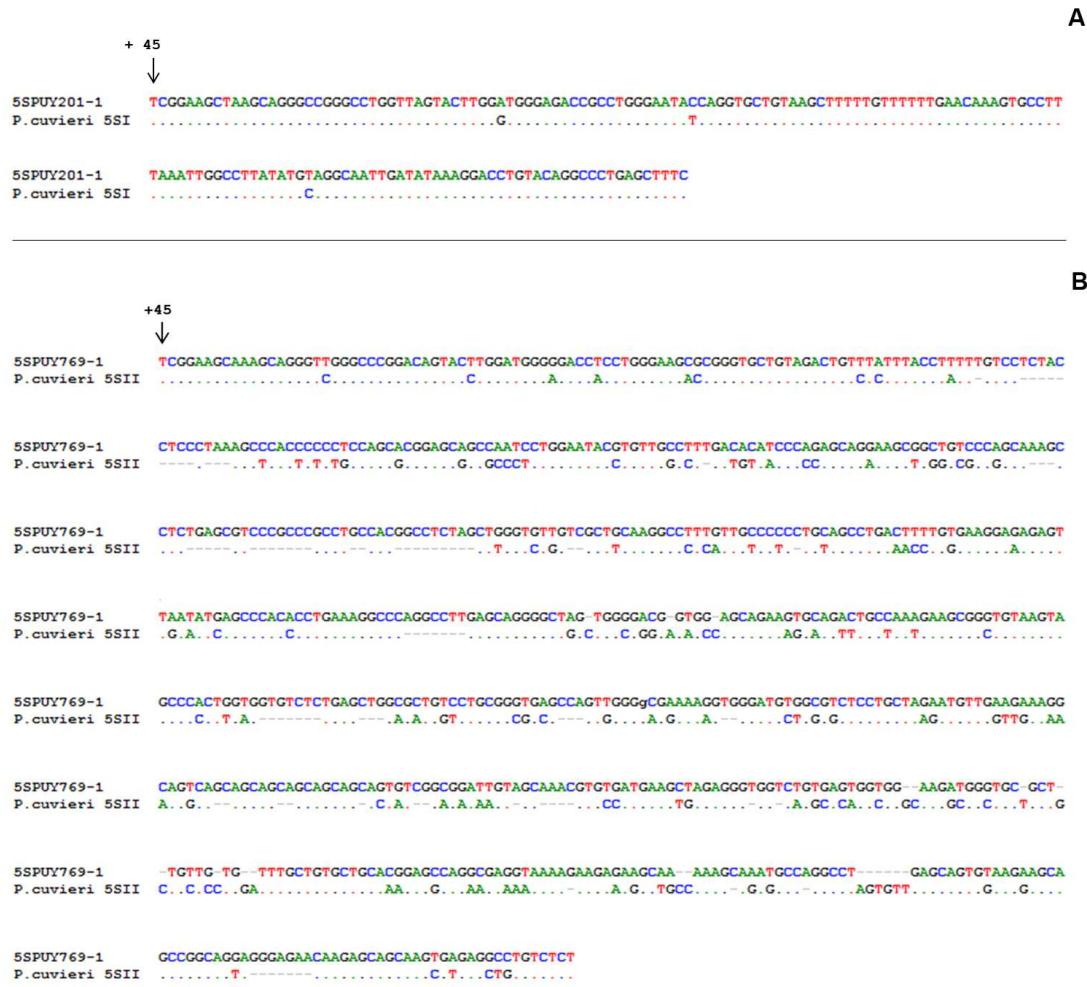


Figure 5. Comparison of the 5S rDNA of *Engystomops petersi* and *Physalaemus cuvieri*.
 Alignment of the type I (A) and II (B) 5S rDNA of *E. petersi* from Puyo (5SPUY769-1) and of *P. cuvieri* (accession numbers: JF281131 and JF281134).

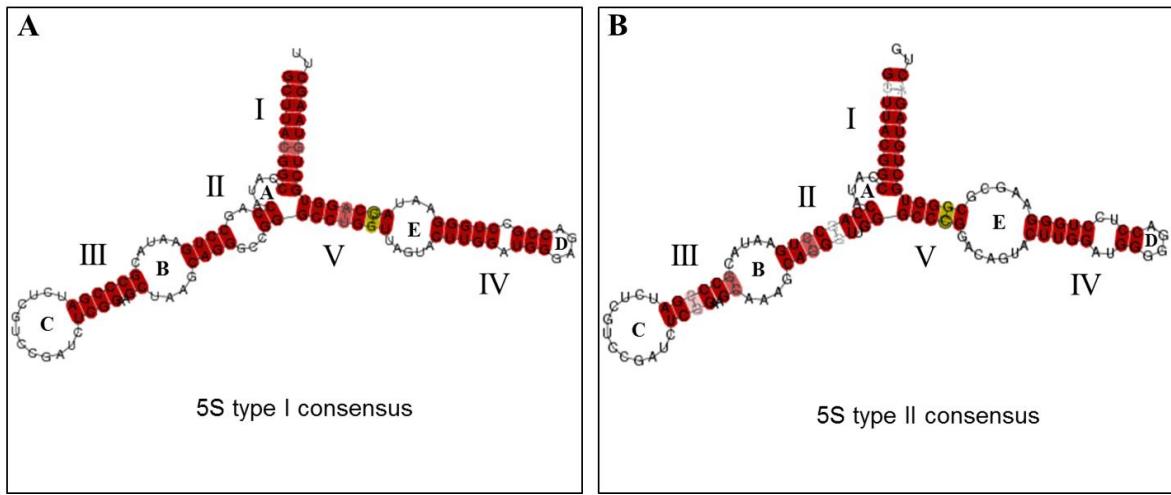


Figure 6: Prediction of the 5S rRNA secondary structure of *Engystomops*.

A. 5S rRNA of the type I consensus secondary structure. **B.** 5S rRNA of the type II consensus secondary structure.

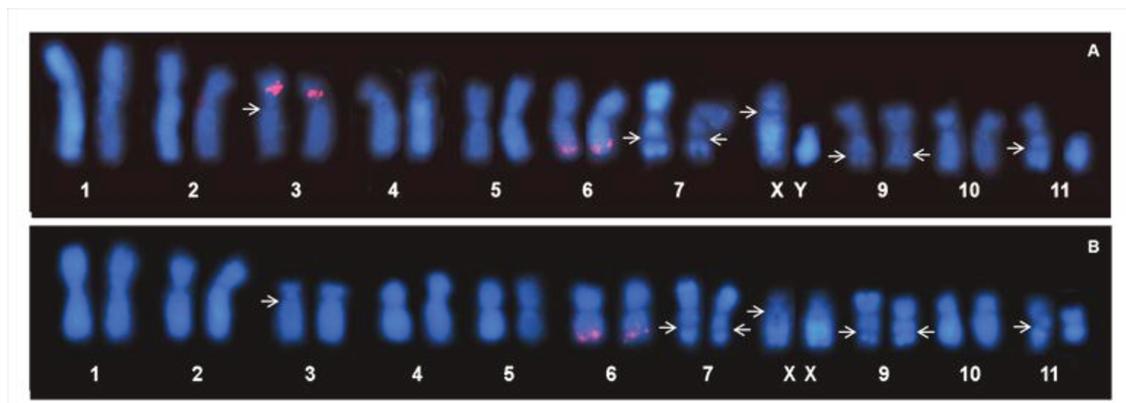


Figure 7. Chromosomal mapping of the 5S and nucleolar rDNA in *Engystomops freibergi*.
 Karyotype of *E. freibergi* hybridized with the probe containing the entire type I 5S rDNA repeating (A), and with the probe containing the entire type II 5S rDNA repeating (B). Arrows indicate the NORs as reported by Targueta *et al.* [33] for the same specimens (A: ZUEC 14440; B: ZUEC 14458). The morphological difference between the homologues of pair 11 in A, and B results from a C-band and NOR heteromorphism, not observed in the ZUEC 14435 female whose karyotype is shown in figure 7A (for details about this heteromorphism, see [34]).

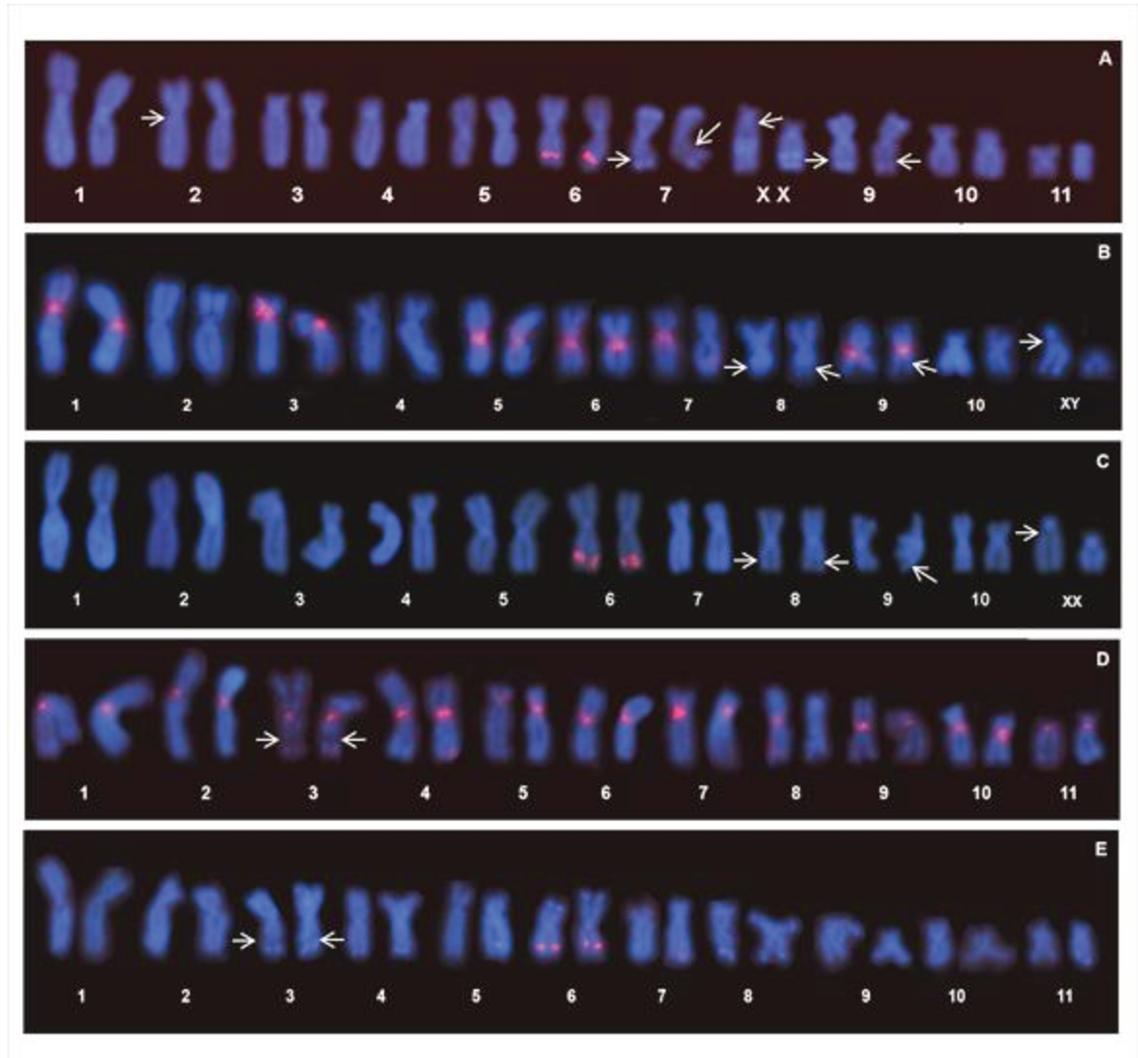


Figure 8. Chromosomal mapping of the type I and II NTS in *Engystomops freibergi* and *Engystomops petersi*.

Karyotypes of *E. freibergi* (A), *E. petersi* from Puyo (B-C), and *E. petersi* from Yasuní (D-E) hybridized with probes for type I (B,D) and type II (A,C,E) NTS. Arrows indicate secondary constrictions of the NORs. The morphological difference between the Chromosomes X in C is due to a heteromorphism of a terminal C-band (for details about this heteromorphism, see [33]).

Conclusões

- Duas classes de DNAr 5S, distintas principalmente pelo tamanho e composição nucleotídica do espaçador não-transcrito, estão presentes no genoma de *E. freibergi* e de *E. petersi* (Puyo e Yasuní).
- A região de 120 pb possivelmente codificadora de RNAr 5S presente no DNAr 5S do tipo II e, principalmente, aquela do DNAr 5S do tipo I de *Engystomops* são bem semelhantes a sequências já descritas para outros organismos.
- Sequências muito semelhantes a regiões consideradas regulatórias da transcrição de RNAr 5S foram encontradas no DNAr 5S do tipo I de *Engystomops* e sugerem sua atividade gênica. São elas: sequência rica em resíduos T na extremidade 3' da região codificadora, domínio “GAACAAA”, TATA-box, região TATA-like, região de controle interna (ICR), resíduo C na posição -1.
- No DNAr 5S do tipo II de *Engystomops*, também foi identificada uma região rica em resíduos T, uma região TATA-like e uma região muito similar à região de controle interna (ICR). Considerando que tais regiões diferem mais em relação às sequências consideradas consensos do que as encontradas no DNAr 5S do tipo I, surgiram dúvidas sobre a sua funcionalidade.
- A localização *in situ* das sequências do DNAr 5S do tipo I e do tipo II nos cariótipos das espécies em estudo possibilitou o reconhecimento de homeologias do cromossomo 3 de *E. freibergi* e de *E. petersi* (Puyo) com o cromossomo 5 de *E. petersi* (Yasuní), e também entre o cromossomo 6 dos três cariótipos.
- A sonda composta apenas pela região de NTS proveniente do DNAr 5S do tipo I detectou a região centromérica de vários cromossomos nos cariótipos de *E. freibergi* e de *E. petersi*. É possível que tais sondas tenham detectado sequências repetitivas derivadas de DNAr 5S, como aquela já descrita para uma espécie *Physalaemus*, gênero-irmão de *Engystomops*.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Dissertação de Mestrado** intitulada “Estudo da organização do gene ribossomal 5S em populações de *Engystomops* da Amazônia (Anura, Leiuperidae)”:

() não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

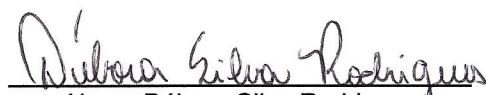
Tem autorização da(s) seguinte(s) Comissão(ões):

() CIBio - Comissão Interna de Biossegurança , projeto nº 2005/03, Instituição: Instituto de Biologia, UNICAMP.

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Prof. Dr. MARCELO LANCELLOTTI
Presidente da Comissão Interna de Biossegurança
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