Condecoración Eugenio Espejo

Postulante: Concejala Soledad Benítez

Postulado: Miryan Rosita Rivera Íñigüez

Fecha de postulación: 03/12/2019

Soledad Benítez Burgos
CONCEJALA
MUNICIPIO DEL DISTRITO METROPOLITANO DE QUITO

Oficio Nro. GADDMQ-DC-SB-2019-0114-O

Quito, D.M., 03 de diciembre de 2019

Asunto: Ordenanza No. 224 Condecoraciones, Premios y reconocimientos

Señor Doctor
Jorge Homero Yunda Machado
Alcalde del Distrito Metropolitano de Quito
GAD DEL DISTRITO METROPOLITANO DE QUITO
En su Despacho

De mi consideración:

Al amparo de las disposiciones establecidas en la Ordenanza N° 224 referente a las condecoraciones, premios y reconocimientos, pongo a su consideración y por su digno intermedio al Concejo Metropolitano de Quito, la postulación de la Magister Miryan Rosita Rivera Íñiguez para hacerse acreedora a la Condecoración"Eugenio Espejo" que se entrega a personalidades destacadas en las ciencias biológicas y naturales.

La Magister Rivera es profesora principal a tiempo completo en la Escuela de Ciencias Biológicas de la Pontificia Universidad Católica del Ecuador y una ilustre investigadora quiteña, fundadora y directora del Laboratorio de Investigaciones de Citogenética y Biomoléculas de Anfibios (LICBA) de la Pontificia Universidad Católica del Ecuador, laboratorio que en la actualidad forma parte del Centro de Investigación de la Salud para América Latina (CISAL. Su investigación de más de 20 años respecto a las potencialidades curativas de las secreciones de la piel de algunas especies de ranas nativas del Ecuador, ha logrado demostrar que se puede desarrollar medicinas para el tratamiento de enfermedades ocasionadas por bacterias y hongos así como también, para el tratamiento del cáncer, especialmente de algunos tipos de leucemias. Su contribución ha entregado a nuestro país y al mundo, una esperanza certera para enfrentar este flagelo de la humanidad minimizando los graves efectos colaterales que suelen ocasionar los tratamientos de quimioterapias.

La Magister Miryan Rivera ha presentado y socializado los resultados de la investigación que dirige, en varios foros internacionales y nacionales recogidos en varias publicaciones de revistas científicas ya sea como textos completos o sus resúmenes, junto a resultados de otras investigaciones de notables científicos de todo el mundo. Fue presidenta de la Sociedad Ecuatoriana de Biología, núcleo de pichincha; ha dirigido 30 tesis de pregrado y 4 de post grado, entre otros aportes y méritos que la caracterizan.

SECRETARIA CONCEJALIA
RECIBIDO: Orónica
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HORA: 16:18
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MUNICIPIO DEL DISTRITO METROPOLITANO DE QUITO

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Quito, D.M., 03 de diciembre de 2019

Por lo expuesto y como un acto de justo reconocimiento a las virtudes que adornan a la Magíster Miryan Rivera y a los aportes que ha hecho a la investigación científica en el campo de la biología aplicada a la medicina, me siento honrada al presentar su candidatura a la condecoración "Eugenio Espejo" que otorga el Municipio del Distrito Metropolitano de Quito a personalidades destacadas en las ciencias biológicas y naturales.

Adjunto documentación que sustenta mi pedido.

Atentamente,

Mgs. Cecilia Soledad Benitez Burgos
CONCEJALA METROPOLITANA

Anexos:
- hoja de vida MG. Mirian Rivera.pdf
- resumen ejecutivo de los principales proyectos.pdf
- publicacion 1.pdf
- publicacion 2.pdf

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TÍTULOS UNIVERSITARIOS:

Licenciada en Ciencias Biológicas. PUCE. 1989.

Especialista en Desarrollo del Pensamiento y Educación. PUCE-SI 2006.

Magíster en Desarrollo del Pensamiento y Educación. PUCE-SI. 2010

TRABAJOS DESEMPEÑADOS:

- Profesora Principal a Tiempo Completo e Investigadora en la Escuela de Ciencias Biológicas de la Pontificia Universidad Católica del Ecuador.
- **Directora** del Programa de Investigación: CARACTERIZACIÓN QUÍMICA Y CITOGENÉTICA DE ANFIBIOS ECUATORIANOS, dentro del cual se han desarrollado los siguientes Proyectos de Investigación:
  - "ACTIVIDAD ANTIMICROBIANA DE PÉPTIDOS SINTÉTICOS OBTENIDOS A PARTIR DE LA PIEL DE RANAS ECUATORIANAS (Proyecto vigente desde marzo de 2019)
  - ELUCIDACIÓN DE LA ESTRUCTURA PRIMARIA DE PÉPTIDOS ANTIMICROBIANOS DE LA PIEL DE *Agalychnis spurrelli* MEDIANTE CLONAJE MOLECULAR (En vigencia desde febrero del 2018
  - "EVALUACIÓN DE LA ACTIVIDAD ANTICANCERÍGENA DE LAS FRACCIONES PEPTÍDICAS PROVENIENTES DE LA SECRECIÓN CUTÁNEA DE *Agalychnis spurrelli* y *Phyllomedusa tomopterna* (ANURA: HYLIDAE) Y SU CARACTERIZACIÓN CITOGENÉTICA. En vigencia desde julio de 2016-2017.
  - "EVALUACIÓN DE LA ACTIVIDAD ANTIMICROBIANA Y ANTICANCERÍGENA DE SECRECIONES CUTÁNEAS DE ANFIBIOS ECUATORIANOS. En vigencia desde febrero de 2015.

- POTENCIAL MICROBIANO DE SECRECIONES PEPTÍDICAS DE ANFIBIOS EN BACTERIAS MULTIRRESISTENTES Y CEPAS TIPO. En vigencia desde febrero de 2015.

- "EFECTO DE PÉPTIDOS ANTIMICROBIANOS DE ANFIBIOS SOBRE LÍNEAS CELULARES CANCERÍGENAS". Proyecto financiado por la PUCE. En vigencia desde marzo del 2010.

- "CARACTERIZACIÓN CITOGENÉTICA DE VARIAS ESPECIES DE ANUROS ECUATORIANOS". Proyecto financiado por la PUCE. En vigencia desde marzo del 2010.

- "PURIFICACIÓN DE PÉPTIDOS ANTIMICROBIANOS DE SECRECIONES CUTÁNEAS DE *AGALYCHNIS SPURELLI* (ANURA: HYLIDAE)". Proyecto financiado con fondos SRI2009. En vigencia desde Julio del 2010.

- "EFECTO DE UN PÉPTIDO ANTIMICROBIANO EXTRAÍDO DE LA PIEL DE *Agalychnis litodryas,* SOBRE EL CRECIMIENTO DE HONGOS FITOPATÓGENOS Y CARACTERIZACIÓN CITOGENÉTICA DE VARIAS ESPECIES DE ANUROS". Proyecto financiado por la PUCE desde enero del 2008 hasta diciembre del 2009.

- "COMPUESTOS ANTIMICROBIALES EN *Agalychnis litodryas* (ANURA: HYLIDAE)" (Continuación), financiado por la PUCE. 2006-2007.

- "ESTUDIOS CITOGENÉTICOS DE VARIAS ESPECIES DE ANUROS ECUATORIANOS DE LAS FAMILIAS LEPTODACTYLIDAE Y DENDROBATIDAE", financiado por la PUCE, 2006-2008.

- "COMPUESTOS ANTIMICROBIALES EN *Agalychnis litodryas* (ANURA: HYLIDAE)", financiado por la PUCE. 2005-2006.

- "ESTUDIO DE CROMOSOMAS MITÓTICOS, RELACIONES FILOGENÉTICAS Y DETECCIÓN DE EPIBATIDINA EN ESPECIES DE DENDROBATIDOS ECUATORIANOS". Financiado por la PUCE, 2003-2006.

- "CARACTERIZACIÓN CITOGENÉTICA *Colostethus machalilla, Epipedobates boulengeri, E. tricolor, E. Spinosai* (ANURA: DENDROBATIDAE), financiado por la PUCE, 2002 – 2003.

- "RELACIONES FILOGENÉTICAS Y CLASIFICACIÓN DE RANAS DE LA FAMILIA DENDROBATIDAE (ANURA) BASADAS EN ANÁLISIS CITOGENÉTICOS Y MOLECULARES", financiado por la PUCE, 2001 – 2002.

- **Investigadora en los proyectos**:

  - ESTUDIO DE PÉPTIDOS DE INTERÉS BIOLÓGICO EXTRAÍDOS DE LA PIEL DE LA RANA *AGALYNCHNIS SPURRELLI* POR MÉTODOS EXPERIMENTALES Y COMPUTACIONALES

  - POTENCIAL ANTIMICROBIANO DE SECRECIONES DE PIEL DE ANUROS Y EXTRACTOS DE HONGOS ENDÓFITOS ECUATORIANOS

PRESENTES EN LAS COLECCIONES QUITO CATÓLICA SOBRE BACTERIAS TIPO Y MULTIRRESISTENTES. PROYECTO MERCEDES 2016.

- CARACTERIZACIÓN Y PURIFICACIÓN DE EXTRACTOS DE HONGOS ENDÓFITOS Y SECRECIONES DE ANFIBIOS PARA LA OBTENCIÓN DE MOLÉCULAS BIOACTIVAS. PROYECTO ALEX 2016

- POTENCIAL ANTIMICROBIANO DE SECRECIONES PEPTÍDICAS DE ANFIBIOS EN BACTERIAS MULTIRRESISTENTES Y CEPAS TIPO. PROYECTO MERCEDES 2015

- GENOTIPAJE DE LA RESISTENCIA A ANTIMICROBIANOS EN BACTERIAS MULTIRRESISTENTES Y SU POSIBLE INHIBICIÓN CON SECRECIONES PEPTÍDICAS DE ANFIBIOS CON POTENCIAL DE USO BIOMÉDICO. PROYECTO MERCEDES 2014

- "INVENTARIO Y CARACTERIZACIÓN GENÉTICA Y MORFOLÓGICA DE LA DIVERSIDAD DE ANFIBIOS, REPTILES Y AVES DE LOS ANDES DEL ECUADOR", financiado por la SENACYT, 2009-2011.

- "BIOCOMPUESTOS SECRETADOS POR LA PIEL DE ANUROS CON POTENCIAL DE USO BIOMÉDICO", financiado por el CONESUP, 2006 – 2008.

- "INVENTARIO Y CARACTERIZACIÓN DE LA FLORA Y FAUNA DE GAMBO ALLPA Y LA SUBCUENCA DEL RIO ITAMBI, auspiciado por INSTRUCT del Canadá (Inter-american Networking for Studies & Training in (Natural) Resource Usage for Comunity y Transformation) y el Departamento de Geografía de la PUCE, febrero del 2001 a noviembre del 2002.

- Profesora y Directora del Área de Ciencias Naturales en el Centro Pedagógico Lev Vygotsky, 1998 – 1999.

- Profesora de Ciencias Naturales en la UNEAL, 1997 – 1998.

- Profesora Interina a tiempo exclusivo e Investigadora en el Departamento de Ciencias Biológicas de la Pontificia Universidad Católica del Ecuador, septiembre de 1988 hasta julio de 1990.

- Profesora Accidental a tiempo exclusivo e Investigadora en el Departamento de Ciencias Biológicas de la Pontificia Universidad Católica del Ecuador, desde septiembre de 1988 hasta julio de 1990.

- Asistente de Investigación en el Proyecto "CITOTAXONOMÍA DE *Drosophila y Gryllus*" Proyecto auspiciado por el CONUEP y la PUCE, desde octubre de 1996 hasta septiembre de 1998.

- Becaria en el Laboratorio de Preparaciones del Departamento de Ciencias Biológicas de la Pontificia Universidad Católica del Ecuador.

International Congress of Genetics. Foz do Iguaçu. Brasil. Septiembre 10 – 14. 2018.

- Caracterización citogenética de *Teratohyla amelie* y *Teratohyla midas* (Anura: Centrolenidae). M.B. Trujillo, A. Blasco, **M. Rivera**. XLI Jornadas Nacionales de Biología. PUCE. Quito. Ecuador. 2017.

- Caracterización cromosómica de salamandras ecuatorianas del género *Bolitoglossa* (Caudata: Plethodontidae). Andrade Verónica, Blasco Ailín, Rivera Miryan. XLI Jornadas Nacionales de Biología. PUCE. Quito. Ecuador. 2017.

- Caracterización funcional de cinco péptidos de la piel de la rana *Agalychnis spurrelli* (Phyllomedusine: Hylidae). Miryan Rivera I., Ailín Blasco-Zúñiga y Carolina Proaño-Bolaños. Conferencia dictada en el XI Congreso Latinoamericano de Herpetología. PUCE. Ecuador. Julio 24-28 de 2017.

- Efecto del cautiverio sobre las bacterias benéficas de la piel de tres especies del género Atelopus (Anura: Bufonidae) Ailin A. Blasco, Sandra Victoria Flechas, Valeria Ramirez, Andres Merino-Viteri, Miryan Rivera, Adolfo Amézquita. Congreso Latinoamericano de herpetología. PUCE-Quito, Ecuador. Julio 24-28 de 2017.

- Estudios cromosómicos en nueve especies del género *Dendropsophus* (Anura, Hylidae). Acosta S, Blasco-Zuñiga A, Ferro JM, Cardozo D, Rivera M, Faivovich J, Baldo D. Póster presentado en el I Congreso Argentino-Paraguayo de Herpetología, 26-30 de septiembre de 2016.

- Análisis citogenéticos en trece especies de la familia Phyllomedusidae (Amphibia: Anura). Díaz Huesa EG, Cardozo D, Suárez P, Kolenc F, Borteiro C, Blasco-Zuñiga A, Rivera M, Berneck B, Faivovich J, Baldo D. Póster presentado en el I Congreso Argentino-Paraguayo de Herpetología, 26-30 de septiembre de 2016.

- Evaluación del efecto antimicótico de bacterias aisladas de la piel de anuros de zonas altas frente al hongo patógeno *Batrachochytrium dendrobatidis*. XL Jornadas Nacionales de Biología, 16-18 de noviembre de 2016.

- Comparación de la eficiencia de dos técnicas citogenéticas para la obtención de cromosomas mitóticos en salamandras. XL Jornadas Nacionales de Biología, 16-18 de noviembre de 2016.

- Evaluación de la actividad antimicrobiana de la secreción cutánea de siete especies de anuros ecuatorianos. XL Jornadas Nacionales de Biología, 16-18 de noviembre de 2016.

- Análisis ontogenético de la actividad antimicrobiana presente en la secreción cutánea de *Agalychnis spurrelli* (Anura: Hylidae). XL Jornadas Nacionales de Biología, 16-18 de noviembre de 2016.

- Expositor en el I Encuentro por el día de la Tierra. Junio 2016, PUCE.

- Efecto de glucocorticoides y estrés sobre la producción de péptidos antimicrobianos en *Agalychnis spurrelli* (Anura: Hylidae). **Conferencia** presentada en el 7º Congreso Brasileño de Herpetología, Gramado-Río Grande

del Sur-Brasil, 11 de septiembre de 2015.

- Potencial uso biomédico de secreciones cutáneas de anfibios. **Conferencia** presentada en el XVII Congreso Sudamericano de Farmacología y III Congreso de Químicos Farmacéuticos y Bioquímicos Farmacéuticos de Pichincha, Quito-Ecuador, 10 de octubre de 2014.

- Cromosomas y Biomoléculas de Anuros Ecuatorianos. **Conferencia** presentada en el Instituto de Biologia de la Universidade Estadual de Campinas (UNICAMP), Campinas-Sao Paulo,Brasil) el 6 de marzo de 2013.

- Andrea Vargas, Oscar Pérez, Iliana Alcocer, Jeannette Zurita, **Miryan Rivera.** Pruebas antifúngicas de secreciones cutáneas de *Agalychnis* sp. (Anura: Hylidae) en cinco especies de levaduras patógenas del género *Candida* y caracterización de las secreciones por medio de técnicas moleculares. **Conferencia** presentada en las XXXVI Jornadas Nacionales de Biología. Guayaquil-Ecuador. 22 de noviembre (2012).

- Andrade Verónica, Blasco-Zúñiga Ailín, Vásquez Elizabeth, **Rivera Miryan**. 2012. Resultados preliminares de los estudios cromosómicos de tres poblaciones de *Epipedobates anthonyi* y una de *Epipedobates tricolor* (ANURA: DENDROBATIDAE). **Póster** presentado en las XXXVI Jornadas Nacionales de Biología. Guayaquil-Ecuador. Noviembre (2012).

- García-Iturralde Santiago, Blasco-Zuñiga Ailín, **Rivera Miryan**. Análisis de los cromosomas mitóticos de 4 especies de ranas de la familia Centrolenidae. **Póster** presentado en las XXXVI Jornadas Nacionales de Biología. Guayaquil-Ecuador. Noviembre (2012).

- Vásquez Elizabeth, Blasco Ailín, **Rivera Miryan**. 2012. Análisis de los cromosomas mitóticos de dos especies de dendrobátidos (Anura: Dendrobatidae) presentes en la Estación Científica Yasuní. **Póster** presentado en las XXXVI Jornadas Nacionales de Biología. Guayaquil-Ecuador. Noviembre (2012).

- Boeris, JM; Blasco-Zuñiga, A; Martí, D; Suarez, P; **Rivera, M**; Baldo, D. Estudios citotaxonómicos en especies de la familia Ceratophryidae Tschudi 1838 (Anura, Neobatrachia). **Póster** presentado en el XIII Congreso Argentino de Herpetología. Mar del Plata – Argentina. Noviembre (2012).

- Barbero, G.A., Cardozo, D., Suarez, P., Nagamachi C., Pieczarka, J., Gomes, A., **Rivera, M.**, Blasco-Zuñiga, A; Faivovich, J., & Baldo, D. Estudios cromosómicos en 23 especies de Hypsiboas (COPHOMANTINI, HYLINAE, HYLIDAE). **Póster** presentado en el XIII Congreso Argentino de Herpetología. Mar del Plata – Argentina. Noviembre (2012).

- Piel de anfibios: Un verdadero arsenal químico con posibles aplicaciones biomédicas. **Conferencia** presentada en el curso de formación continua del Proyecto ALUMNI PUCE: "La biodiversidad una oportunidad para el desarrollo de proyectos sustentables", PUCE, Quito-Ecuador, Octubre (2012).

- Secreciones cutáneas de anfibios y su potencial uso antimicrobiano y anticancerígeno. **Conferencia** dictada en el Simposio Interdisciplinar de Investigación, Postgrados y Vinculación con la Colectividad. PUCE. Quito-

Ecuador. Septiembre (2012).

- Bruschi, D. P., Busin, C. S., Lima, A. P., **Rivera, M.**, Blasco-Zúñiga, A., Recco-Pimentel, S. M. Cytogenetic studies in *Phyllomedusa vaillantii* and *P. ayeaye* with descriptions of multiple NORs. **Póster** presentado en el 7th World Congress of Herpetology. Vancouver, Agosto (2012).

- Rodrigues, D. S., **Rivera, M.**, Lourenco, L. B. The use of the 5s ribosomal dna enables the recognition of chromosomal homeologies in the amazonian *Engystomops* karyotypes (ANURA, LEIUPERIDAE). In: 18th International Chromosome Conference - Delegate Book. Manchester-Reino Unido. 29 Agosto-1 Septiembre (2011).

- Vargas, A., Pérez, O., Ortega, D., Dután, L., Vargas D., **Rivera, M.** Caracterización de péptidos antimicrobianos de (*Agalychnis* sp.) por medio de técnicas moleculares. XXXV Jornadas Nacionales de Biología y I Congreso Ecuatoriano de Mastozoología. Quito. Ecuador. Noviembre, 2011.

- Ramírez G., Alcocer I., Zurita J. & Rivera M. Detección de fracciones peptídicas con actividad antimicrobiana extraídas de secreciones cutáneas de *Agalychnis* sp. (ANURA: HYLIDAE). XXXV Jornadas Nacionales de Biología y I Congreso Ecuatoriano de Mastozoología. Quito. Ecuador. Noviembre, 2011.

- Blasco-Zúñiga, A., García. S., **Rivera. M.**, Estudios cromosómicos de ranas de cristal ecuatorianas (ANURA: CENTROLENIDAE). XXXV Jornadas Nacionales de Biología y I Congreso Ecuatoriano de Mastozoología. Quito. Ecuador. Noviembre, 2011.

- Inventores ecuatorianos: sus experiencias en el ámbito de la propiedad industrial. **Ponencia** con la que participé en la Mesa Redonda que se desarrolló durante el Seminario Internacional: "La propiedad Intelectual en la Investigación, Innovación y Transferencia de Tecnología de la Universidades". Universidad Central del Ecuador. 16 de Marzo de 2011.

- Andrea P. Vargas, Carolina Proaño, Andrés Caicedo, Nory González, Gabriela Ramírez, Po Hao Chuang, Mercedes Rodríguez-Riglos, Jeannette Zurita, Iliana Alcocer, **Miryan Rivera**. ANTIMICROBIAL ACTIVITY OF SKIN SECRETIONS OF ECUADORIAN FROGS ON MULTI-RESISTANT BACTERIA AND FITOPATOGEN. **Póster** presentado en el 17º Congreso del Grupo Francés de Péptidos y Proteínas realizado en Aussois, Francia. Febrero 2011.

- Rodrigues, D.S., **Rivera, M.R.** & Lourenço, L.B. DNA RIBOSSOMAL 5S: UM NOVO MARCADOR PARA ESTUDOS CROMOSSÔMICOS DE *ENGYSTOMOPS* DA AMAZÔNIA. XI Congreso Argentino de Herpetología. Buenos Aires-Argentina. Octubre 2010.

- Rodrigues, D. S., **Rivera, M.**, Lourenco, L. B. LOCALIZAÇÃO CROMOSSÔMICA DIFERENCIAL DE SEQUÊNCIAS DE DNAR 5S DO TIPO I E DO TIPO II EM CARIÓTIPOS DE ANUROS DO GÊNERO ENGYSTOMOPS. In: Resumos do 56º Congresso Brasileiro de Genética. Sociedade Brasileira de Genética, p. 1-361. Guarujá – Sao Paulo - Brasil. Septiembre (2010).

- DESCRIPCIÓN CARIOTÍPICA DE DOS ESPECIES ECUATORIANAS DEL GÉNERO *Engystomops* (ANURA: LEIUPERIDAE). **Conferencia** presentada en

el Workshop – Anfíbios: Uma abordagem cromossômica e molecular. Campinas-Sao Paulo-Brasil, 7 de agosto de 2009.

- Participación en la **mesa redonda** "EVOLUÇÃO CROMOSSÔMICA EM LEIUPERIDAE E LEPTODACTYLIDAE", en el Workshop – Anfíbios: Uma abordagem cromossômica e molecular. Campinas-Sao Paulo-Brasil, 7 de agosto de 2009.

- COMPARATIVE KARYOLOGICAL ANALYSIS OF *EPIPEDOBATES* AND *HYLOXALUS* ECUADORIAN SPECIES (ANURA: Dendrobatidae). **Póster** presentado en el Primer Simposio Internacional PUCE-Fulbright. Enero de 2008. Quito - Ecuador.

- ANALYSIS OF THE PEPTIDES SECRETIONS OF ECUADORIANS AMPHIBIAN IN PATHOGENIC BACTERIA WITH SUSCEPTIBILITY TEST. **Póster** presentado en el Primer Simposio Internacional PUCE-Fulbright. Enero de 2008. Quito - Ecuador.

- Participación en la **mesa redonda: "**RELEVANCIA DE LOS BIOPOLÍMEROS EN AMÉRICA LATINA". 1st. International Conference BiopMat: Biopolymers: Sources, Transformation, Production an Innovating Applications. 7 Marzo de 2008. Veracruz – México.

- EFECTO DE BIOPOLÍMEROS SECRETADOS POR LA PIEL DE ANFIBIOS ECUATORIANOS SOBRE BACTERIAS PATÓGENAS. **Conferencia** dictada en: 1st. International Conference BiopMat: Biopolymers: Sources, Transformation, Production an Innovating Applications. 7 de marzo de 2008. Veracruz – México.

- Análisis citogenético del clado *Epipedobates* (ANURA: DENDROBATIDAE). Blasco, A. & **Rivera M.**, **Póster** presentado en VIII Congreso Latinoamericano de Herpetología. 8th Latin-American Congress of Herpetology. 24-29 de noviembre de 2008. Varadero, Cuba.

- Actividad biológica de las secreciones cutáneas de anfibios ecuatorianos. **Conferencia** presentada por una hora en la UDLA por petición del IEPI, Diciembre de 2008. Quito, Ecuador.

- Fluorochrome stainings show heterochromatin differences among karyotypes of three populations of *Engytomops petersi.* **Póster** presentado en el 6th World Congress of Herpetology, 19 de agosto, 2008, Manaos, Brasil.

- Chromosome characterization of nine ecuadorian poison frogs from *Epipedobates* and *Hyloxalus* genera. **Póster** presentado en el 6th World Congress of Herpetology, 22 de agosto, 2008, Manaos, Brasil.

- Análisis de las secreciones peptídicas de anfibios ecuatorianos en bacterias patógenas con pruebas de susceptibilidad. **Póster** presentado en el 1º

Encuentro Internacional en Biotecnología y 1ª Feria de Exhibición Biotecnológica. 10 – 14 de Marzo 2008. Medellín – Colombia.

- Análisis cariotípico de una especie nueva de anuro de la familia LEPTODACTYLIDAE., **conferencia** dictada en la XXIX Jornadas Ecuatorianas de Biología, Manta- Ecuador. 24 de noviembre de 2005.

- Estado de la investigación de anfibios en el ecuador. **Conferencia** dictada en el auditorio de la FUNDACYT. Quito-Ecuador. Abril, 2005.

- Métodos para estudios cariológicos. **Conferencia** dictada dentro del curso "Introducción a las Técnicas de Campo para el Estudio de los Mamíferos en Ecuador", Departamento de Ciencias Biológicas, PUCE, Quito-Ecuador. 22 de mayo del 2002.

- Inventario y caracterización de la flora y fauna de Huambo Allpa y la subcuenca del río Itambi. **Conferencia** dictada durante el Primer Encuentro Internacional, Intercultural para la Gestión Integral de Cuencas y Asentamientos Humanos: Imbakucha 2002, Otavalo, 28 de mayo del 2002.

- FLORA Y FAUNA DE HUAMBO ALLPA Y LA SUBCUENCA DEL RÍO ITAMBI, **conferencia** dictada ante el INSTRUCT del Canadá en Otavalo, noviembre del 2002.

- ANALISIS DE UNA GENOTECA DE EXPRESIÓN TESTICULAR HUMANA MEDIANTE ANTICUERPOS MONOCLONALES ANTI-ACROSINA HUMANA, presentado ante la Sociedad Ecuatoriana de Biología, Núcleo de Pichincha, 4 de enero de 1992.

- INTERACCIOINES GAMÉTICAS EN HUMANOS: ESTUDIO MEDIANTE ANTICUERPOS MONOCLONALES: Capote, C., Giacaman, A., Becker, M:I:, **Rivera M.**, Sepúlveda, M. S., y De Ioannes A., **Póster** presentado en la XIII Reunión Annual de la Sociedad Chilena de Inmunología, 14 de diciembre de 1990.

- ANALISIS CITOGENÉTICO DE LOS CROMOSOMAS MITÓTICOS DE *Drosophila guayllabambae* (sp.n.) y *D. hydei*, presentado en el Primer Congreso Nacional de Ciencias, 26 de marzo de 1987, Quito – Ecuador.

- ESTUDIO DE LOS CROMOSOMAS MITÓTICOS Y POLITÉNICOS DE *Drosophila guayllabambae* (spn:), X Jornadas Nacionales de Biología, 20 de noviembre de 1986, Guayaquil – Ecuador.

ASISTENCIA A CURSOS y CONFERENCIAS:

- Ciclo de Conferencias Amor Naturae 2018 organizadas por el Colegio de Ciencias Biológicas y Ambientales de la USFQ. Teatro Calderón de la Barca, USFQ y en el Auditorio del Hotel Reina Isabel. 21-22 de fefrero de 2018.

- Semimnario organizado por BIOMOL: Secuenciación Masiva NGS (Next-Generation-Sequencing). Hotel Crown Plaza.Quito-Ecuador. 6 de abril de 2017.

- Curso teórico: Parasitología de anfibios y reptiles: Métodos de colecta, análisis y estudios. Gramado-Río Grande del Sur-Brasil, 7 de noviembre de 2015.

- Curso teórico práctico de Proteómica y bases para la Espectrometría de masas. Guayaquil-Ecuador, del 1 al 4 de diciembre de 2014.

- Academic programs review workshop. Quito y Tena-Ecuador, del 24 al 26 de septiembre de 2014

- XVII Congreso Internacional de Farmacología y III Congreso de Químicos Farmacéuticos y Bioquímicos Farmacéuticos de Pichincha: "Las Ciencias Bioquímicas y Farmacéuticas al servicio de la salud y la vida". Quito-Ecuador, del 9 a 11 de octubre de 2014.

- Simposio: "Genética – Día del ADN, 28 de abril de 2014. Universidad de las Américas (UDLA)". Quito – Ecuador.

- Curso: "Bases y Aplicaciones de Bioinformática", del 16 al 20 de diciembre de 2013 (40 horas). PUCE. Quito – Ecuador.

- V Simposio Andino de Laboratorio y Medicina: "Los genes nos ponen a prueba". 6 y 7 de septiembre de 2013. Quito – Ecuador.

- Curso: "Introducción al PhotoShop" dictado por Verónica Yépez R, del 7 al 20 de marzo de 2012 (16 horas). PUCE.

- Curso: "Fundamentos de la PCR en Tiempo real y Aplicaciones en Biología y Medicina". Abril 2011. PUCE. Quito. Ecuador.

- Curso: Actualización en las aplicaciones de la Versión Windows 7 y Office 2010, dictado por el Ing. Darwin Jiménez, del 23 de mayo al 3 de junio de 2011 (20 horas). PUCE.

- Curso: "Planeación y evaluación efectivas de proyectos de investigación en biomedicina", dictado por la Mst. Rosa Chiriboga, el Lcdo. Esteban Báus y el Dr. Andrés Jaramillo, del 24 al 27 de abril del 2011 (32 horas). PUCE.

- 1º Seminario Taller sobre Propiedad Intelectual: "Protección y valoración de la propiedad intelectual en la investigación y transferencia".. 15 de enero de 2010 (20 HORAS). PUCE/IEPI.

- Curso: "Espectrometría de Masas (GC/MS) acoplado a Cromatografía de Gases y sus aplicaciones en análisis ambiental, de alimentos y forense", dictado por la Dra. Elena Stashenko, del 3 al 8 de abril del 2009 (30 horas). PUCE.

- Curso: "Aplicación de la Citometría de Flujo al Manejo de Recursos Genéticos", dictado por la Dra. Guadalupe Palomino (UNAM, México) del 15 al 18 de agosto de 2007 (20 horas). Universidad Nacional de Colombia. Palmira – Colombia.

- Curso: "Prácticas de Enseñanza en la Educación Superior. V Versión", dictado por la Mtr. Juliana Jaramillo Pabón, del 6 al 11 de Octubre de 2007 (20 horas). PUCE.

- Curso: "Uso práctico y creativo de las nuevas técnicas en el aula", dictado por el Ing. Pedro Ponce, del 13 de noviembre al 18 de diciembre de 2006 (40 horas). PUCE.

- Curso de capacitación "Herramientas de Productividad de Microsoft: Windows XP Professional y Office 2003" dictado en la PUCE. Enero-Febrero de 2005.

- Seminario – Taller "Lecto Escritura Avanzada" , dictado por el Dr. Alejandro De Zubiría, del 3 al 5 de marzo del 2004 (20 horas), Quito – Ecuador.

- Seminario – Taller "Didáctica y Evaluación Universitaria", dirigido por el Dr. Alejandro De Zubiría, del 12 al 13 de abril de 2002 (8 horas), Pontificia Universidad Católica del Ecuador, Quito – Ecuador.

- Seminario – Taller de "Refuerzo y Capacitación en Innovaciones Curriculares", del 07 al 11 de septiembre de 1998, Quito – Ecuador.

- Curso de Perfeccionamiento Docente Universitario II, del 14 al 16 de enero de 1994, Pontificia Universidad Católica del Ecuador, Quito - Ecuador.

- Curso de Perfeccionamiento Docente Universitario I, del 26 al 28 de noviembre de 1993, Pontificia Universidad Católica del Ecuador, Quito – Ecuador.

- Curso Teórico – Práctico de Ingeniería Genética, del 6 al 17 de septiembre de 1993 en Caracas –Venezuela.

- Curso Teórico – Práctico de Inmunología del Cáncer dictado por la Dra. Vivian Rumajanek del Instituto del Cáncer de Brasil, del 14 al 23 de septiembre de 1992, Quito –Ecuador.

- Curso de Adiestramiento en Biología de la Reproducción (Inmunología de la Reproducción), del **13 de agosto de 1990 al 13 de agosto de 1991, Santiago – Chile**.

- VIII Curso Iberoamericano de Biología Celular, dictado por el Dr. Gonzalo Giménez Martín, Dr. Julio Sánchez Rufas, Lcdo. Juan Giménez Avián y la Dra. Consuelo De La Torre, del 29 de noviembre al 13 de diciembre de 1988. Quito – Ecuador.

- Curso Teórico – Práctico de Bioquímica y Biología Molecular, dictado por el Dr. Horace B. Gray de la University of Houston, del 13 al 24 de junio de 1988.

- VII Curso Iberoamericano de Biología Celular, dictado por el Dr. Gonzalo Giménez Martín y la Dra. Consuelo De La Torre, del 14 al 28 de octubre de 1987, Santo Domingo – República Dominicana.

- Curso de Aislamiento e Identificación de Hongos, dictado Dr., John Hedger, del 13 al 22 de septiembre de 1985. Quito – Ecuador.

- Curso Teórico – Práctico de Biología Celular Proliferación Celular, dictados por el Dr. Gonzalo Giménez Martín y la Dra. Consuelo de la Torre, del 5 al 15 de diciembre de 1983. Quito – Ecuador.

- Ecología de Hongos. Quito – Ecuador del 16 al 18 de junio de 1983, dictado por el Dr. John Hedger del Universtity College of Wales, de Gran Bretaña.

ACTIVIDADES ADICIONALES

Presidenta de la Sociedad Ecuatoriana de Biología Núcleo de Pichincha 2002-2004, actual Secretaria de la misma organización y Tesorera de SEB Nacional.

Editora principal de la Revista de la Pontificia Universidad Católica del Ecuador Nº 71, Septiembre 2003, Quito - Ecuador

Vocal del Consejo de la Facultad de Ciencias Exactas y Naturales de la PUCE por tres períodos consecutivos y Vocal del Consejo de la Escuela de Ciencias Biológicas de la PUCE por cinco períodos consecutivos, hasta la presente fecha.

RESUMEN DE INVESTIGACIONES DE LA MASTER MIRYAN RIVERA IÑIGUEZ

La máster Miryan Rivera es fundadora y directora del Laboratorio de Investigaciones de Citogenética y Biomoléculas de Anfibios (LICBA) de la Pontificia Universidad Católica del Ecuador, laboratorio que en la actualidad forma parte del Centro de Investigación de la Salud para América Latina (CISeAL).

La máster Rivera es la mentora del programa de investigación: Caracterización química y citogenética de Anfibios del Ecuador, en el que se han desarrollado 25 proyectos centrados básicamente en dos importantes líneas de investigación:

1.- Búsqueda de moléculas provenientes de la piel de ranas ecuatorianas con potencial uso biomédico, con la finalidad de contribuir a dar solución a dos graves problemas de salud pública como son: La resistencia bacteriana y el cáncer.

2.- Descripción de los cromosomas mitóticos de anfibios del Ecuador con fines taxonómicos y filogenéticos, conducente a incrementar el conocimiento sobre la biología de este importante grupo de vertebrados.

El interés por estudiar las aplicaciones biomédicas de las secreciones cutáneas de ranas, surge por el **desenfrenado incremento de la resistencia que presentan microorganismos infecciosos frente a los antibióticos convencionales**. El creciente número de cepas de bacterianas multirresisstentes constituye un grave problema de salud pública a nivel mundial, pues los antibióticos que hasta hace poco salvavan vidas, hoy han perdido su efectividad, por lo que la opción para curar infecciones, ha disminuído drásticamente. De acuerdo a reportes emitidos por la BBC en base a informes emitidos por el gobierno británico, si no se encuentran nuevos antibióticos hasta el año 2050, se podría alcanzar en el mundo, 10 millones de muertes por año.

Ante esta alarmante realidad, en el LICBA se emprendió desde hace aproximadamente 10 años la búsqueda de nuevas drogas que constituyan alternativas para la terapia de infecciones. Una de las posibles fuentes de nuevas drogas es la piel de los anfibios ya que este importante grupo de vertebrados ha desarrollado, como parte de su sistema inmune innato, una serie de moléculas peptídicas para defenderse de microorganismos patógenos. Estas moléculas

pueden proveer las estructuras claves para el desarrollo de nuevos antimicrobianos.

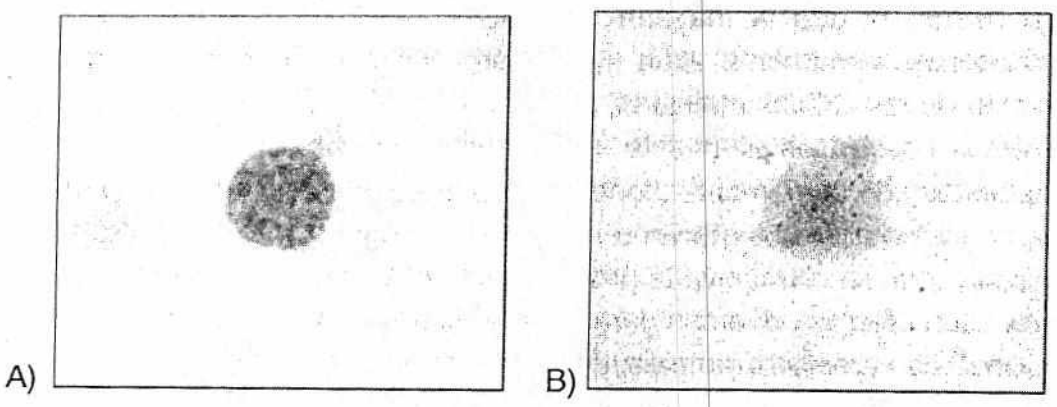
En efecto, el equipo liderado por la máster Miryan Rivera ha logrado determinar que la secreción de la piel de algunas ranitas ecuatorianas, son capaces de controlar el crecimiento de bacterias Gram positivas, Gram negativas pero sobre todo a bacterias mulrresistentes a antibióticos convencionales. En varios de los estudios realizados "*in vitro*", se ha podido constatar que cepas bacterianas que resultaron ser resistentes a 17 diferentes tipos de antibióticos de uso comercial, son altamente sensibles a la secreción de una ranita de la familia Hylidae. Este efecto antimicrobiano se probó también contra cinco especies de hongos del género *Candida*, causante de importantes infecciones nosocomiales. Y tal como se puede constatar en la siguiente imagen, éstos patógenos son incapaces de sobrevivir frente a la secreción cutánea de la ranita. En definitiva, se ha logrado probar *in vitro* la acción antibacteriana y antifúngica de la secreción de la piel de ranitas ecuatorianas.

La mencionada acción se atribuye a péptidos antimicrobianos producidos en glándulas cutáneas de estos anfibios y que actuarían lisando o perforando la membrana celular de los microorganismos por una interacción electrostática, pues son moléculas cargadas positivamente que interactuarían con las cargas negativas de la superficie de los patógenos, produciendo verdaderos poros, con la consecuente muerte del patógeno.

Tomando en cuenta que las células cancerosas presentan en su superficie celular una carga neta negativa, en el LICBA también se ha probado la actividad anticancerígena de los péptidos antimicrobianos, esperándose que, gracias a la carga positiva de los péptidos, se ocasione similar efecto sobre ellas.

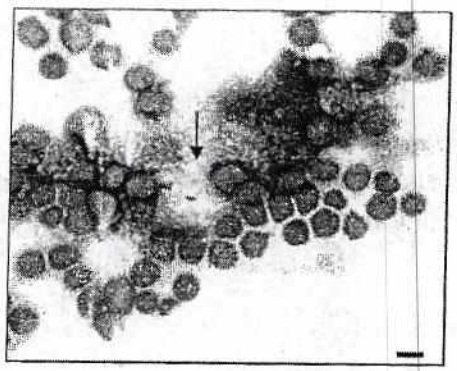
Se realizaron experimentos en los que se probó "*in vitro*"el efecto que la secreción de la piel de la ranita tiene sobre células malignas aisladas de pacientes

con diferentes tipos de leucemias, entre ellas: Leucemia linfocítica aguda (LLA), leucemia mieloide aguda (LMA) y leucemia mieloide crónica (LMC). Y tal como se puede constatar en la siguiente figura, el péptido antimicrobiano destruye totalmente a la célula leucémica.



Leucocitos de la leucemia linfocítica aguda, pre y pos tratamiento con secreción peptídica total de *Agalychnis spurrelli*. A. Célula sin tratamiento. **B.** Célula tratada con una dosis peptídica de 1000 µg/ml.

Reportes sobre el efecto cancerígeno de la secreción cutánea de anfibios se ha reportado ya en la literatura, pero lo interesante es que se pudo constatar que la secreción de esta ranita ecuatoriana, mató exclusivamente las células malignas dejando intactas a las células sanas.



Sangre de Leucemia Mielocítica Aguda, tratada con una dosis de 500 µg/ml. La flecha negra indica los restos de los blastos malignos lisados mientras que la flecha verde indica los eritrocitos intactos.

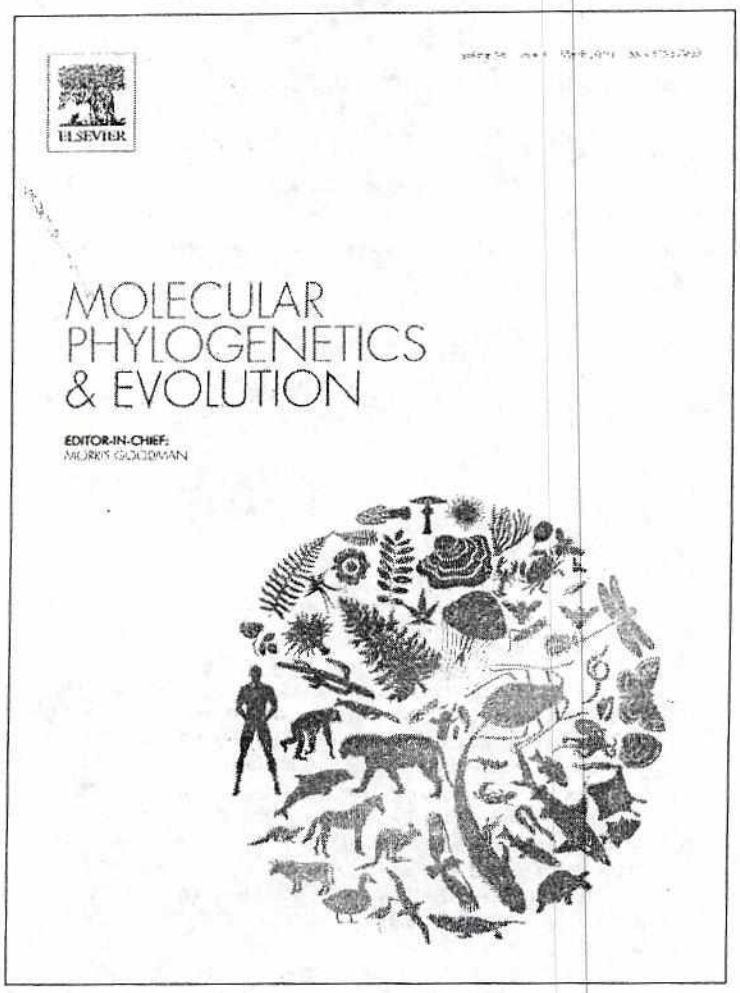
El tratamiento convencional más utilizado contra el cáncer es la quimioterapia, cuyo principal inconveniente es que, a largo plazo, las células

cancerígenas pueden desarrollar resistencia a las drogas terapéuticas. A esto se suma el hecho de que la mayoría de drogas usadas deben ser aplicadas en concentraciones sumamente altas lo que provoca que, además de controlar la proliferación de las células malignas, afecten a las células sanas, causando por lo tanto efectos colaterales altamente indeseables. Es por esto que los resultados aquí expuestos son sumamente alentadores porque al verificarse que la secreción cutánea de las ranitas solo atacan a las células malignas y no a las células sanas se esperaría que se disminuya la probabilidad de generar resistencia al péptido y que no causen efectos adversos para el organismo. Con estos resultados se abre la posibilidad de caracterizar moléculas que podrían dar solución al cáncer que es una de las principales causas de mortalidad prematura en la población humana mundial. Al momento ha identificado ya, no menos de una veintena de moléculas peptídicas efectivas en el control del crecimiento de células malignas de leucemias, sino también cáncer de pulmón, cerebro y melanoma metastásico (cáncer de piel).

La otra línea de investigación dearrollado por el equipo liderado por Miryan Rivera pretende contribuir al conocimiento de los anfibios desde un punto de vista citogenético, centrándose en el estudio de los cromosomas mitóticos de distintas especies de ranas y salamandras *Ecuatorianas*. La información que se está recopilan, está contribuyendo notoriamente a clarificar las controversiales relaciones filogenéticas y las posiciones taxonómicas de este grupo de vertebrados.

En el Ecuador, este tipo de estudios ha sido abordado exclusivamente en LICBA desde hace aproximadamente una década, y año tras año se incrementa el número de especies descritas cariotípicamente, a tal punto que hasta el momento se ha descrito la composición cromosómica de alrededor 30 especies de ranas ecuatorianas. Los resultados de estas investigaciones han sido publicados y presentados en prestigiosas revistas, congresos y jornadas científicas nacionales e internacionales. Los datos hasta aquí recopilados han motivado la revisión taxonómica de varias especies de anuros ecuatorianos, lo cual demuestra la trascendental importancia de este tipo de estudios, que merecen continuar desarrollándose.

EJEMPLO DE DOS PUBLICACIONES CIENTÍFICAS INDEXADAS



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Cytogenetic contributions for the study of the Amazonian *Engystomops* (Anura; Leiuperidae) assessed in the light of phylogenetic relationships

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ABSTRACT

Genetic divergence and speciation mechanisms of the Amazonian *Engystomops* frog have been inferred mainly from mtDNA sequences, microsatellite and male advertisement call. Although many aspects of this divergence remain unclear, cytogenetic analyses of Amazonian *Engystomops* populations are described and are compared to the relationships inferred from mitochondrial and nuclear DNA sequence data. High cytogenetic variation distinguished karyotypic patterns among the populations, even between populations which had no prezygotic isolation previously inferred from mating call analysis. Interestingly, the Puyo and Acre populations, which are in different clades, showed heteromorphic sex chromosomes (XX/XY), not identified in the other Ecuatorian populations analyzed. The analysis of a specimen collected in a site near Yasuní (Ecuador) which was cytogenetically related to the specimens from Puyo (Ecuador), was also phylogenetically closely related these specimens. In the rhodopsin nucleotide sequences, six polymorphic sites were identified and various specimens were heterozygous for them. All the data presented herein, in conjunction with those previously reported, corroborate the hypothesis that *Engystomops petersi* is a complex of distinct species. It also indicates that karyotypic evolution patterns may have played a substantial role in the *Engystomops* speciation and the occurrence of sporadic mating events between divergent evolutive lineages is discussed.

1. Introduction

The Amazonian *Engystomops* frogs are considered particularly interesting for speciation studies. Their geographical distribution extends from the Andean foothills of Ecuador, Peru, and Bolivia to the Colombian, Brazilian, and French Guiana Amazon basin (based on Funk et al., 2008; Frost, 2009). The wide geographical distribution of these mainland frogs 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 influential investigation, Funk et al. (2007) provided 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 populations in Ecuador and northern Peru composed one clade, referred as the 'northwestern clade'. The second clade, referred as the '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 which had been identified as *E. petersi*. In addition, Funk et al. (2007, 2008) suggested that *E. petersi* (named *Physalaemus petersi* by those authors) encompass the northwestern clade while the southwestern Amazon clade would be *E. freibergi* (termed *Physalaemus freibergi* by them) corroborating the proposal of Ron et al. (2006).

Based on analyses of the male advertisement call, Boul et al. (2007) and Guerra and 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 regions. For instance, an interesting variation was found between La Selva and Yasuní, two populations in opposing sides of the Napo River, in Ecuador (Boul and Ryan, 2004; Ron et al., 2006; Boul et al., 2007; Guerra and Ron, 2008). Females from La Selva and Yasuní prefer calls from males of the same population instead of calls from foreign populations in crossing experiments produced in laboratory setting according to Boul et al. (2007). However, mating preference driven 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 and Ron, 2008). Conversely, females from Puyo did not recognize the call from La Selva, although both populations had simple calls. In a study on the male advertisement call and gene flow assessed with

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microsatellite loci, Boul et al. (2007) proposed that sexual selection is driving behavioural isolation and speciation in the Amazonian *Engystomops*. In addition, Guerra and Ron (2008) suggested that additional factors are involved in the 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, leading to reproductive isolation.

Cytogenetic analyses in three *Engystomops* populations from the Acre State, in the Brazilian Amazon, revealed the sympathy 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 substantial progress that resulted from recent investigations, many cytogenetic, taxonomic and evolutionary aspects of the Amazonian *Engystomops* populations remain unclear. To contribute to the understanding of these anurans, this study describes 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 and Duellman, 1984). [illegible] also analyzed specimens from Yasuní and La Selva, two populations from Ecuador that show prezygotic isolation (Boul et al., 2007; Guerra and Ron, 2008). A population not yet analyzed from the state of Acre, in Brazil, located in the southwestern Amazonia, in the distribution area of *E. freibergi* according to Funk et al. (2008), was also studied. Since important cytogenetic variations were observed among specimens of the same or adjacent localities, we included the individuals analyzed cytogenetically in a phylogenetic study, together with those samples already used by Funk et al. (2007), to allow a proper evolutionary interpretation of the karyotypical data. The phylogenetic study provided here also includes a data set of nuclear genes, not used in previous inferences.

2. Materials and methods

2.1. 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 Y[illegible], Província of Orellana, Ecuador; one male ('Yasuní-km 20' specimen) sampled about 20 km 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, state of Acre, Brazil ('UFAC-Acre' 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. The voucher numbers of all these specimens are in Appendix 1.

2.2. Chromosome preparation

Chromosomes were obtained from metaphasic cells from intestines and testes of animals previously treated with colchicine, according to Schmid (1978) and Schmid et al. (1979) with a few modifications. Prior to intestine and testes removal, the animals were deeply anesthetized. Cell suspensions were dripped in clean [illegible] Giemsa 10%, silver stained by the Ag–NOR method (Howell and Black, 1980) and submitted to C-banding (King, 1980). As for heterochromatin characterization, the karyotypes were sequentially stained with two fluorochromes: 4′-6-diamidino-2-fenilidone (DAPI) and mytramycin (MM). First, several plates were 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, Bethesda, MD, USA). Chromosomes were ordered and classified according to Green and Sessions (1991).

2.3. Taxon sampling for molecular analysis

The sequences used in molecular analyses were obtained from some of the specimens analyzed cytogenetically and others collected at identical localities and from GenBank, as shown in Fig. 1 and Appendix 1. Specimens collected at Rio Tejo, Acre, Brazil, a population previously studied by Lourenço et al. (1998, 1999), were also included in the molecular analyses.

2.4. DNA extraction, amplification and sequencing

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 h 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 mitochondrial tRNA-Val, 12S and 16S ribosomal genes were 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 nuclear genes RAG-1 and rhodopsin were amplified using the primers RAG-1R and RAG-1F (Faivovich et al., 2005), and Rhod1A and Rhod1C (Bossuyt and Milinkovitch, 2000), respectively. The PCR amplified products were purified with a GFX PCR and Gel Band DNA Purification kit (GE Healthcare, England) and directly used as templates for sequencing in an automatic DNA sequencer ABI/Prism (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator kit (Applyed Biosystems, Foster City, CA, USA), as recommended by the manufacturer. DNA sequences were bi-directionally sequenced and edited using Bioedit version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using ClustalW.

2.5. Phylogenetic inferences from mitochondrial genes

Approximately 927 bp of the 12S ribosomal gene, 69 bp of the tRNA-Val gene and 1475 bp of the 16S ribosomal gene of 27 Amazonian *Engystomops* specimens were sequenced as described above. The concatenated sequences of the12S, tRNA-Val and 16S genes together with those already available in GenBank for Amazonian *Engystomops* and for *E. coloradorum*, *E. guayaco*, *E. montubio*, *E. pustulosus*, *E. randi*, *Engystomops* sp., *Engystomops* sp. 1, *Engystomops* sp. 2 were used to compose a data matrix with 117 OTUs. The GenBank accession numbers for all of the sequences used are presented in Appendix 1. To measure character congruence between the data sets of different genes, the partition homogeneity test (ILD) (Farris et al., 1995) was applied to the matrix, using PAUP [illegible]

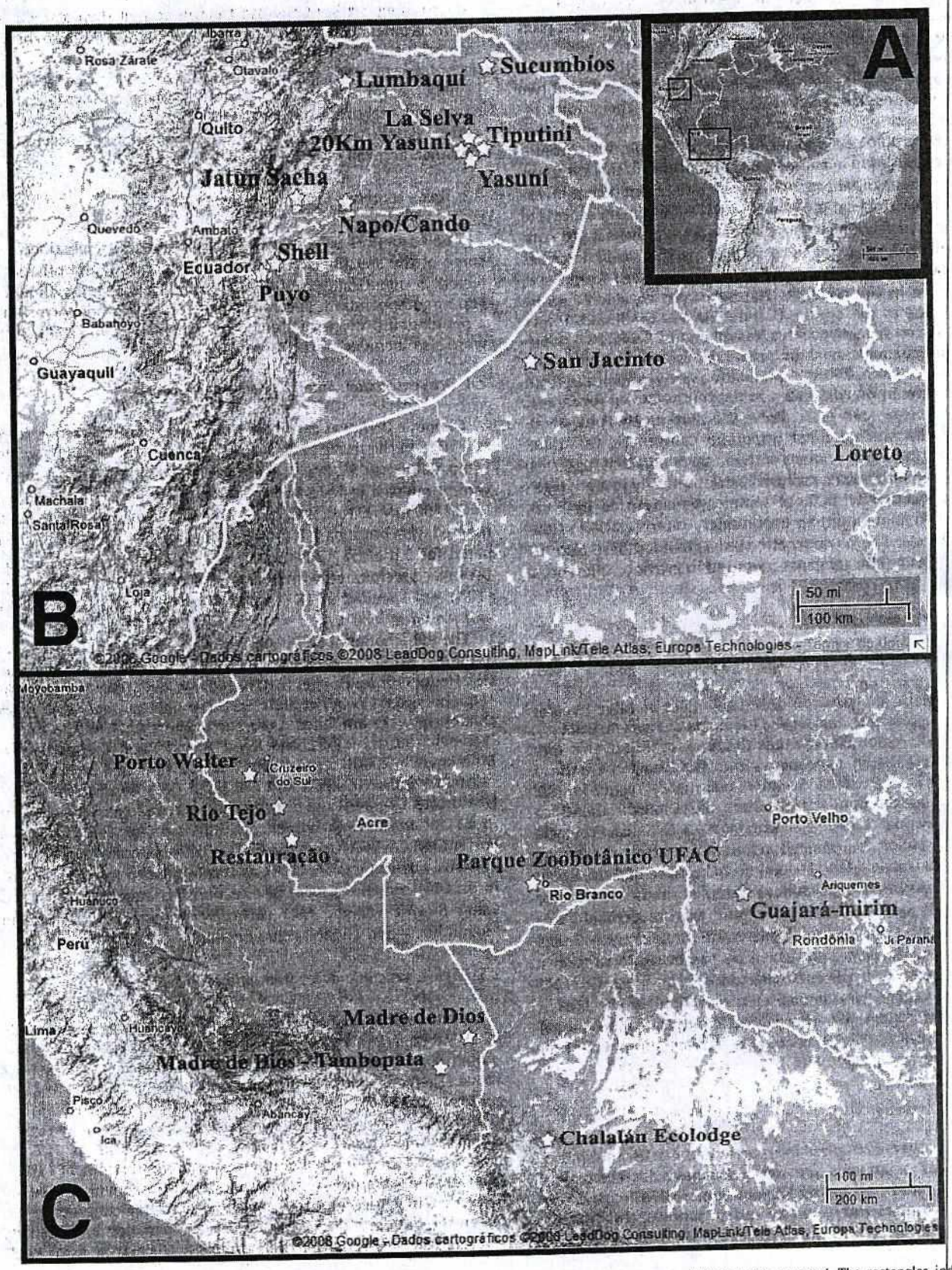

Fig. 1. (A) Partial South America map in small scale displaying the site locations (stars) where *Engystomops* populations were surveyed. The rectangles indicate the geographical distribution of the *Engystomops* northwestern and southwestern clades, which are shown in large scale in (B and C), respectively. (B and C) Geographical distributions of the *Engystomops* northwestern and southwestern clades, respectively. Stars: sampling sites.

cated that the data sets were not incongruent, supporting the use of the concatenated matrix for phylogenetic analyses.

Phylogenetic relationships were inferred from the concatenated matrix of mtDNA sequences by the Maximum Parsimony (MP) method (Camin and Sokal, 1965), using PAUP* 4.0β10 (Swofford, 2000). To perform a parsimony ratchet analysis in PAUP, we used PAUPRat (Sikes and Lewis, 2001). We started 10 independent ratchets and a strict consensus tree was calculated from the best trees in each of these inferences, using PAUP. These 10 consensus trees were used to obtain the majority-rule consensus tree in PAUP. Transitions and transversions were considered to have the same weight, gaps were considered as the fifth state and the phylogenetic trees were searched using a heuristic algorithm, with 10 random addition-sequence replicates. Nodal support for the MP arrangement was assessed through non-parametric bootstrap analysis (Felsenstein, 1985), with a heuristic search based on 1000 pseudoreplicates. The ACCTRAN option was used to optimize the characters.

Maximum Likelihood (ML) analysis using PAUP* 4.0β10 (Swofford, 2000) was also conducted with the concatenated matrix. The most appropriate evolution model for ML analysis was selected by Modeltest 3.7 (Posada and Crandall, 1998). Because bootstrap analysis using ML is not computationally feasible for large data sets, the nodal support was assessed through Bayesian analysis using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2005).

The following *Engystomops* species were used as outgroup in all the analyses: *E. coloradorum*, *E. guayaco*, *E. montubio*, *E. pustulosus*, *E. randi*, *Engystomops* sp., *Engystomops* sp. 1, *Engystomops* sp. 2.

2.6. Nuclear genes analyses

Sequences of 433 bp of the RAG-1 gene and of 312 bp of the rhodopsin gene were obtained for specimens from Acrean populations and from Ecuatorian populations of *Engystomops*, as shown in Appendix 1. A data matrix of 48 OTUs was generated with the rhodopsin sequences and another with 31 OTUs with the RAG-1 sequences. These data sets were not combined nor were they combined with those of mitochondrial genes, since the same specimens were not used to obtain the sequences. Therefore, the phylogenetic relationships were inferred separately for each of these nuclear genes by Maximum Parsimony (MP) (Camin and Sokal, 1965) and Maximum Likelihood (ML) methods, using PAUP* 4.0β10 (Swofford, 2000). In the MP analyses, transitions and transversions were considered to have the same weight, gaps were considered as the fifth state and the phylogenetic trees were searched using a heuristic algorithm, with 10 random addition-sequence replicates. Nodal support for the MP arrangement was assessed through non-parametric bootstrap analysis (Felsenstein, 1985), with a heuristic search based on 1000 pseudoreplicates. The ACCTRAN option was used to optimize the characters.

The most appropriate evolution model for likelihood analyses was selected by Modeltest 3.7 (Posada and Crandall, 1998). The nodal support in this case was assessed through non-parametric bootstrap analysis (Felsenstein, 1985) with a heuristic search based on 100 pseudoreplicates. We also conducted a Bayesian analysis using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2005) to provide statistical support for the inferred topology.

3. Results

3.1. 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 1 subtelocentric (pair 11), as shown in Fig. 2. Pair 11 was characterized as the sex chromosome pair of the system XX/XY. The data showed that X chromosomes were homomorphic in three females and all the males exhibited a Y chromosome. Two females showed a heteromorphic pair X, but the difference concerned the terminal C-band, which in one chromosome was larger than in the other (insets in Fig. 2A, B and 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 X sex chromosome (Fig. 2A). These regions were identified as NORs by the Ag–NOR method (Fig. 2B) and *in situ* hybridization (Fig. 2C and D). 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 (Fig. 2D).

Centromeric regions were identified by C-banding in all chromosomes, including the sex pair (Fig. 2E), as well as the terminal region of both arms of pairs 8 and 9, and of the long arm of pair 10 and chromosome 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 pair 8 was apparently adjacent to the NOR (Fig. 2B and C). In the NOR-bearing homologue 9, the terminal C-band in the long arm was adjacent to the NOR.

Chromosome preparations 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 pair 8 and of the long arm of pair 9 remained unstained (Fig. 2F). The centromeric regions were not distinguishable by fluorochrome staining. The previously C-banded plates showed a different result, since they had the centromeric and terminal C-bands of the pairs 8, 9, 10 and chromosome 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 (Fig. 2G). Most likely, this heteromorphism justifies the differences found in the morphology of the homologues of this pair (Fig. 2A and G).

MM staining revealed pericentromeric regions in some chromosome pairs, including the NOR-bearing X chromosome (Fig. 2G). A large telomeric region, coincident with a DAPI-positive region, was strongly stained with the MM fluorochrome on 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 (Fig. 2G).

A pericentromeric region of the short arm of pair 1 was observed as a secondary constriction after Giemsa or fluorochrome staining without previous treatment (Fig. 2A and F) and after silver staining (Fig. 2B) or C-banding (Fig. 2E). In C-banded metaphases stained with MM and especially with DAPI, this pericentromeric region seemed stained (Fig. 2G), which may 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 (Fig. 2H).

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

3.2. 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 and 9), submetacentric (pair 10) and subtelocentric (pairs 5 and 11). Pair 11 was not heteromorphic in the five males analyzed, in contrast with the observed in Puyo karyotypes (Fig. 2A).

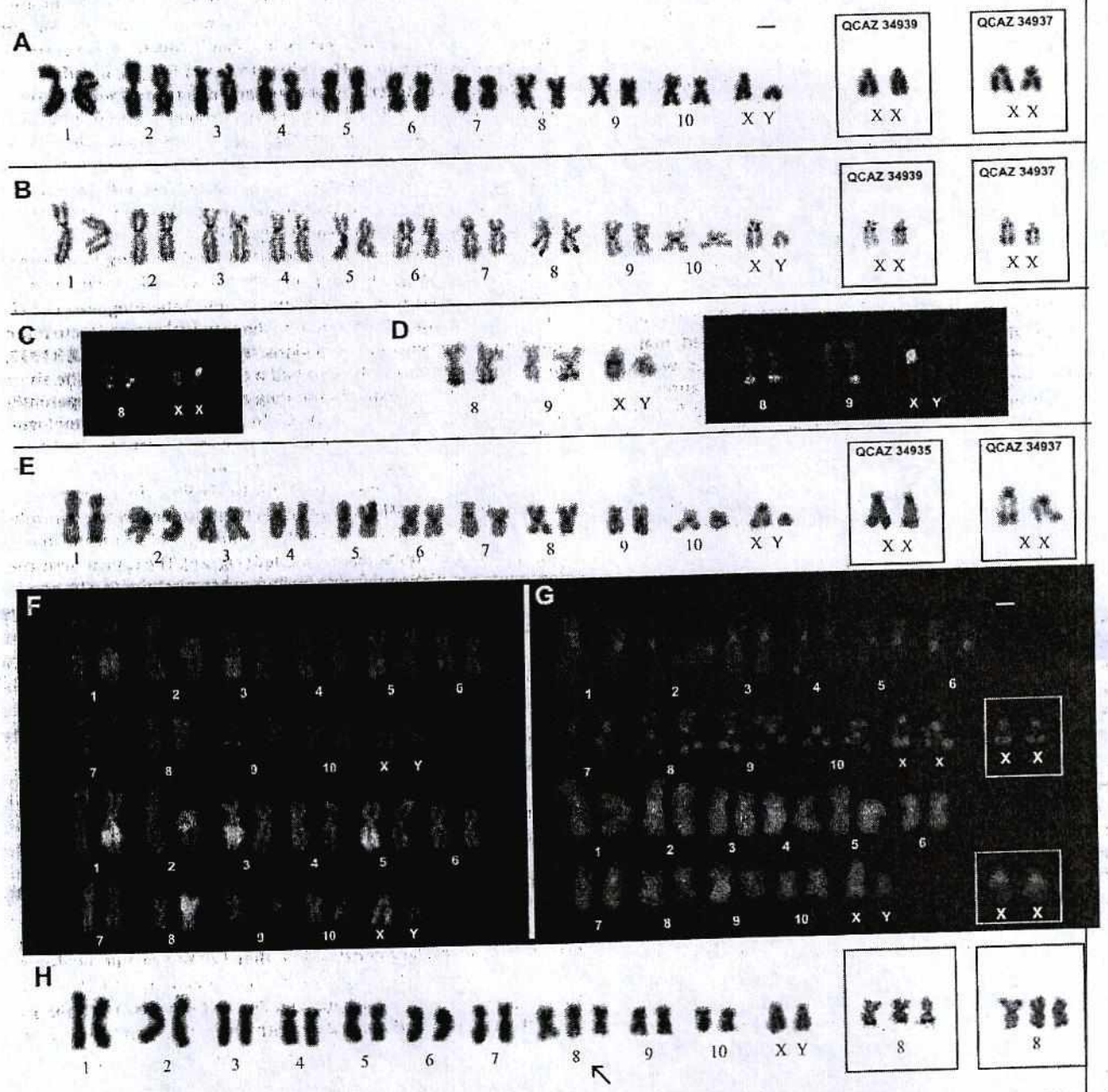

Fig. 2. Karyotypes of male specimens of *Engystomops petersi* from Puyo (A) stained with Giemsa, (B) submitted to the Ag–NOR technique, (E) C-banded, (F) DAPI-stained (top) and MM-stained (bottom), (G) DAPI-stained (top) and MM-stained (bottom) after C-banding technique. The insets in (A, B and E) show the homomorphic and the heteromorphic pair XX from the females ZUEC 34939 (A and 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, chromosome 8 after the Ag–NOR technique (left) and C-banding (right). Bar = 2 μm.

Giemsa staining discriminated pairs 3 and 8, with secondary constrictions in their long arm (Fig. 3A). The secondary constriction of pair 3 was the only onerecognized as NOR using the Ag–NOR technique (Fig. 3B). This secondary constriction exhibited a unique feature, characterizing an intraindividual variation. This secondary constriction was observed as a small region of constriction 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 (Fig. 3A inset). All of those regions were also stained by the Ag–NOR technique (Fig. 3B).

The centromere in all chromosome pairs was detected after C-banding (Fig. 3C and D). In addition, terminal C-bands were observed in the following areas: both arms of pairs 3 and 8, in the long arm of pairs 4, 10 and 11, and in the short arm of pair 6. A weakly stained C-band could be distinguished at the short arm of one homologue of pair 10 (Fig. 3C). In pair 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 pairs 3, 4, 8, 9 and 11 (Fig. 3E). After C-banding, all centromeres and terminal C-bands of pairs 3, 4 and 8 were evidenced by DAPI (Fig. 3F). The MM fluo-

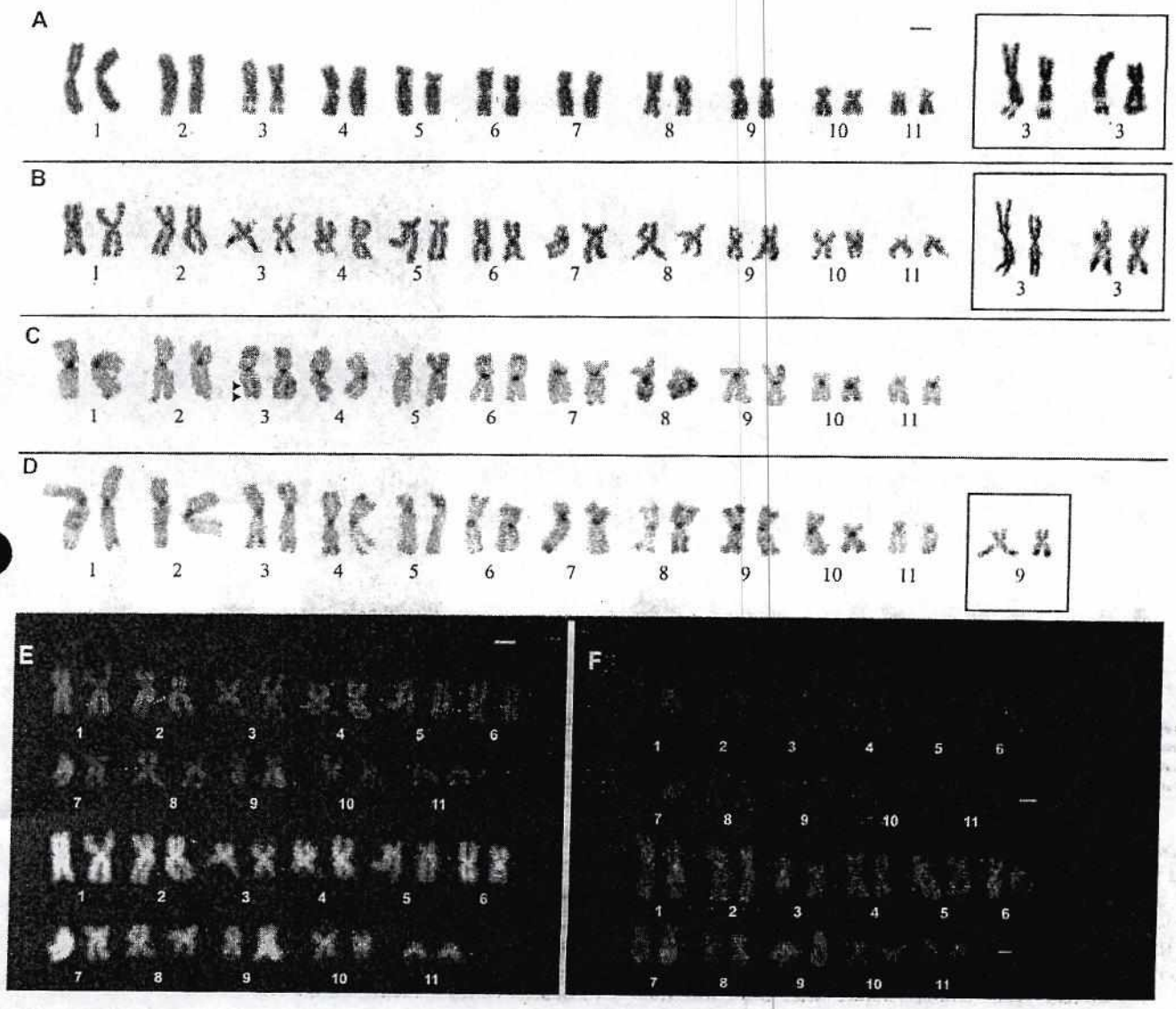

Fig. 3. Karyotypes of the *E. petersi* Yasuní specimens QCAZ 34944, QCAZ 34947 and QCAZ 34946 submitted to (A) Giemsa staining, (B) the Ag-NOR technique and (C) C-banding, respectively. The arrowheads in (C) indicate the two heterochromatic bands that flank the NOR. The insets in (A and B) show chromosome 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.

centromeres were weakly stained in metaphases previously submitted to C-banding (Fig. 3F).

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

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

3.3. Karyotype of the specimen Yasuní-km 20

The karyotype of the Yasuní-km 20 specimen (QCAZ 30826) was remarkably distinct from the other analyzed *E. petersi* specimens of this geographic region (Fig. 4A). Interestingly, this karyotype was highly similar to the Puyo specimens, differing only in NOR distribution (Fig. 4B and C). In the metaphases of the Yasuní-km 20 specimen 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 pair 4, long arm of pair 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 heterochromatic regions (Fig. 4D, E and F).

3.4. 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 and 11), one pair of submetacentric (pair 5) and one pair of subtelocentric (pair 2) (Fig. 5). A secondary constriction was observed in the short arm of pair 6 (Fig. 5A), which was revealed as NOR by the Ag-NOR method (Fig. 5C). In 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 (Fig. 5).

The C-banding revealed conspicuous centromeric heterochromatic regions in all chromosomes (Fig. 5B). In addition, terminal

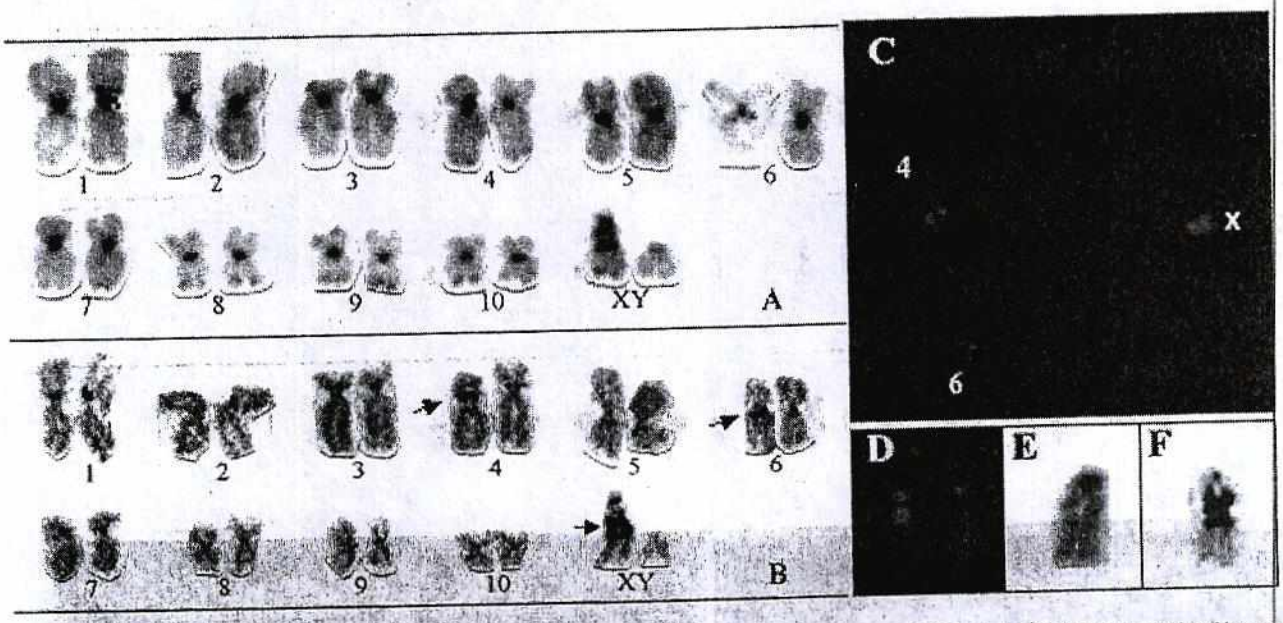

Fig. 4. Cytogenetics of the Yasuní specimen QCAZ 30826. (A) Karyotype submitted to C-banding. (B) Karyotype after Ag–NOR staining. The NORs were pointed by arrows. (C) Mitotic metaphase hybridized with the rDNA probe HM 123 and stained with propidium iodide. Note that some heterochromatic regions seen 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).

C-bands were observed at the telomeres of both arms of pairs 6, 7, 10 and 11. Telomeric bands could also be seen in the long arm of pair 3, and in the short arms of pairs 8 and 9. Two additional C-bands were observed in interstitial regions in the long arm of pair 5 and the short arm of pair 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 pair 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 (Fig. 5C–G).

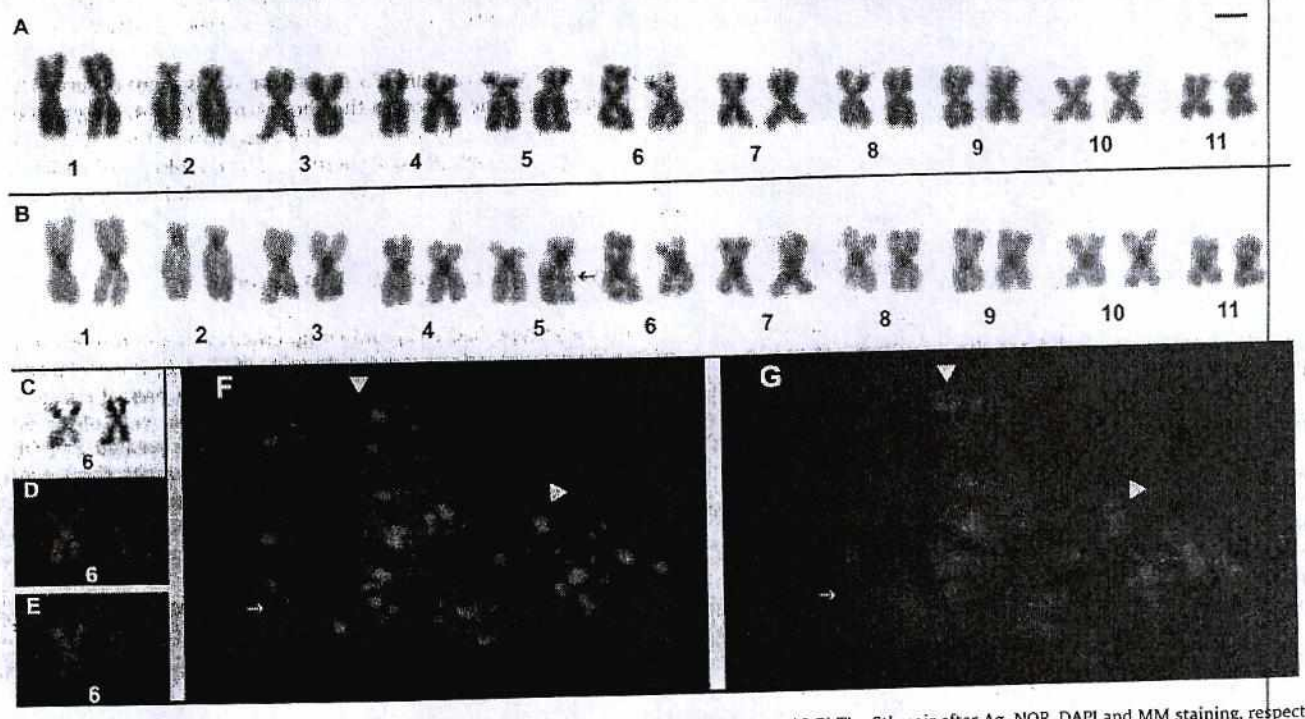

Fig. 5. Cytogenetics of the La Selva specimen. (A) Giemsa-stained karyotype. (B) C-banded karyotype. (C–E) The 6th pair after Ag–NOR, DAPI and MM staining, respectively. Note the heterochromatic blocks flanking the NOR. (F–G) The same metaphase stained with DAPI and MM, respectively, after C-banding. Note the different regions stained by

3.5. Cytogenetics of the UFAC-Acre population

In all UFAC-Acre specimens the karyotype was $2n = 22$ and consisted of six metacentric chromosome pairs (1, 2, 4, 6, 7 and 9), 3 submetacentric pairs (3, 10 and 11) and 2 subtelocentric pairs (5 and 8) (Fig. 6A). Because pair 8 was heteromorphic in all males and homomorphic in females, it was classified as a sex chromosome pair (Figs. 6 and 7). Four specimens showed another heteromorphic pair, although it was present in both male and female specimens and so it was considered unrelated to sex determination (Figs. 6 and 7).

NOR sites, totaling five, were individually detected in pericentromeric regions in the long arm in one homologue of pair 3, in a distal region in the long arm of pair 7, terminally in the long arm of pair 9, and interstitially in the long arm of pair 11 (Figs. 6B and 7). The only fixed NOR was observed in pair 7. The remaining NOR sites were not always present, further evidencing NOR polymorphism detected in this population (Figs. 6B and 7). In the UFAC-Acre specimens, a maximum of eight NOR-bearing chromosomes was observed (Fig. 7C).

All the centromeres were C-banded and terminal C-bands were present in the short arms of pair 4, 7 and 9, and also at the long arms of pairs 7, 9 and chromosome X, while interstitial C-bands were seen in at the long arm of pair 11 and chromosome X. The terminal C-bands in pairs 7 and 9, and the interstitial C-band in pair 11 were adjacent to NOR sites. The interstitial C-band at the long arm of pair 11 was occasionally absent, similar to its adjacent NOR, which implied a morphological chromosome difference between the homologues (Fig. 7). The sex chromosome pair of female ZUEC 14435 was heteromorphic according to its C-band pattern (Fig. 7A).

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

3.6. Molecular inferences from mtDNA

In MP analysis, 571 of the 2418 sites were informative. The parsimony ratchet approach yielded 2010 trees, with 1471 steps (CI = 0.6524; RI = 0.9315; RC = 0.6078). Fig. 8 shows the majority-rule consensus tree generated by this analysis and the clades that were not found in all of the MPT are indicated. The ML analysis used a GTR+I+G model of sequence evolution. The likelihood score was 6768.5049 (estimated base frequencies: A 0.3671, C 0.1868, G 0.1645, T 0.2816; rate matrix: A–C 7.3164, A–G 30.4400, A–T

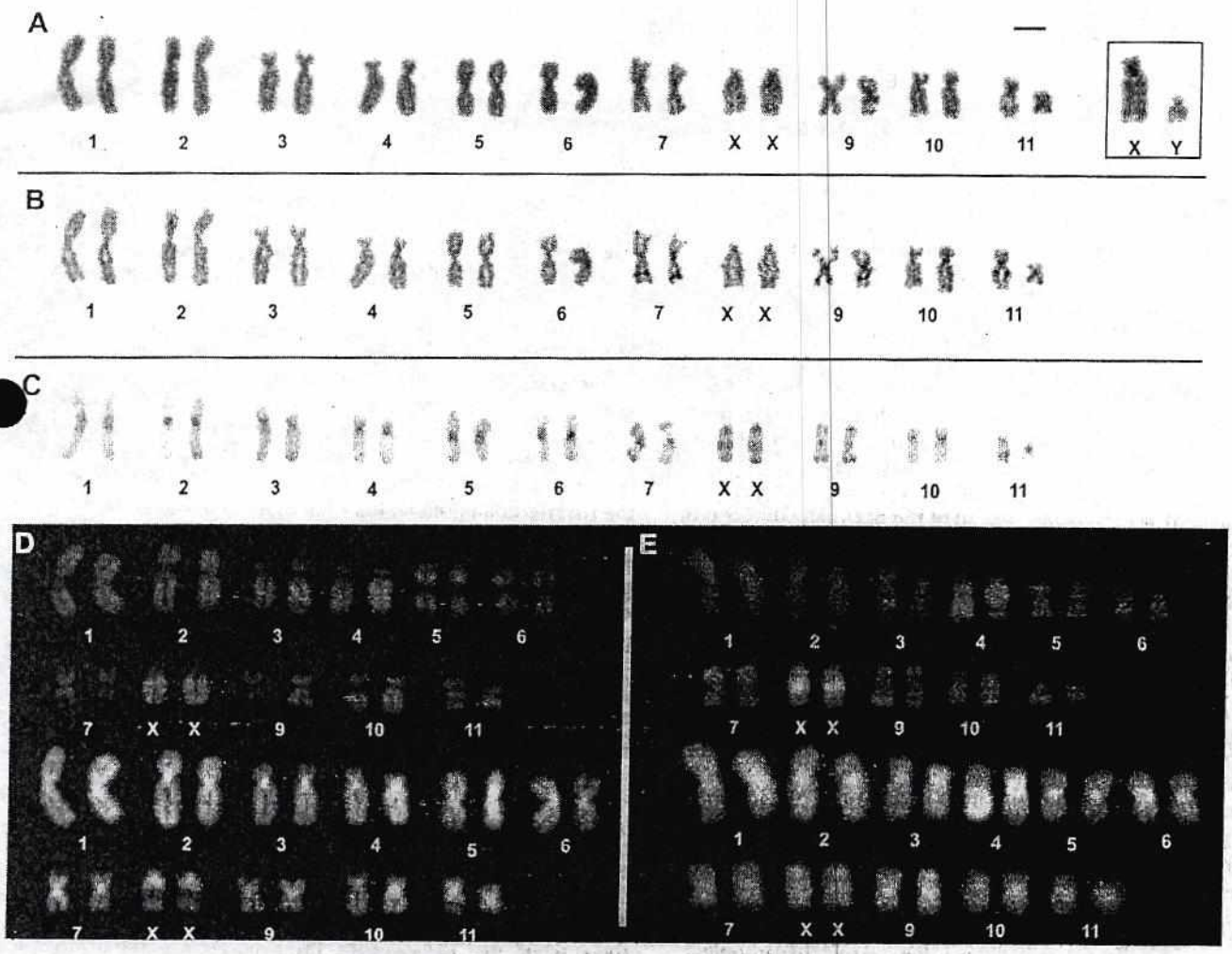

Fig. 6. Giemsa-stained (A), silver stained (B) and C-banded (C) karyotypes from the female ZUEC 14432 from UFAC-Acre population. The inset in A shows the heteromorphic

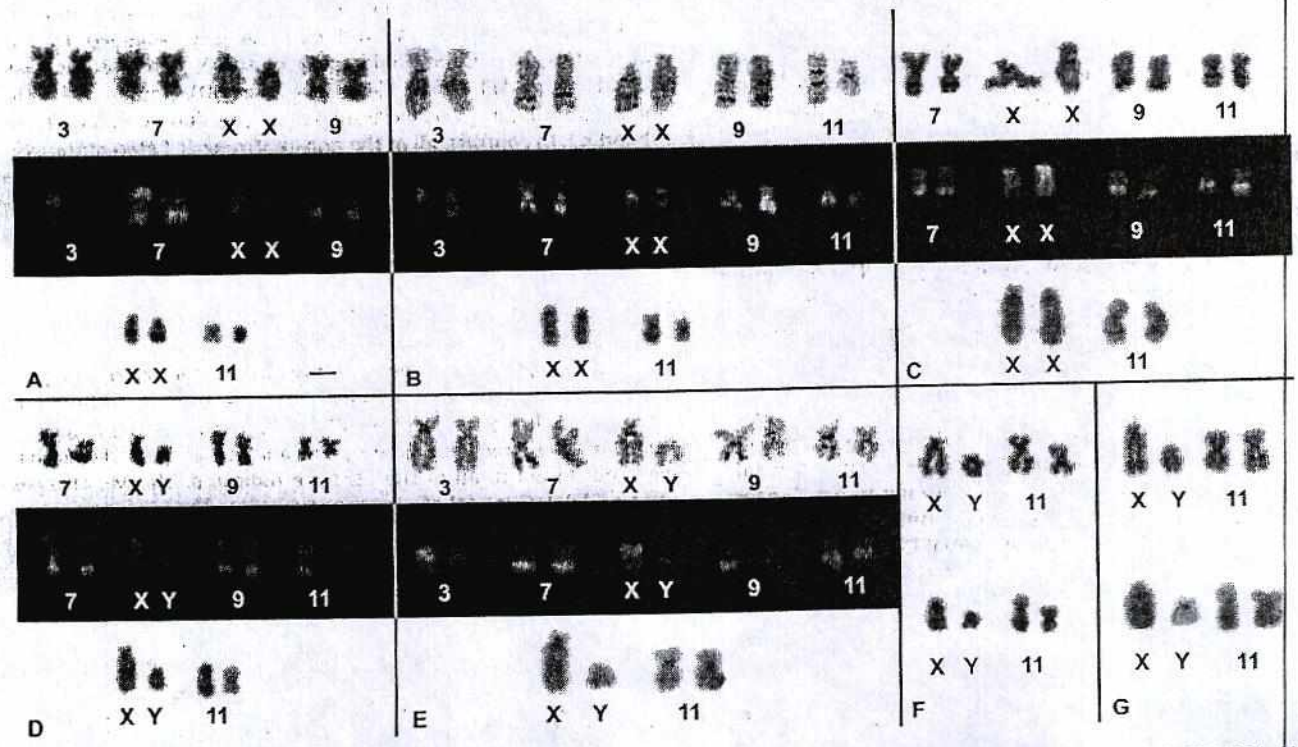

Fig. 7. NOR-bearing chromosome pairs from seven different UFAC-Acre specimens (A–G) stained by Ag–NOR (top) and hybridized with the rDNA probe HM 123 (middle). In the bottom, the C-banded pair 11 and sex chromosomes of the seven specimens. Bars = 2 μm.

10.8909, C–G 2.3206, C–T 68.7585, G–T 1.000; shape parameter for gamma distribution: 0.6529; proportion of invariant sites: 0.5513). This analysis generated a tree topology (Fig. 9) in which the clades higly supported in MP analyses were also seen. The average sequence divergence within the Amazonian *Engystomops* was 4.4%, between this and the outgroup divergence climbed to 31.6%.

The Amazonian *Engystomops* were clustered in three major clades within the ML tree and also in 1996 of the MPT in the MP analysis (Figs. 8 and 9). The first clade was composed by the *Engystomops* specimens from Pará, which was highly supported in all the analyses. The second major clade (southwestern clade) was weakly supported by bootstrap analysis but its Bayesian probability was high (0.98) and comprised all of the Brazilian populations, the specimens from the North to the South banks of the Madre de Dios River, in Peru, and the population from Bolivia. The remaining populations were grouped in a third clade (northwestern clade), supported by a bootstrap of 81% in MP analysis and 0.98 of Bayesian probability in ML inference.

Inside the southwestern clade, the Brazilian UFAC-Acre population, not sampled by Funk et al. (2007), was clustered together with the population from Rondônia. The specimens from Rio Tejo were confidently clustered together with the populations from Porto Walter, Restauração and the specimen *Engystomops freibergi* (GenBank DQ337229).

Sub-groups were also observed inside the northwestern clade. An interesting finding related to this clade was observed for the specimen QCAZ 30826 from the *E. petersi*, refered as Yasuní-km 20 specimen, which in all the analyses was grouped together with the specimens from Puyo and Shell regardless of their geographical distance. This group (Puyo + Shell + Yasuní-km 20 specimen) combined with Jatun Sacha, Cando and Napo also composed a well-supported clade. Except for the Yasuní-km 20 specimen, this clade uses the name as the Upper Napo clade by Funk et al. (2007).

Among the remaining populations in the northwestern clade, only the Yasuní and Tiputini populations (Ecuador) remained together in a same sub-group with high confidence. The Lower Napo clade was recovered only in the ML analyses described herein (Fig. 9) and by Funk et al. (2007), but not by the MP inferences (Fig. 8 and Funk et al., 2007).

The La Selva population was grouped with the specimen from Lumbaqui–Sucumbios (Ecuador) and with some specimens from Puerto Bolivar–Sucumbios (Ecuador) in a well-supported clade in MP and ML analyses. Interestingly, not all of the haplotypes found in Puerto Bolivar–Sucumbios were in this clade. Instead, they were grouped with some specimens from the Amazon Conservancy for Tropical Studies-Loreto (Peru), in another well-supported clade (Figs. 8 and 9), as previously described by Funk et al. (2007).

The clade composed by La Selva + Sucumbios in the ML tree, is the sister-group of the Yasuní + Tiputini clade (Fig. 9). However, in MP analyses the relationships among these clades and other Ecuatorian clades were not resolved. Although in some MPT La Selva + Sucumbios is the sister-group of the Yasuní + Tiputini clade, in others it was more related to that clade which included Puyo + Shell + Yasuní-km 20 specimens.

In all the inferences, San Jacinto–Loreto (Peru) haplotypes were not grouped according to their locality, as already observed by Funk et al. (2007). Instead, they were mixed with haplotypes of specimens from other populations, but in arrangements not well-supported (Figs. 8 and 9).

3.7. Nuclear genes analyses

For the RAG-1 sequence, 14 polymorphic sites and six haplotypes were recognized among the 23 specimens of the Acrean group, while five polymorphic sites and four haplotypes were found in the Ecuatorian group. The average sequence divergence within the Acrean group was 0.09% for this gene, while in the Ecuatorian group, it was 0.49%. Between these groups, the sequence divergence averaged 0.44%.

In MP analysis for RAG-1 sequences, 11 of the 433 nucleotides were informative. A Hundred MPT was generated, with 26 steps (CI = 0.923; RI = 0.950). The ML analysis used a HKY model of

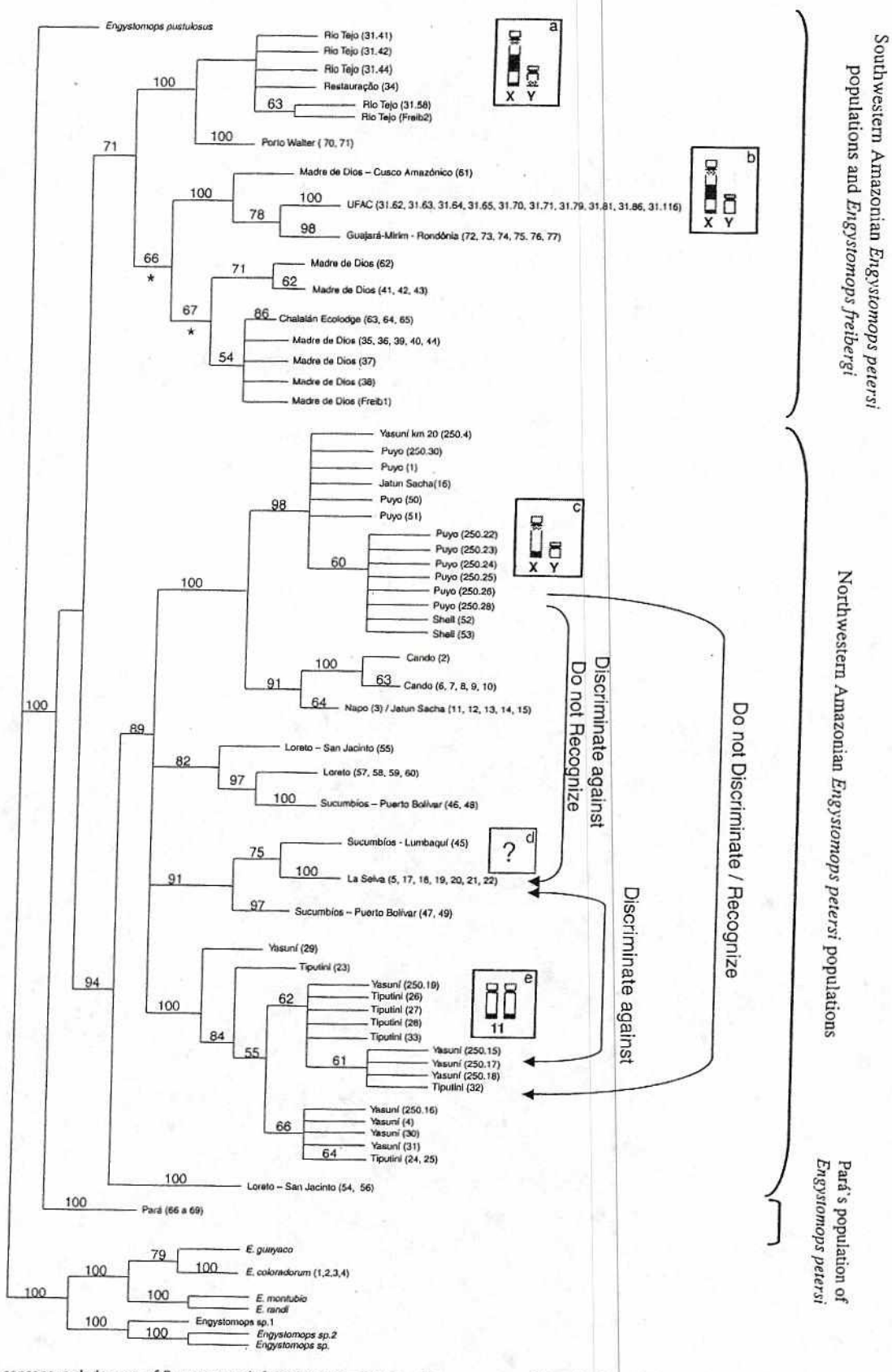

Fig. 8. Majority-rule consensus cladogram of *Engystomops* inferred by MP analyses using sequences of the mitochondrial genes 12S rDNA, val-tRNA and 16S rDNA. Numbers above the branches are bootstrap values and the asterisks indicate the clades that were not found in all of the MPT. Available data of vocal calls (Guerra and Ron, 2008) are indicated for the cytogenetically studied populations. The sex chromosomes are schematized for the groups studied cytogenetically (gray boxes). To clarify the comparison between the cytogenetic results, the available data of mating calls (Boul et al., 2007) are indicated.

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MOLECULAR CYTOGENETICS

RESEARCH Open Access

Interstitial Telomeric Sequences (ITS) and major rDNA mapping reveal insights into the karyotypical evolution of Neotropical leaf frogs species (*Phyllomedusa*, Hylidae, Anura)

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Abstract

Background: The combination of classical cytogenetics with molecular techniques represents a powerful approach for the comparative analysis of the genome, providing data for the systematic identification of chromosomal homologies among species and insights into patterns of chromosomal evolution within phylogenetically related groups. Here, we present cytogenetic data on four species of Neotropical treefrogs of the genus *Phyllomedusa* (*P. vaillantii*, *P. tarsius*, *P. distincta*, and *P. bahiana*), collected in Brazil and Ecuador, with the aim of contributing to the understanding of the chromosomal diversification of this genus.

Results: With the exception of *P. tarsius*, which presented three telocentric pairs, all the species analyzed had conservative karyotypic features. Heterochromatic patterns in the genomes of these species revealed by C-banding and fluorochrome staining indicated the presence of a large number of non-centromeric blocks. Using the Ag-NOR method and FISH with an rDNA 28S probe, we detected NOR in the pericentromeric region of the short arm of pair 7 in *P. vaillantii*, pair 1 in *P. tarsius*, chromosomes 1 and 9 in *P. distincta*, and in chromosome 9 in *P. bahiana*, in addition to the presence of NOR in one homologue of chromosome pair 10 in some individuals of this species. As expected, the telomeric probe detected the terminal regions of the chromosomes of these four species, although it also detected Interstitial Telomeric Sequences (ITS) in some chromosomes of the *P. vaillantii*, *P. distincta* and *P. bahiana* karyotypes.

Conclusion: A number of conservative chromosomal structures permitted the recognition of karyotypic homologies. The data indicate that the presence of a NOR-bearing chromosome in pair 9 is the plesiomorphic condition in the *P. burmeisteri* group. The interspecific and intraspecific variation in the number and location of rDNA sites reflects the rapid rate of evolution of this character in *Phyllomedusa*. The ITS detected in this study does not appear to be a remnant of structural chromosome rearrangements. Telomeric repeats were frequently found in association with heterochromatin regions, primarily in the centromeres, which suggests that (TTAGGG)n repeats might be an important component of this heterochromatin. We propose that the ITSs originated independently during the chromosomal evolution of these species and may provide important insights into the role of these repeats in vertebrate karyotype diversification.

Keywords: *Phyllomedusa*, Karyotypes, rDNA, Interstitial telomeric sequences (ITS)

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Background

Comparative cytogenetic studies provide scenarios of the chromosomal evolution of related taxa and represent an important approach to the identification of chromosomal homologies among species [1]. In many organisms, karyological features have been widely accessed by classical methods, and advances in molecular cytogenetics based on Fluorescence *in situ* hybridization (FISH) experiments have resulted in improved chromosomal mapping of large numbers of sequences and permitted the study of chromosomal variation.

The ribosomal RNA gene, which is a repetitive DNA sequence that is organized in tandem, is widely used in chromosomal investigations and provides a good chromosomal marker for comparative cytogenetic studies [2]. This sequence shows several features of 'hotspots' of chromosomal recombination because it consists of a clustered organization of repeats and is frequently located in pericentromeric and subtelomeric regions [3]. A high rate of mutation/homogenization of intergenic spacer regions is observed (e.g. see references [4-7]), and these modifications have an important role in chromosomal reorganization during karyotype evolution. The association of the NOR repositioning events due to the presence of transposable elements has already been noted [8,9].

Chromosomal mapping of telomeric sequences has been widely used to identify chromosomal rearrangements among karyotypes of vertebrates and to detect fusion and/or fission, inversion or translocation events [10-16]. Many recent studies have emphasized the important role of this sequence in chromosomal evolution [17-19], and many studies have reported that sequences related to telomeric sequences form a component of satellite DNA [20-22].

Cytogenetic studies have demonstrated the presence of interstitial telomeric sequences (ITS) in many phylogenetic groups (for references, see reference [23]). In most cases, these sequences are associated with heterochromatin regions that do not appear to represent remnants of ancient chromosomal rearrangements [24-29]. Non-telomeric repeats of the sequence (TTAGGG)n in heterochromatin regions or in the margins of these blocks, which have been termed 'het-ITS' are easily detected in FISH experiments [23]. However, fine-scale studies in mammals have also documented the extensive occurrence of short telomeric repeats within the internal regions of chromosomes (s-ITSs) [18,30-32], and, according to Ruiz-Herrera [19], this feature is presumably present in all vertebrate species. The presence of s-ITS in other vertebrates could be underestimated due to the presence of fewer repeats; such repeats may not be detectable at the resolution of conventional FISH experiments.

The *Phyllomedusa* genus is an interesting group within which to conduct comparative cytogenetic analyses. In addition to the fact that the intrageneric relationships of some of the species remain unclear, this Neotropical treefrog genus raises many taxonomic questions at the species level [33]. The genus currently includes 30 species [34]. Molecular phylogenetic inferences support the presence of four species groups [33]: the *P. hypochondrialis* group [35], the *P. tarsius* group [36], the *P. burmeisteri* group [37] and the *P. perinesos* group [38]. The species *P. atelopoides*, *P. bicolor*, *P. boliviana*, *P. vaillantii*, *P. sauvaggi*, and *P. tomopterna* [33,34] are not included in any of these groups. Cytogenetic data show extensive multiple NOR [39-44] and interspecific NOR variation [40,41,43,45] in the species that have been karyotyped.

Our goal in this work was to investigate the karyotypes of four species of the *Phyllomedusa* genus: *P. vaillantii*, species that remain unassigned to any species group, *P. tarsius*, include in the *P. tarsius* group and *P. distincta* and *P. bahiana* which are included in the *P. burmeisteri* group. We used multiple chromosomal markers to better understand the chromosomal evolution of this genus. In addition to providing insights concerning the evolution of rDNA clusters, we report an interesting distribution pattern of non-terminal telomeric repeats in karyotypes of this genus.

Results

The chromosome diploid number in all species karyotyped showed 26 chromosomes. With the exception of *P. tarsius*, which showed three telocentric pairs (pairs 7, 10 and 12), the remaining karyotypes consisted of four metacentric pairs (1, 4, 8 and 11), six submetacentric pairs (2, 3, 5, 6, 12 and 13) and three subtelocentric pairs (7, 9 and 10) (Figure 1A-G). Secondary constrictions was observed in the pericentromeric region of the short arm of pair 7 in *P. vaillantii* karyotype, in the pair 1 in *P. tarsius*, pairs 1 and 9 in *P. distincta*, and in chromosomes 9 in *P. bahiana* karyotype, besides secondary constriction in one homologue of the pair 10 (see Figure 2).

C-banding detected centromeric heterochromatin in all chromosome pairs, with difference in amount of heterochromatin among pairs: some chromosomes pairs exhibits weak and almost absence heterochromatin block while others pairs exhibits salient marker in this region. Non-centromeric heterochromatin blocks were widely found in *P. vaillantii* chromosomes of the three populations sampled (Figure 3A-C). Remarkable interstitial heterochromatin blocks were detected in both arms of the chromosomes of pair 8 in addition to interstitial C-bands in homologs of pairs 1, 6, 7, 9 and 11 (Figure 3A-C). The karyotype of *P. tarsius* showed C-bands in the pericentromeric region of the short arm of chromosome pairs 3 and 6 and in the long arms of pairs 1, 4 and 11 (Figure 3D-E). The same C-banding pattern was observed in Brazilian and in Ecuadorian populations. Heterochromatin was also

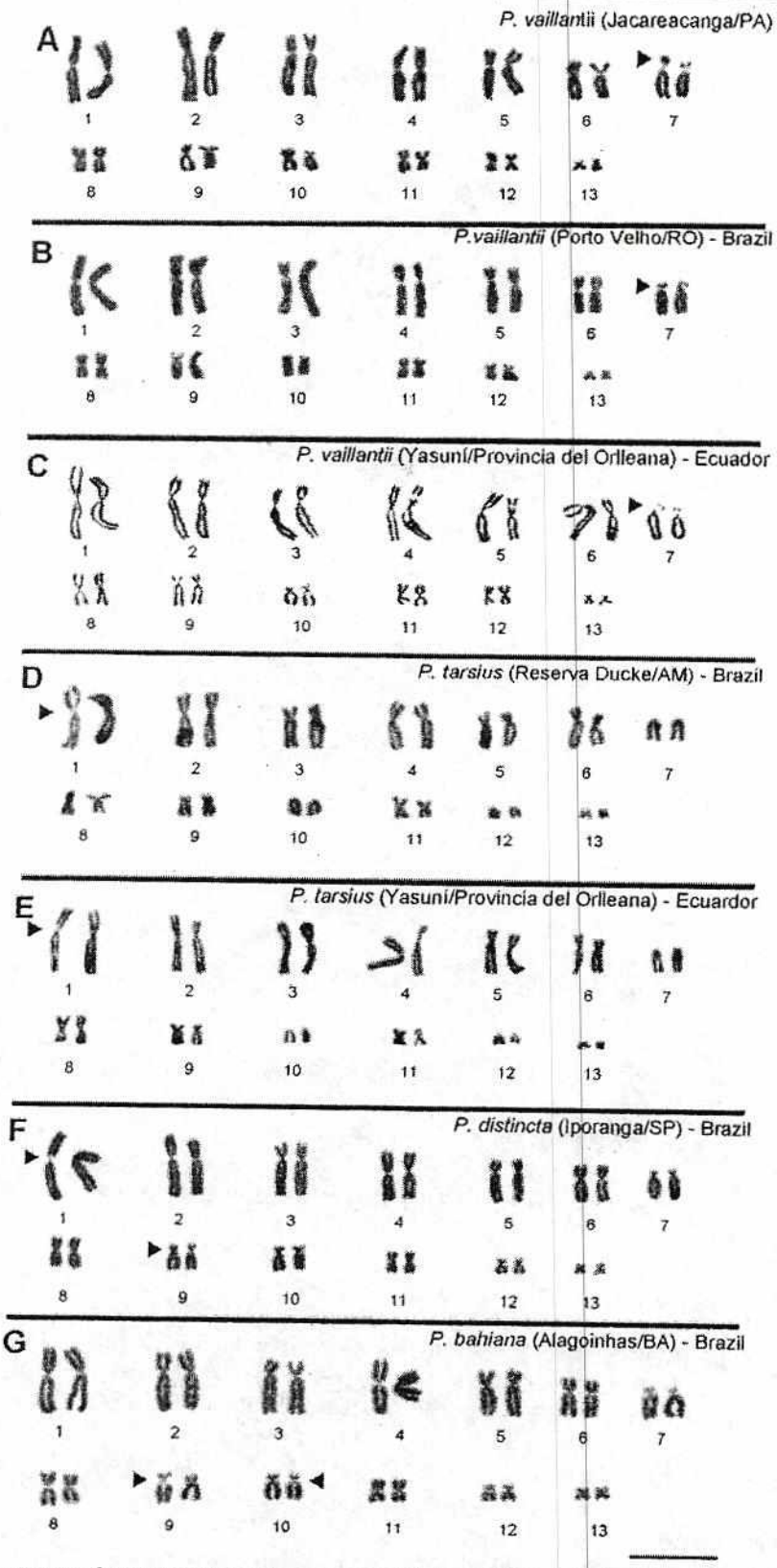

Figure 1 Giemsa-stained karyotypes of *P. vaillantii* from (A) Jacareacanga/PA, Brazil, (B) Porto Velho/RO, Brazil and (C) Yasuní/ Provincia del Orlleana, Ecuador; *P. tarsius* from (D) Reserva Ducke/AM, Brazil and from (E) Yasuní/Provincia del Orlleana, Ecuador; (F) *P. distincta* from Iporanga/SP, Brazil and (G) *P. bahiana* from Alagoinhas/BA, Brazil. The arrowhead indicates secondary constrictions. Bar = 3 μm.

Figure 2 Topographic map of South America showing sampling localities throughout Brazil and Ecuador for populations included in this study. L1: Jacareacanga/PA, Brazil; L2: Yasuní/Provincia del Orlleana, Ecuador; L3: Porto Velho/RO, Brazil; L4: Alagoinhas/BA, Brazil; L5: Iporanga/SP, Brazil; L6: Reserva Ducke/AM, Brazil.

detected in both arms of the chromosomes of pair 8 and in the pericentromeric region (Figure 3D-E). *P. distincta* exhibited the presence of C-bands in the pericentromeric region of the short arms of chromosome pairs 3 and 6 and in the long arms of pairs 1 and 11 (Figure 3F). In this species, a C-positive pericentromeric block was also detected in both arms of the chromosomes of pair 8 (Figure 3F). The heterochromatin pattern of *P. bahiana* revealed C-bands in the pericentromeric region of the short arms in homologs of pairs 3 and 6 and in the long arms of pairs 1 and 11 (Figure 3G). The homologs of pair 8 exhibited pericentromeric blocks in both arms.

The use of base-specific fluorochrome staining after C-banding of chromosomes improved the detection of heterochromatin patterns and revealed additional interesting features of the karyotypes studied. In the case of *P. vaillantii*, pericentromeric C-bands showed DAPI-positive patterns (pairs 1, 6, 7, 8 and 11) (Figure 4A, top). In addition to the blocks detected by the C-banding method, bright interstitial signals were observed. Mithramycin staining resulted in brilliant signals in regions coincident with secondary constrictions in the short arms of the homologs of pair 7 visualized by Giemsa staining (Figure 4A, bottom). In metaphase chromosomes of *P. tarsius*, DAPI staining exhibited a pattern coincident with C-banding, with outstanding bright signals in the heterochromatin of both arms of chromosome pairs 8 and 11 (Figure 4B), whereas MM staining produced only a modest signal coincident with the region containing secondary constrictions in the homologs of chromosome pair 1 (Figure 4B, bottom). The DAPI pattern showed centromeric fluorescence in almost all chromosomes of

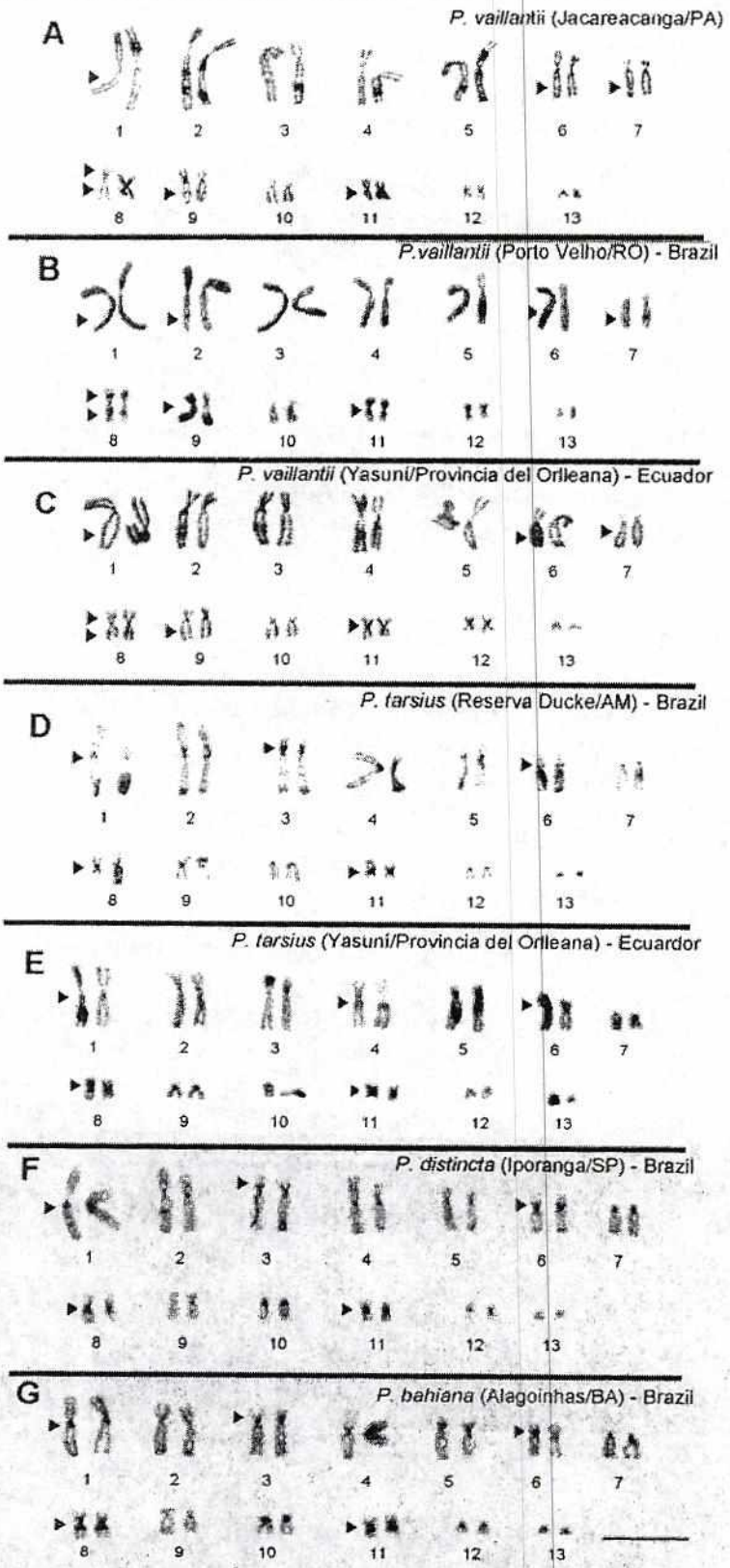

Figure 3 (See legend on next page.)

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Figure 3 Karyotypes defined by C-banding of *P. vaillantii* from (A) Jacareacanga/PA, Brazil, (B) Porto Velho/RO, Brazil and (C) Yasuní/Provincia del Orlleana, Ecuador; *P. tarsius* from (D) Reserva Ducke/AM, Brazil and from (E) Yasuní/Provincia del Orlleana, Ecuador; (F) *P. distincta* from Iporanga/SP, Brazil and (G) *P. bahiana* from Alagoinhas/BA, Brazil. The arrowhead indicates interstitial heterochromatin blocks. Bar = 3 μm.

P. distincta in addition to some fluorescence in the region of the secondary constriction in chromosome pair 9 (Figure 4C). In chromosome pair 8, a brilliant signal was evident in the pericentromeric block of the long arm (Figure 4C). The MM pattern was evident in the centromeres of almost all chromosomes as well as in the region coincident with the secondary constriction in the short arms of the homologs of pair 9 (Figure 4C, bottom). The heterochromatin of the centromeres of chromosome pairs 5 and 13 did not show a strong signal with any fluorochrome staining (Figure 4C). Specimens of *P. bahiana* exhibited relatively weak fluorescence in the centromeric regions of the majority of the chromosome pairs (Figure 4D); in these specimens, the pericentromeric heterochromatin was more easily detected by C-banding. The secondary constrictions in the short arm of chromosome pair 9 showed fluorescence signals by MM-staining, and some centromeres were stained (Figure 4D).

In all karyotypes, the secondary constrictions observed in conventional Giemsa staining were coincident with NOR sites detected by the Ag-NOR method and this was confirmed by FISH experiments (Figure 5). In the three sampled populations of *P. vaillantii*, NORs were detected in the pericentromeric region of the short arm of chromosome pair 7 (Figure 5A-C). The Ag-NOR method also revealed NOR in the pericentromeric region of the short arm of chromosome pair 1 in *P. tarsius* from Reserva Ducke (Manaus, Brazil) and from Yasuní (Província del Orellana, Ecuador) (Figure 5D-E). Two NOR-bearing chromosome pairs were detected in all specimens of *P. distincta* analyzed; the NORs were located in the pericentromeric region of the short arm of chromosome pair 1 and in the pericentromeric region of the long arm of chromosome pair 9 (Figure 5F). Specimens of *P. bahiana* displayed a NOR that was fixed in the pericentromeric region of the long arms of the chromosomes of pair 9 (Figure 5G). In this population, two specimens presented one additional NOR in one of the homologs of chromosome pair 10 (Figure 5G). This conditional was also detected in FISH experiments using an rDNA 28S probe.

The telomeric probe hybridized to all telomeres in the chromosomes of all karyotypes analyzed but showed a differential pattern of interstitial signals in the four species examined. The *P. vaillantii* karyotype exhibited conspicuous signs of ITS in the centromeric regions of chromosome pairs 4 and 6 (Figure 6A), whereas a brighter hybridization signal was detected in the short arms of the homologs of chromosome pair 13 (Figure 6A), the complete short arm being marked by the telomeric

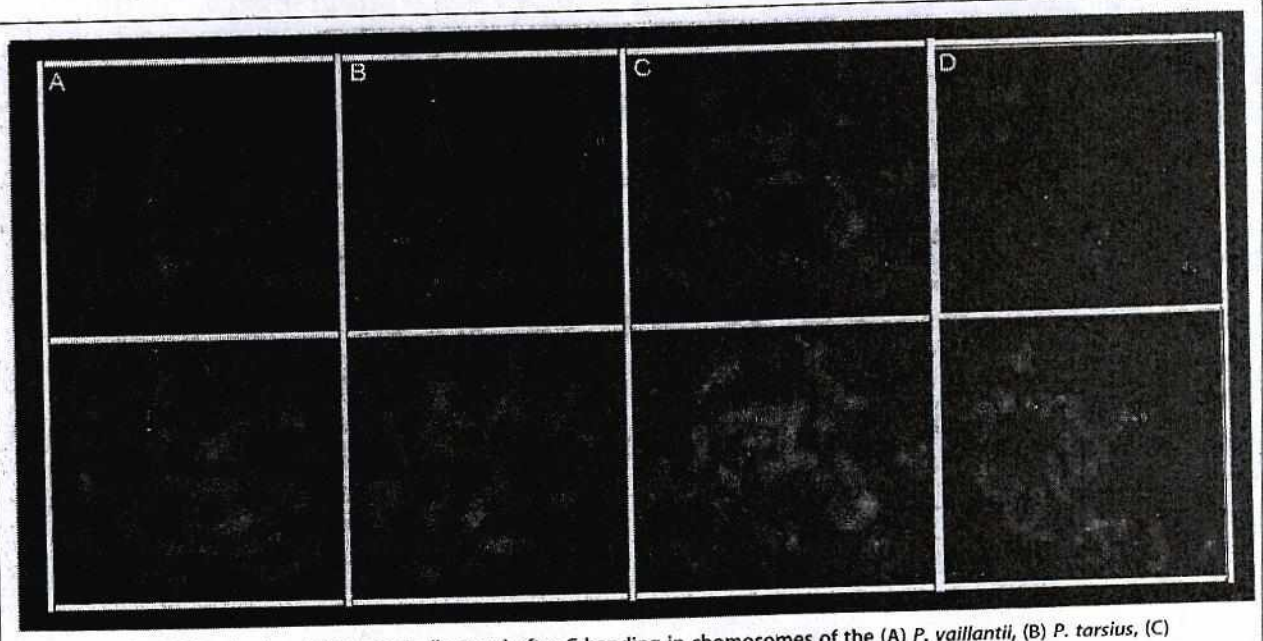

Figure 4 DAPI staining (top) and Mitramycin (bottom) after C-banding in chomosomes of the (A) *P. vaillantii*, (B) *P. tarsius*, (C) *P. distincta* and (D) *P. bahiana*. The arrows highlighters specific chromosome pairs of each species according to results section.

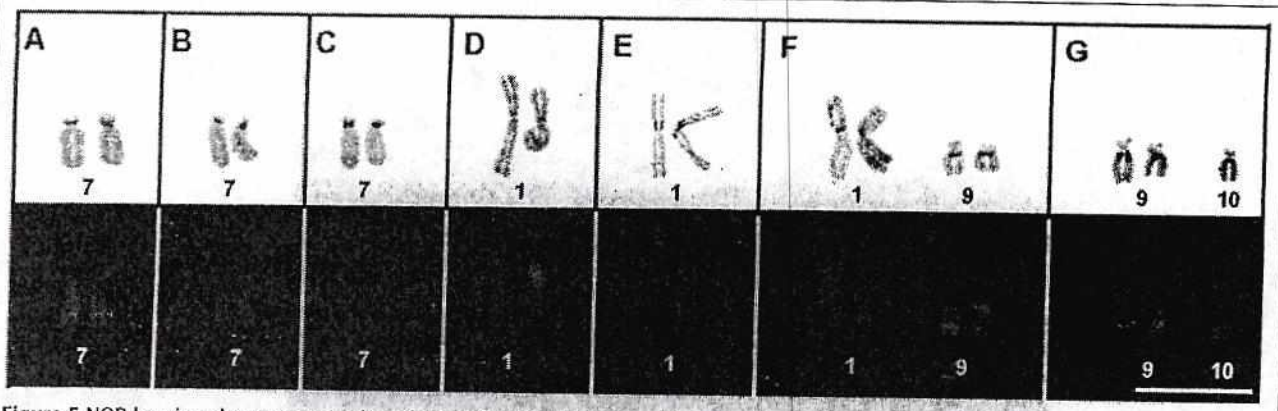

Figure 5 NOR-bearing chromosome pairs submitted to silver impregnation using Ag-NOR method (top) and hybridized with 28S rDNA probe (bottom): *P. vaillantii* from (A) Jacareacanga/PA, Brazil, (B) Porto Velho/RO, Brazil and (C) Yasuní/ Província de Orllana, Ecuador; *P. tarsius* from (D) Reserva Ducke/AM, Brazil and from (E) Yasuní/Província de Orllana; (F) *P. distincta* and (G) *P. bahiana*. Bar = 3 μm.

probe. Although *P. tarsius* did not exhibit interstitial signals in any chromosomes, the telocentric morphology of chromosome pairs 7, 9 and 10 was most evident through this approach (Figure 6B). ITS was also detected in the centromeric regions of chromosome pairs 8 and 11 of *P. distincta* (Figure 6C). Finally, in the karyotype of *P. bahiana*, the telomeric probe hybridized to the centromeric region of chromosomes pairs 4 and 6, and stronger hybridization that extended to the pericentromeric region of the arms was detected in the centromeric region of chromosome pair 11 (Figure 6D).

Discussion

Chromosomal analysis of four representatives of the genus *Phyllomedusa* revealed conservative karyotypic features, including primarily diploid chromosome number (2n = 26), a finding that is consistent with previous reports [39-46]. One special case that showed a deviation in chromosome number was *P. tetraploidea* (2n = 52) [47], a polyploid species with a karyotype clearly derived from 2n = 26.

The four species of the *Phyllomedusa* genus karyotyped in this study showed conserved chromosomal morphology. Despite the different numeric classification of some chromosome pairs among karyotype descriptions in the literature, it is possible to recognize homologies among almost all chromosomes. In this context, the pair 7 subtelocentric and 8 metacentric describes here correspond to, respectively, the pairs 8 subtelocentric and 7 metacentric of the karyotype described by Gruber et al. [44] and Barth et al. [42]. The chromosomal classification used in this study is based on the karyotype ordination described by Bruschi et al. [43] and Bruschi et al. [45]. However, this difference in chromosomal ordination did not represent a real cytogenetic variation among karyotypes of the *P. distincta* and *P. bahiana*.

The telocentric chromosome pairs observed in the *P. tarsius* karyotype are apparently restricted to species of the *P. tarsius* group. These karyotypic traits were detected in *P. camba*, another species within this phenetic group, and this condition was noted as indicative of a possible karyological synapomorphy for the *P. tarsius* group [41]. Currently, the *P. tarsius* group includes *P. camba*, *P. neildi*, *P. trinitatis* and *P. tarsius* [34], although cytogenetic data from *P. neildi* and *P. trinitatis* are necessary to confirm this hypothesis. However, the chromosome complement of one population of *P. tarsius* from Peru described by Bogart [47] showed exclusively bi-armed pairs. If the population of *P. tarsius* from Peru analyzed in that study corresponds to the same taxonomic unit as the Brazilian and Ecuadorian populations analyzed here, the synapomorphy proposed by Paiva et al. [41] should be reevaluated. We have not discarded the possibility that this inconsistency may be due to misidentification of the Peruvian population.

The study of the distribution of heterochromatin in the genomes of these four species using the C-banding method and fluorochrome-specific base staining revealed the presence of a considerable number of non-centromeric blocks, primarily in the *P. vaillantii* karyotype. The C-banding pattern of *P. distincta* observed here is coincident with the arrangement of heterochromatic regions described by Gruber et al. [44]. The metacentric chromosome pair 8 showed pericentromeric heterochromatin in both arms in karyotypes of the *P. tarsius*, *P. distincta* and *P. bahiana* analyzed here and could be a chromosome marker in karyotype of this species. In the *P. vaillantii*, the pair 8 showed C-positive band in interstitial region, noticeable feature of this karyotype.

Fluorochrome staining after C-banding permitted the identification of an AT-rich class of heterochromatin in the C-positive blocks of the karyotype of *P. vaillantii* as well as in the centromeric regions of the majority of *P. tarsius* and *P. bahiana* chromosomes. Consistent with previous suggestions, the MM markers were coincident

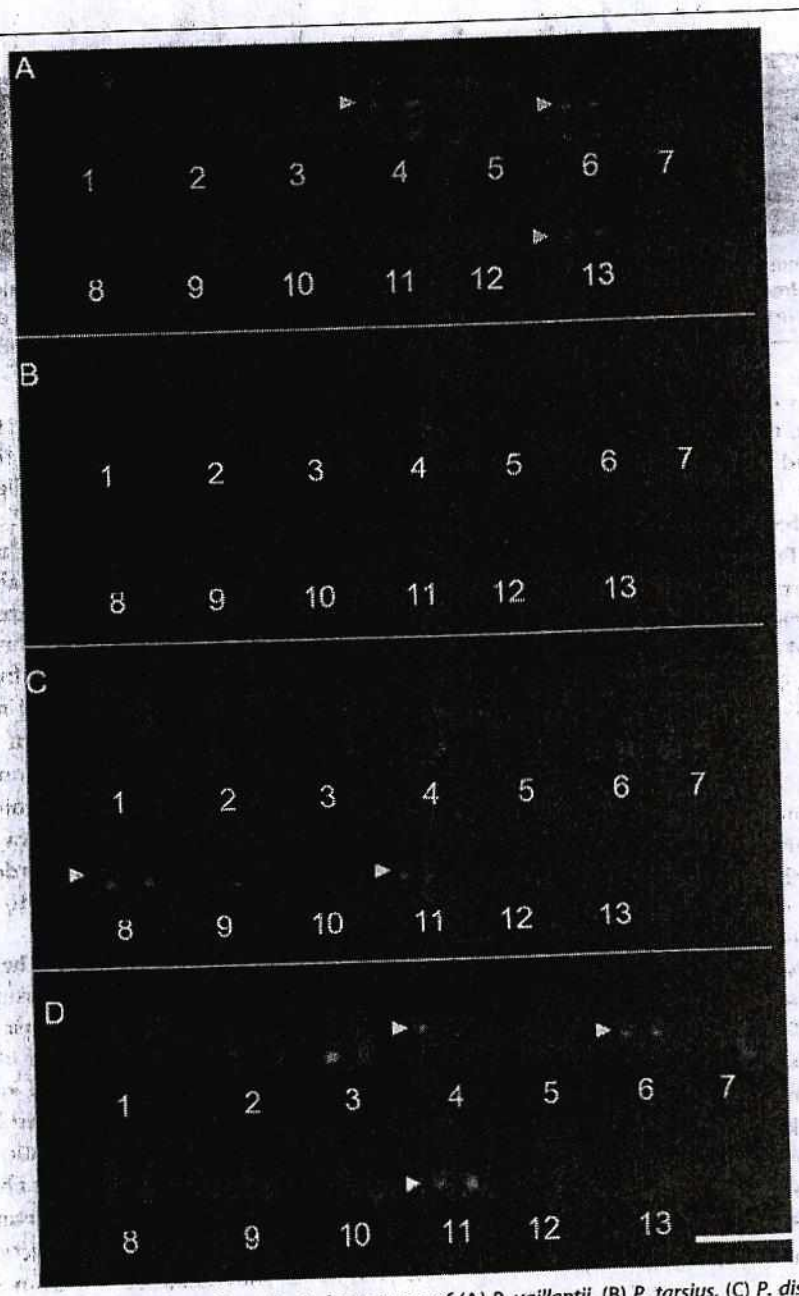

Figure 6 ***In situ*** **hybridization with the telomeric probe in karyotypes of (A)** ***P. vaillantii,*** **(B)** ***P. tarsius,*** **(C)** ***P. distincta*** **and (D)** ***P. bahiana.*** The arrowhead indicates interstitial telomeric sequence (ITS) adjacent to constitutive heterochromatin (het-ITSs). Bar = 3 μm.

with secondary regions. Similar brilliant signals with DAPI and MM staining in the centromeric regions of the majority of chromosome pairs in the *P. distincta* karyotype and in some chromosome pairs of *P. bahiana* could be explained by the presence of similar amounts of AT and GC bases within the repetitive sequences present in this region of heterochromatin. Similar labeling was reported in *Eleutherodactylus atkinsi* [14], *E. pantone* and *Pristimantis terraebolivaris* and in *Sphaenorhynchus lacteus* [16].

P. tarsius showed rDNA cluster detected in chromosome 1, as well as, *P. camba,* another species in the *P. tarsius* group, also showed NOR in same chromosome pair and in addition the clusters detected in chromosome pair 5 [41]. Future cytogenetic analysis in *P. neildi* and *P. trinitatis* could be provides better compression about evolutionary dynamics of this chromosomal marker in this group.

The *P. distincta* karyotype showed NOR in chromosome pairs 1 and 9, corroborating a previous report [44], whereas *P. bahiana* showed NOR in pair 9, consistent

with the findings of Barth et al. [42], as well as an additional marker in one homolog of pair 10 found in in two of eight specimens sampled in this study. Based in this scenario, Gruber et al. [44] proposed that the NOR in chromosome pairs 1 and 9 are conserved in the *P. burmesteri* group. The *P. bahiana* karyotype features reported in the present study provide novel insights into NOR evolution within this group. Based on the phylogenetic inferences [33], the *P. burmeisteri* group represents a monophyletic clade in which *P. bahiana* is a sister species of the remaining species within the group (*P. distincta*, *P. burmeisteri*, *P. iheringii* and *P. tetraploidea*). *Phyllomedusa sauvagii*, which remains unassigned to any phenetic group, is a sister species of the *P. burmeisteri* group. Here, we suggest that the NOR in chromosome pair 9 is the plesiomorphic karyotype condition. This idea is supported by the presence of this condition in *P. bahiana* and in the karyotype of *P. sauvagii* [39], both of which carry NOR on chromosome pair 9.

The NOR patterns of the species karyotyped in this work are consistent with the known notable characteristics of rDNA clusters in the *Phyllomedusa* genus; many cases of multiple NOR sites among the karyotyped species have been reported by other authors [39-44]. NOR position in the genome has been successfully used as a chromosomal marker in comparative cytogenetic studies in many vertebrates groups, and the possible role of NORs as hotspots of recombination during evolution has been widely discussed [3,9,48,49].

The origins of the ITSs detected in our analysis cannot be explained by assuming that ITSs represent remnants of structural chromosome rearrangements that occurred during the evolution of these karyotypes. Our arguments denote of expressive evolutionary chromosome conservation observed among the species of this genus with known karyotypes. If we consider the karyotype data in the light of the phylogeny proposed for the genus [33], the presence of ITSs in these karyotypes cannot be imputed to any traits of the chromosome rearrangements that are perceptible by classical cytogenetic. Indeed, the presence of ITSs in vertebrate genomes has been explained as a relic of the reorganization of chromosome architecture that occurred during the evolution of individual karyotypes. Through study of the comparative cytogenetic of many groups, it is possible to discern the remnants of chromosomal rearrangements from fission and/or fusion events [10,50,13-16] or chromosomal inversions [10,11] inferred from ITS signals. In amphibians, the presence of ITSs provides evidence of rearrangements that occurred during karyotype evolution in species of the Terrarana group [14] and has been invoked recently to explain the reduction in chromosome number in Dendropsophini [16].

Despite the fact that these suggestions are strongly supported by evidence from a number of organisms, the intrachromosomal telomeric repeats observed in the karyotypes of *P. vaillantii, P. distincta and P. bahiana* could be the result of amplifications of (TTAGGG)n repeats that occurred independently during the chromosomal evolution of these species. In opposition to the idea that the distribution of ITS in the karyotype represents remnants of ancient rearrangements, their distribution has usually been considered to be a result of the occurrence of double-strand breaks in the germ line [31]. Despite the fact that the precise molecular model accounting for these features is unclear, many studies have attributed the presence of widely distributed intrachromosomal ITSs to the insertion of telomeric DNA during the repair of double-strand breaks by the non-homologous end-joining pathway (NHEJ) [18,22,31,51]. Telomeric repeats are subject to evolutionary forces that can amplify the number of repeats or homogenize the repeat sequences according to the dynamics of evolution of the repetitive DNA sequences [19].

Non-terminal telomeric repeats were primarily detected in centromeric regions and coincided with regions of heterochromatin blocks (het-ITS). This interesting pattern has previously been reported in amphibians of the *Aplastodiscus* genus [26,28] and has been widely reported in rodents [24,52] and in plants of the *Solanum* genus [29,53].

ITS are frequently associated with heterochromatin regions, and previous investigations have shown that these repeats represent a primordial component of the repetitive DNA in cetacean [20,54], fishes [21] and rodents [22,27]. The conspicuous hybridized signals detected in some chromosome pairs such as the homologs of pair 13 of *P. vaillantii* indicate that (TTAGGG)n repeats represent a major motif in repetitive DNA.

Conclusion

The presence of telocentric pairs in species of the *P. tarsius* group is an interesting feature observed within this genus: this species showed the same chromosomal number as another species, and the telocentric pairs found in species of this group are homologs of the subtelocentric pairs found in other karyotypes. In this case, it is possible that the short arms were lacking in the *P. tarsius* clade. Unlike the other species analyzed in this paper, the *P. tarsius* karyotype was the only karyotype that did not exhibit a hybridization signal for ITS. We suggest that additional experiments, including flow cytometric analysis and chromosomal painting should be conducted to better clarify the origins of this apparent autoapomorphy within the *P. tarsius* group.

In the karyotypes of *P. vaillantii, P. distincta and P. bahiana*, the most parsimonious explanation to the presence of ITS could be results of the amplifications of

Table 1 Species of *Phyllomedusa* analyzed, sample number (N) their respective sampling localities and voucher number

Species	N	Locality	Voucher
P. vaillantti	01	Jacareacanga, Pará, Brazil.	ZUEC 15998
P. vaillantti	05	Yasuní, Provincia del Orellana, Ecuador	QCAZ 43241-43247
P. vaillantti	04	Porto Velho, Rondônia, Brazil	ZEUC 17035; 17036
P. tarsius	05	Reserva Ducke, Manaus, Brazil	ZUEC 16201-16204
P. tarsius	05	Yasuní/Provincia del Orellana, Ecuador	QCAZ 47276; 47278-47280
P. distincta	03	Iporanga, São Paulo, Brazil	ZUEC 17033-17035
P. bahiana	08	Alagoinhas, Bahia, Brazil	ZUEC 20656-20662

ZUEC: Museu de Zoologia "Prof. Dr. Adão Cardoso", do Instituto de Biologia da Universidade Estadual de Campinas (UNICAMP), São Paulo, Brazil; QCAZ: Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ), Quito, Ecuador.

(TTAGGG)n repeats that occurred independently during the chromosomal evolution of these species. The results presented in this study will contribute to the understanding of the mechanisms of chromosomal evolution that have operated in *Phyllomedusa* genus, and provides evidences about the role of repetitive sequences in karyotypes diversification in vertebrates.

Methods

We analyzed populations of *P. vaillantii*, *P. tarsius*, *P. distincta* and *P. bahiana* sampled from Brazil and Ecuador localities (Table 1). The collection of specimens from Brazil was authorized by SISBIO/ Instituto Chico Mendes de Conservação da Biodiversidade under number 20266–1. Specimens sampled in Brazil were deposited in the Museu de Zoologia "Prof. Adão José Cardoso" (ZUEC), at Universidade Estadual de Campinas, São Paulo, Brazil and the vouchers of populations sampled in Ecuador were deposited in the Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ), Quito, Ecuador. The complete list of the species, localities sampled, number of individuals examined, and voucher numbers are provide in Table 1.

The chromosomal preparations were obtained from intestinal and testicular cells of individuals previously treated with colchicine (2%) for 4 h following procedures modified from King and Rofe [55] and Schmid [56]. The mitotic metaphases were stained with 10% Giemsa to karyotyping determination. The identification of heterochromatic regions was performed using C-banding technical followed Sumner [57] with modifications. To better characterize the heterochromatic regions, C-banded chromosomes were stained with fluorochrome AT-specific DAPI and GC-specific Mytramycin (MM). We detected the NORs positions using the Ag-NOR method [58].

The physical map of the rDNA genes and telomeric sequences were detected by Fluorescent "in situ hybridization" (FISH) experiments using specific probes and protocols. To detected rDNA genes, we used 28S fragment isolated by Bruschi et al. [45]. The probe was PCR-labeled with digoxigenin, hybridized according to Viegas-Péquignot [59] and the hybridized signal was detected with an anti-digoxigenin antibody conjugated with rhodamine (Roche). Telomeric sequences was detected using the telomeric PNA probe $(CCCTAA)_3$ (peptide nucleic acid - PNA -Applied Biosystems), kit performed following the manufactures' manual. Metaphases were photographed under n Olympus BX-60 microscope and analyzed using the Image Pro-Plus software, version 4 (Media Cybernetics, Bethesda, MD, USA). The chromosomes were measured and the centromere index (CI), relative length (RL), and centromere ratio (CR) were estimated. The chromosomes were ranked and classified according to the scheme of Green and Sessions [60].

Abbreviations

rDNA: Ribosomal DNA; ITS: Interstitial telomeric sequences; DAPI: 4 6-diamidino-2-phenylindole; NOR: Nucleolus organizer region; FISH: Fluorescence *in situ* hybridization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DPB prepare and analysis of chromosomal data and drafted the manuscript. MR and ABZ helped prepare for the cytogenetic analysis. APL helped to collect and identify the specimens. SMRP designed and coordinated the study and helped draft the manuscript. All authors have read and approved the final manuscript.

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