Tesis de Doctorado en Ciencias Biológicas

PEDECIBA

Subárea Genética

Caracterización genética del bagre negro *Rhamdia quelen* en cuencas de Uruguay

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Fuentes de financiación que permitieron el desarrollo de esta Tesis de Doctorado

Programa de Desarrollo de las Ciencias Básicas (PEDECIBA), a través de Alícuotas Anuales y el programa de financiación de Cursos en el Exterior.

Agencia Nacional de Investigación e Innovación, a través de los Proyectos María Viñas PR_FMV_2009_1_2793 y FMV_2014_104718, Beca de Doctorado y el Sistema Nacional de Investigadores.

Comisión Sectorial de Investigación Científica a través del Proyecto de iniciación "Identificación de unidades evolutivas de *Rhamdia quelen* con aplicación en acuicultura y conservación de la biodiversidad en el Uruguay" y el Programa de Movilidad e Intercambios Académicos 2018.

Agradecimientos

Esta Tesis es el producto de varios años de formación, por lo tanto, hay varias personas a las que agradecer.

- En primer lugar, a Carmen y Graciela por su dedicación, apoyo constante a mi formación académica, también estando siempre atentas al plano personal. Destacar el compromiso demostrado por Carmen que pese a estar a miles de kilómetros, siempre estuvo pendiente de mi proceso e intentando ayudar en la medida de lo posible.
- A los Dres. Enrique Lessa, Fernando Pérez Miles y Marcelo Loureiro por aceptar ser parte del Tribunal y por la disposición a la hora de la corrección de la Tesis y la coordinación de la defensa. A la Dra. Susana González y Héctor Romero por formar parte de mi Comisión de Seguimiento. Al Dr. Arley Camargo y nuevamente a la Dra. Susana González por aceptar ser candidatos a integrar el Tribunal.
- A Cristhian y Wilson por dedicar parte de su tiempo, horas de sueño y recursos a la colecta de bagres. A Verónica por el apoyo en Laboratorio y siempre estar a la orden para dar una mano en lo que haga falta.
- A todos los compañeros de la Sección Genética Evolutiva.
- Al Dr. Paulino Martínez y Dra. Belén Gómez Pardo por colaborar y aceptar participar en los dos Proyectos María Viñas, que fueron parte fundamental de que esta investigación sea llevada a cabo. Al grupo de ACUIGEN que desde 2012 me ha abierto la puerta y además de permitirme crecer mucho desde lo académico, me brindó la oportunidad de conocer un grupo humano excelente. A Susana Sánchez, Lucía Insúa, Vanessa Pérez, María Portela Vázquez y María López Villar, que me enseñaron, me ayudaron y me apoyaron en el trabajo de Laboratorio realizado en la Universidad de Santiago de Compostela, Campus de Lugo. A los Dres. Manuel Vera, Miguel Hermida, Francesco Maroso y Adrián Millán, así como también a Adrián Chiclana y Andrés Blanco que fueron fundamentales para poder realizar el trabajo de RADseq y explotar a fondo sus resultados. También agradecerle al Dr. Carlos Fernández y al Dr. José Antonio Álvarez Dios que me están apoyando en el trabajo de Transcriptómica. A los Dres. Raquel Fernández y Jorge Guerra por compartir conmigo sus conocimientos. A Mónica Otero y Sonia Gómez por hacer el trabajo en el Departamento más fácil.
- Agradecerles especialmente a mis padres, que me apoyaron siempre, fueron indispensables para que pueda dedicarme a lo que me gusta y mal o bien vivir de ello (por ahora). Además, por cuidarme y soportarme en las malas.
- A Ileana, que desde que está en mi vida me ha apoyado en lo académico y en lo personal. Incluso le ha tocado ser pierna derecha. Además, agradecerles a mis suegros y cuñados.
- A mis hermanos, pero sobre todo a los sobrinos que me han dado.
- A amigos en general (a los del fútbol, a los de facultad, a los de Lugo, a los de Italia, a los de REGENEC).
- Finalmente, a la DINARA por los ejemplares donados y a todos aquellos que se acuerdan de mi y me traen algún trozo de bagre, especialmente a Marcelo Loureiro y Franco Teixeira que realizaron aportes importantes.

Resumen

Rhamdia quelen pertenece al Orden Siluriformes y como varios representantes del Orden, estos peces constituyen recursos acuáticos valiosos de la región Neotropical, de importancia en pesquerías y para el desarrollo de la acuicultura. La sistemática de esta especie ha sido muy controvertida entre los distintos estudios de tipo sistemático basados en rasgos morfológicos contrapuestos a los moleculares y citogenéticos. En este marco se justifica la necesidad de desarrollar un estudio con el objetivo de caracterizar la estructura genética de las poblaciones de *R. quelen* en las mayores cuencas de Uruguay.

En este estudio se realizó un primer abordaje filogeográfico basado en distintos marcadores de ADN mitocondrial y microsatélite, con el objetivo de investigar el patrón de diferenciación genética de *R. quelen* a escalas macro, meso y microgeográfica. A escala macrogeográfica se identificaron siete linajes mitocondriales que componen el complejo de especies *R. quelen*, cuya divergencia es explicada principalmente por eventos vicariantes dentro y entre las principales cuencas de la región. A mesoescala se constató la presencia de cinco de estos linajes en el sistema de cuencas LP-PM-AO (Río de la Plata, sistema de lagunas Patos-Merín, más arroyos y lagunas que desembocan en la costa suroeste del Océano Atlántico), tres de ellos (*Rq2, Rq4 y Rq6*) presentes en las cuencas de Uruguay. A microescala geográfica se identificaron tres poblaciones bien diferenciadas en las lagunas costeras analizadas (L. del Sauce, L. de Rocha y L. Castillos).

Con el objeto de generar loci microsatélites específicos de *R. quelen* se realizó un rastreo genómico de estos marcadores mediante pirosecuenciación. El análisis de un 0,02 % del genoma identificó 13.552 secuencias de tipo microsatélite. El ensayo de 30 marcadores seleccionados permitió generar un panel de 10 loci informativos en *R. quelen*, de utilidad en abordajes de diversidad genética y de parentesco en estudios evolutivos y aplicaciones en acuicultura en esta especie.

En un tercer abordaje se realizó un análisis de diversidad genética poblacional en el sistema de cuencas LP-PM-AO, basado en los 10 loci microsatélite nucleares generados en el marco de esta Tesis. Se hallaron evidencias de hibridación histórica entre los linajes mitocondriales *Rq4* y *Rq6* de *R. quelen*, previo a la conformación geológica actual de las cuencas. El patrón de diversidad genética de *R. quelen* en las cuencas de Uruguay se ajustó a la distribución geográfica de las cuencas de Norte a Sur y de Este a Oeste. Además, se constató que las poblaciones de *R. quelen*

de Uruguay analizadas en las lagunas costeras presentan características de diversidad genética e histórico demográficas diferentes a las poblaciones asociadas a ambientes fluviales.

Además, se realizó un análisis genómico poblacional de *R. quelen* en Uruguay a través de la técnica de 2bRAD-seq con el objetivo de evidenciar huellas de selección. Este análisis permitió genotipar 17.575 loci polimórficos, a partir de los cuales se identificaron 75 loci con huellas de selección divergente entre las muestras del Norte (Río Uruguay) y Sur (lagunas costeras) del país. A su vez, el análisis de estructuración genética permitió identificar dos clústeres genómicos altamente diferenciados en las cuencas de Uruguay, asociados al patrón de distribución geográfico, siguiendo un eje Norte-Sur. Además, se han identificado genes candidatos ligados a huellas de selección divergente asociados al desarrollo, a funciones celulares y reproducción. Finalmente, la subestructura genética hallada en base marcadores genómicos de 2bRADseq, en comparación con los análisis de marcadores microsatélites, permitió confirmar la presencia de cinco poblaciones bien diferenciadas en las cuencas de Uruguay.

Esta Tesis permitió confirmar que el complejo de especies *R. quelen* está compuesto por varios linajes mitocondriales que habrían hibridado en el pasado. Sin embargo, la estructura poblacional estaría principalmente determinada por dos clústeres genómicos en incipiente especiación, que presentan un patrón de distribución semejante al de los linajes mitocondriales.

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Introducción

Antecedentes de *Rhamdia quelen*

Dentro de los Siluriformes Neotropicales, el género *Rhamdia* se distribuye desde el norte de México al centro de Argentina (Perdices et al., 2002). El género, antes considerado como parte de la familia Pimelodidae, fue reubicado en la familia Heptapteridae por Lundberg & McDade (1986). Esta familia tiene representantes en todas las áreas de endemismo del Neotrópico, lo que la convierte en un grupo potencial para análisis de tipo biogeográfico (de Pinna, 1998), resultando de utilidad a la hora de definir las unidades básicas de estudios biogeográficos y sus relaciones. Sin embargo, hasta la fecha no ha sido tomada en cuenta en este tipo de análisis debido al escaso conocimiento que se tiene de la relación intergénero en varios de los taxa que la integran (Bockmann & Guazzelli, 2003).

Las sinapomorfías que definen el género *Rhamdia*, según Silfvergrip (1996), son: 1) cuerpo revestido por piel o cuero, sin escamas; 2) tres pares de barbas (bigotes) sensitivos de diferente tamaño entre sí; 3) una larga aleta adiposa con el extremo posterior libre; 4) fontanelas posteriores cerradas; 5) procesos humerales bien desarrollados y ausencia de dientes en el vómer.

Adicionalmente, su coloración puede variar desde un amarillo pálido hasta el gris, con el vientre siempre más claro, pudiendo presentar manchas. Los ejemplares adultos de las especies del género *Rhamdia* pueden alcanzar los 40 cm de largo total y 2 Kg de masa corporal. No presentan dimorfismo sexual aparente, sin embargo, en épocas reproductivas el orificio urogenital presenta diferencias entre sexo (Gomes et al., 2000, García, 2009).

La sistemática del género ha sido controvertida desde la primera revisión sistemática basada en caracteres morfológicos. En dicho trabajo, Silfvergrip (1996) sinonimizó más de 100 especies dentro del género, de las cuales 46 fueron sinonimizadas a la especie *Rhamdia quelen* (Quoy & Gaimard, 1824). Posteriormente, Perdices et al. (2002) realizaron un análisis filogeográfico en especies de *Rhamdia* de Mesoamérica, mediante marcadores mitocondriales (citocromo b (cyt b) y ATP sintasa 8 y 6 (ATPasa 8/6)). En el mismo concluyeron en la existencia de dos grupos monofiléticamente recíprocos, conformados por las especies de *Rhamdia* trans-andinas y cis-andinas, que habrían divergido a causa del levantamiento de los Andes. Como consecuencia de este resultado, se restringe el límite norte de la distribución de *R. quelen* a la cuenca del Orinoco, ya que esta especie ha sido originalmente descrita para la región cis-andina. Además, en este

trabajo se propone la validez de *R. cinerascens* (Günther, 1864) y *R. guatemalensis* (Günther, 1864), ya que éstas forman clados independientes, quedando externas a *R. quelen* y *R. laticauda*. Tanto *R. cinerascens*, como *R. guatemalensis*, habían sido sinonimizadas previamente por Silfvergrip (1996). A su vez, Hernández et al. (2015) en un análisis morfológico y molecular (basados en el gen cyt b) confirmaron la validez de estas dos últimas especies y también propusieron la validez de *R. saijaensis* (Rendahl, 1941) (también sinonimizada a *R. quelen* por Silfvergrip (1996)). Estos autores enfocaron su análisis en la región trans-andina y el extremo noroeste de la región cis-andina. Por otro lado, en la región cis-andina específicamente en la cuenca del Río Iguazú, Garavello & Shibatta (2016) basados en evidencia morfológica y citogenética proponen quitar a *R. branneri* (Haseman, 1911) y *R. voulezi* (Haseman, 1911) de la sinonimia de *R. quelen* y considerarlas como especies válidas del género *Rhamdia*. Por otro lado, el estudio a nivel citogenético de poblaciones de *R. quelen* de Brasil por Martinez et al. (2011), constató un número cromosómico constante (58 cromosomas), aunque con diferencias cariotípicas entre poblaciones del Paraná alto ((22 metacéntricos (m) + 18 submetacéntricos (sm) + 12 subtelocéntricos (st) +6 acrocéntricos (a)) y las de Araguaia (18m + 18sm + 14st + 8a). Además, estos autores encontraron variaciones en la ubicación del ADN ribosomal 18S y 5S, evidenciando la existencia de reordenamientos cromosómicos generados por inversiones pericéntricas. Según estos autores, estas variaciones cromosómicas fijadas en las poblaciones serían la causa de la divergencia de estas poblaciones de *Rhamdia*. Las contradicciones a nivel taxonómico junto a las evidencias de variación cariotípica, conducen a la hipótesis de que *R. quelen* es un complejo de especies (Martinez et al., 2011). Los análisis filogenéticos basados en marcadores moleculares publicados hasta el momento se centraron en la región norte de la distribución de *R. quelen*, especialmente la región trans-andina. Sin embargo, el sistema de cuencas integrado por el Río de la Plata, el sistema de lagunas Patos-Merín, así como arroyos y lagunas que desembocan en la costa suroeste del Océano Atlántico (LP-PM-AO; extremo sur de la distribución de *R. quelen*); aún no ha sido abordado.

Relevancia de *R. quelen* para la pesca artesanal y la acuicultura

Rhamdia quelen comúnmente conocido como “bagre negro”, es explotado a través de la acuicultura y la pesca artesanal a lo largo de toda su distribución (Vaz et al., 2010, Scaranto et al., 2018); presentando características que lo hacen un buen modelo de cultivo, con alto rendimiento

de la carcasa, alta tasa de crecimiento, fácil manejo reproductivo y una alta relación proteína/grasa (Luchini, 1988; Gomes et al., 2000; Vaz et al., 2010). A su vez, Infante & Manchado (2007) han planteado que el desarrollo de una acuicultura sostenible requiere la evaluación y el conocimiento de la diversidad genética de las poblaciones en cautiverio, a fin de evitar los efectos negativos derivados de la endogamia. Khaw et al. (2012) afirman que el comenzar con una población base con amplia variación genética es casi la marca registrada para el éxito en un programa de mejora genética. Sin embargo, pocos trabajos han evaluado la estructura poblacional de *R. quelen*, lo que permitiría una correcta explotación del recurso (Vaz et al., 2010). En este sentido, Loureiro et al. (2013) propusieron a esta especie como prioritaria para la conservación en base a su explotación en la acuicultura y su valor cultural.

Filogeografía

La filogeografía es la disciplina que estudia los principios y procesos que inciden en el patrón de distribución de los distintos linajes genéticos, por tanto, es considerada como una disciplina puente entre los procesos micro y macroevolutivos (Avise et al., 1987; Avise, 2004; Piorski et al., 2008). De ahí que fenómenos a nivel poblacional tales como mutación, selección natural o deriva génica pueden evidenciar patrones demográficos y relaciones filogenéticas a nivel de especie y niveles taxonómicos más altos (Bermingham & Moritz, 1998). Aunque existen excepciones, el fenómeno de estructuración genética se da en la mayoría de las especies, en mayor o en menor medida (Balloux & Lugon-Moulin, 2002; Avise, 2009). Dicha estructuración, puede darse tanto en animales de baja dispersión, en donde la divergencia entre individuos suele estar relacionada directamente con la distancia geográfica, como en especies con alta capacidad de dispersión, donde la estructura poblacional puede estar determinada por barreras geográficas o por filopatría (Avise, 2009). Dentro del escenario de especiación alopátrica existen dos hipótesis que explican la estructura encontrada en los diferentes taxa, dispersión y vicarianza. La primera ocurre cuando un conjunto de individuos supera una barrera preexistente pudiendo diferenciarse del taxón original, generando una nueva población o subpoblación (Avise, 2000). Mientras que la vicarianza implica la subdivisión y fragmentación poblacional de un taxón por el surgimiento de una barrera que puede conducir a la diversificación en dos o más grupos (Crisci et al., 2009). En relación a esto, Lieberman (2000) introduce el término geodispersión para referirse a los eventos geológicos que

generan la dispersión de un grupo de taxa, por ejemplo, la captura de cuencas. Análisis filogeográficos basados en datos moleculares de estas poblaciones pueden dar cuenta de la estructura poblacional, pudiendo delimitar la distribución de las especies (Barluenga & Meyer, 2005; Piorski et al., 2008), reconocer poblaciones híbridas e introgresión (Vera et al., 2013), así como también, dar respuesta a preguntas del tipo biogeográfico, tales como: evidenciar fenómenos de vicarianza crípticas en la historia biológica de la biota (eventos pasados de fragmentación) expansión del rango de distribución y colonización (Avise, 2000; Frankham et al., 2010; Allendorf et al., 2013).

Unidades prioritarias para la conservación

La pérdida de hábitat para las especies y la fragmentación ha puesto en riesgo la diversidad biológica en general, a través de un incremento en el riesgo de deriva genética, reducción de la variabilidad genética a través de cuellos de botella, inhibiendo de este modo el flujo génico y perjudicando los procesos de adaptación de las especies (Templeton et al., 2001). Es así que surge la necesidad de implementar políticas de conservación, para esto es necesario conocer la estructura poblacional del taxón. El desconocimiento de los parámetros poblacionales específicos o la toma de decisiones erróneas al definir las unidades prioritarias, pueden conducir a no tomar medidas para proteger un taxón desconocido en peligro de extinción, el derroche de esfuerzo en proteger especies abundantes o híbridos, o a la no conservación de poblaciones altamente divergentes que podrían ser utilizadas para contrarrestar efectos negativos de la endogamia (Frankham et al., 2004). Además, cuando se trata de una especie explotada en pesquerías y acuicultura la conservación de la diversidad es prioritaria para contrarrestar los efectos de la deriva génica y endogamia, asegurar el progreso de respuesta a la selección en programas de mejora y para mantener la integridad genética de los stocks naturales (Welcomme & Barg, 1997; Infante & Manchado, 2007; Matusse et al., 2016). A su vez, se ha propuesto que niveles bajos de diversidad genética podrían representar un riesgo para la eficacia biológica de la especie o la viabilidad de las poblaciones (Vrijenhoek, 1994; Pauls et al., 2013).

En este sentido, los estudios clásicos de enfoque filogeográfico permiten identificar ESUs (del inglés, "Evolutionarily Significant Units"). Este término ha sido originalmente propuesto por Ryder (1986), desde entonces, la definición y delimitación de estas unidades se ha basado en distintos criterios, que varían sobre todo en el énfasis evolutivo y ecológico (Casacci et al., 2014; Jensen et al., 2014). El criterio de Moritz (1994) es el más comúnmente aplicado (de Guia & Saitoh, 2006; Jensen et al., 2014), en donde se definen a las ESUs como un grupo de organismos que han estado aislados de sus conspecíficos, evolucionando de forma independiente y presentando una diversificación genética importante al resto de las poblaciones de la especie. También, podemos detectar MU ("Management Units"), definidas como aquellas poblaciones en las que su dinámica está marcada por las tasas de nacimientos y muertes, no tanto por las migraciones (Moritz, 1994; Paetkau, 1999; Jensen et al., 2014). De esta manera podemos determinar subpoblaciones o stocks, ya que según Moritz (1994), genéticamente éstos tendrían las mismas características que las MUs. La identificación de estas unidades es prioritaria para la conservación, así como también es conocimiento básico para el desarrollo de emprendimientos de mejora genética, debido a que las distintas unidades podrían presentar distintas adaptaciones locales, poseer diferencias genéticas que promuevan distintas tasas de crecimiento, resistencia a enfermedades, fertilidad y a nivel de especie adaptabilidad o supervivencia (Hoarau et al., 2002; Jensen et al., 2014; Abdul-Muneer et al., 2017). La aplicación de distintos marcadores genéticos moleculares en los últimos años ha permitido identificar unidades de conservación en distintos taxa (Ardura et al., 2013; Kocovsky et al., 2013; Yannic et al., 2016; van Vuuren et al., 2017).

Marcadores mitocondriales permiten evidenciar linajes genéticos ancestrales

El ADN mitocondrial en vertebrados posee aproximadamente 17 kb, conteniendo, además de la región control, 37 secuencias donde 22 corresponden a ARN de transferencia, dos ARN ribosomales (12s y 16s) y 13 ARN mensajeros, por lo que aproximadamente todo el genoma se encuentra destinado a funciones codificantes, siendo muy raro encontrar intrones, secuencias repetidas o pseudogenes (Boore, 1999; Avise, 2000). Desde los inicios de la aplicación de marcadores moleculares, secuencias de ADN mitocondrial han sido elegido tradicionalmente en varios análisis filogeográficos (Avise et al., 2016) debido a su rápida evolución, entre 5 y 10 veces mayor que los genes nucleares (Avise, 2000). Éste es de herencia predominantemente matrilineal

en eucariotas, aunque existen algunas evidencias de ocurrencia de heteroplasmia (Peng et al., 2018). Su rápida evolución se debe a una alta tasa de mutación a causa de un ineficaz sistema de reparación de errores en la replicación y a los altos niveles de concentración de especies reactivas del oxígeno en la mitocondria que generan un ambiente inestable para la molécula de ADN, así como a la general ausencia de recombinación y de proteínas histónicas (Avise, 2009; Castellana et al., 2011). Este tipo de marcador de rápida evolución es indicado para determinar relaciones entre organismos estrechamente relacionados, es decir, entre especies hermanas o poblaciones (Brown et al. 1979; Hey & Machado 2003; Jia et al., 2012). El gen citocromo b (cyt b), de nivel moderado de conservación evolutiva, es uno de los marcadores mitocondriales más comúnmente utilizados (Duchêne et al., 2011). Este marcador ha sido extensamente usado en análisis filogenéticos y filogeográficos en varias especies animales (Pons et al., 2006), así como también en peces (Koblmüller et al., 2006; Concheiro Pérez et al., 2007; Ma et al., 2010), y particularmente en Siluriformes (Perdices et al., 2002; Singh et al., 2012; Unmark et al., 2012; Hernández et al., 2015; Ochoa et al., 2015). El gen codificante para la subunidad I de la citocromo c oxidasa (COI) es también un marcador empleado en varios estudios evolutivos, ya que ha sido propuesto como un marcador ideal para "barcoding" de especies (Duchêne et al., 2011). Varios análisis filogenéticos en COI han permitido detectar patrones de diferenciación dentro de especies y unidades de conservación en diferentes grupos de peces (Ardura et al., 2010; García et al., 2014; Rodríguez-Rey et al., 2017) y especialmente en Siluriformes (Paixão et al., 2018; Carvalho & Beheregaray, 2018).

Microsatélites, marcadores altamente polimórficos

Los microsatélites o SSR ("Single Sequence Repeat") son motivos de secuencias de ADN de 1-6 nucleótidos repetidos en tándem, con un largo de 20 a unos pocos cientos de bases, dispersos por el genoma. Éstos se caracterizan por ser codominantes, con herencia mendeliana, altamente polimórficos, con una tasa evolutiva por locus por generación entre 10^{-2}-10^{-6} (Chistiakov et al., 2006; Piorski et al., 2008). Estos marcadores han sido utilizados en análisis filogeográficos, en especies que han divergido recientemente o en análisis intraespecíficos, ya que a niveles taxonómicos mayores la homoplasia representa un riesgo debido a su alta tasa evolutiva. De esta forma los SSR han contribuido a identificar ESU y MU en estudios de conservación y gestión para

distintos organismos, en particular peces (Carvalho et al., 2012; Montes et al., 2012; Song et al., 2016). Sin embargo, se recomienda complementar con otro tipo de marcadores moleculares (por ejemplo: genes mitocondriales) para la identificación de ESUs (Chistiakov et al., 2006). Por otro lado, los SSR también tienen sus aplicaciones en los emprendimientos de mejoramiento genético, utilizados como herramienta en análisis de paternidad, análisis de trazabilidad en productos pesqueros, elaboración de mapas de ligamiento y estudios de asociación con caracteres de interés (Chistiakov et al., 2006; Miah et al., 2013; Abdul-Muneer, 2014). A su vez, los SSR permiten detectar híbridos e incluso una estimación de la introgresión, esto tiene su implicancia en la conservación de la diversidad genética, por ejemplo, en eventos de translocación de una población a otra y la posibilidad de que estas hibriden (Vera et al., 2013; Abdul-Muneer, 2014). Existen escasos estudios basados en microsatélites en *R. quelen* y hasta el momento se han basado mayormente en loci de amplificación cruzada, aislados en otras especies de Siluriformes (Ribolli & Zaniboni-Filho, 2009): *Pimellodela chagresi* (PC17 y PC97; Moeser & Bermingham, 2005); *Pseudoplatystoma corruscans* (Pcor y Pcor2; Revaldaves et al., 2005). En este sentido, las técnicas de secuenciación masiva de nueva generación (NGS, del inglés, "Next-Generation Sequencing") han permitido acceder a miles de marcadores de tipo microsatélite en una buena relación costo-eficiente en distintos organismos (Perry & Rowe, 2010).

RAD-seq una importante herramienta para genética de poblaciones

El estudio de los patrones o procesos de diferenciación genómica que aporte información útil a la conservación se denomina Genómica de la conservación (Avise, 2010). En este sentido, la principal diferencia entre genómica y genética de la conservación es el uso de un gran número de marcadores. El desarrollo de las plataformas de secuenciación masiva permitió el surgimiento de herramientas asociadas al desarrollo de miles de marcadores moleculares, particularmente SNP (en inglés, "Single Nucleotide Polymorfism") distribuidos por todo el genoma. A su vez, estás técnicas permiten el genotipado de cientos a miles de SNPs para varias muestras, esto es conocido como genotipado por medio de secuenciación (en inglés, "Genotyping By Sequencing" (GBS)). Una de las aproximaciones GBS más empleadas es la tecnología RAD-seq, cuyo término se refiere a la secuenciación de ADN asociado con sitios de restricción (en inglés, "Restriction site-Associated DNA sequencing"). La técnica básicamente consiste en la digestión del ADN total con

enzimas de restricción, la construcción de las bibliotecas y la secuenciación conjunta de gran número de individuos identificados mediante etiquetas de secuencia específicas (Rochette & Catchen, 2017). De este modo se reduce el genoma a una fracción sensiblemente menor que permite, por medio de las nuevas plataformas de secuenciación, acceder a la misma información genómica para un número importante de individuos a una efectiva relación costo-beneficio (Robledo et al., 2017). Una de sus principales características, es que para su desarrollo y genotipado no es necesario tener conocimiento de la secuencia completa del genoma de la especie estudiada, lo que la convierte en una herramienta potente de rastreo de variabilidad genética a escala genómica en especies no modelo. La tecnología tipo RAD-seq ha sido exitosamente utilizada en el campo de la genética de poblaciones, filogeografía, demografía, estudios de hibridación, filogenética, en la construcción de mapas genéticos y en análisis de asociación de rasgos de interés para la producción (Robledo et al., 2017; Rochette & Catchen, 2017). A su vez, esta técnica permite el escaneo genómico de variabilidad adaptativa y huellas de selección, una de las prioridades para la genómica de la conservación (Ouborg et al., 2010).

Hipótesis

El complejo de especies *R. quelen* en la región sur de su distribución está compuesto por diferentes linajes mitocondriales con distinta distribución geográfica determinando la estructura poblacional de este taxón.

Objetivo

Caracterizar la estructura genética de las poblaciones de *R. quelen* en las mayores cuencas de Uruguay mediante distintos marcadores moleculares.

Objetivos específicos

- Evidenciar la existencia de linajes mitocondriales dentro del complejo de especies *R. quelen* mediante secuencias de cyt b.
- Desarrollar marcadores de tipo microsatélite en *R. quelen*, a partir de Pirosecuenciación 454 de Roche de una fracción del genoma.
- Relevar el patrón de diferenciación genética de las poblaciones y linajes mitocondriales de *R. quelen* en Uruguay mediante marcadores de tipo microsatélites y SNPs.
- Evidenciar huellas de selección en las poblaciones de *R. quelen* en Uruguay por medio del análisis genómico de marcadores de tipo SNPs.

Capítulo I

Delimitación de linajes mitocondriales en el complejo de especies *R. quelen*

Environ Biol Fish
DOI 10.1007/s10641-017-0622-1

Species complex delimitation and patterns of population structure at different geographic scales in Neotropical silver catfish (*Rhamdia*: Heptapteridae)

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Received: 30 September 2016 / Accepted: 17 May 2017

Abstract The Neotropical catfish genus *Rhamdia* inhabits rivers and lakes from Mexico to Argentina. Previous studies have found that the taxonomy of this genus, as well as that of *R. quelen,* remains controversial. The present study aims to contribute to the understanding of *Rhamdia* systematics by delimiting putative species, and to elucidate the pattern of genetic differentiation of *Rhamdia* at different geographic levels within the cis-andean region. Species boundaries were defined by Generalized Mixed Yule Coalescent and Automatic Barcode Gap Discovery methods, and by phylogenetic analyses of *cytochrome b* (*cyt b*) sequences. Moreover, we performed phylogeographic analyses based on *cyt b* sequences and microsatellite markers. Patterns of differentiation were analyzed at three nested geographic levels: in the main cis-andean basins (macrogeographic scale); in the second major Neotropical basin system (mesogeographic scale), which encompasses La Plata basin, Patos-Merin basin, and the coastal lagoons draining to SW Atlantic Ocean; and finally, in the three most important coastal lagoons for artisanal fisheries in Uruguay (microgeographic scale). Sixteen species were found within *Rhamdia,* divided into two clades (cis- and trans-andean clades), each composed of eight putative species. Cis-andean *Rhamdia* species have probably diverged due to vicariance events occurring between and within basins since late Miocene-Pleistocene. Microgeographic scale analysis based on *cyt b* and microsatellite data revealed a high genetic structuring among the studied coastal lagoons. Mitochondrial and microsatellite markers enabled to identify three different populations, corresponding to the three coastal lagoons analyzed, which would have diverged recently and could be considered as different Management Units.

Keywords Phylogeography · *Rhamdia quelen* · Conservation units · Microsatellite loci · Mitochondrial marker

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Introduction

Neotropical catfish constitute a polyphyletic group including more than half of the Siluriformes species (de Pinna 1998; Sullivan et al. 2006). Within this order, the genus *Rhamdia* (Heptapteridae) is widely distributed across the Neotropical region, from South of Mexico to Argentina (Perdices et al. 2002). The Neotropical region can be subdivided into cis- and trans- andean, east and to the west of

Published online: 14 June 2017

the Andes chain of mountains, respectively (Albert and Reis 2011).

The taxonomic status of the genus *Rhamdia* is still controversial and not well established. Two decades ago, a morphological systematic revision relocated more than 100 species into 11 species (Silfvergrip 1996). This author synonymized 46 nominal species of *R. quelen* highlighting their intrapopulation diversity. However, Perdices et al. (2002), in a molecular systematic analysis of *Rhamdia* from Central America, proved that *R. cinerascens* and *R. guatemalensis* were valid species. Recently, morphological and molecular analyses of trans-andean species validated these two species and proposed, in addition, to remove *R. saijaensis* from the *R. quelen* synonymy (Hernandez et al. 2015). Also, Garavello and Shibatta (2016) based on morphological and cytogenetic evidences, reappraised *R. branneri* and *R. voulezi* from Iguazu basin. *Rhamdia quelen* has been proposed as a species complex based on a cytogenetic study done on three Brazilian populations by Martinez et al. (2011), who found that different chromosomal rearrangements created distinct karyotypic formulae, with particular geographic distribution. Although the cis-andean *Rhamdia* was previously studied by Hernandez et al. (2015), their work focused in the NE of this region (Colombian Pacific coast, Magdalena and Maracaibo basins). Thus, in the second major Neotropical basin system, which encompasses La Plata basin (LP), Patos-Merin basin system (PM), and the coastal lagoons draining to SW Atlantic Ocean (AO) –altogether, LP-PM-AO-, the *R. quelen* genetic structure remains unknown.

Rhamdia quelen, popularly known as silver catfish bears commercial relevance as resource for artisanal fisheries and aquaculture all along South America, and especially in Argentina, Brazil and Uruguay (Vaz et al. 2010). In this sense, *R. quelen* populations of the La Plata basin and south-western Atlantic Ocean coastal lagoons represent a valuable fishery resource for the local residents. However, few studies have been conducted to evaluate genetic structure of wild populations and to select aquaculture broodstocks (Vaz et al. 2010). Due to the aquaculture exploitation and fishery management, *R. quelen* was designated as a priority species for conservation programs (Loureiro et al. 2013). In this regard, species delimitation is crucial, not only to the fields of systematics and taxonomy, but also for studies of natural history and ecology, fishery management, aquaculture and traceability (Zhang and Hanner 2012). Fortunately, over the last years several algorithms that aid in delimiting species boundaries using DNA data have been developed (Kekkonen et al. 2015). Additionally, it is critical to delineate conservation units, which are defined as populations of organisms that can be managed as distinct entities, and ought to be a priority for conservation purposes. In this context, the present phylogeographic analysis will allow us to identify Management Units (MU) at a microgeographic scale (sensu Moritz 1994; Hurd et al. 2016).

The correct choice of molecular markers is critical in order to obtain reliable information and to draw valid conclusions from the analyses. Several phylogenetic analyses and population genetic studies have been carried out in fishes using the mitochondrial gene *cytochrome b* (*cyt b*) (Concheiro Pérez et al. 2007; Ma et al. 2010). This gene has also been employed specifically in catfish with the purpose of analysing phylogenetic relationships among families (Hardman 2005) and species (Perdices et al. 2002; Singh et al. 2012; Hernandez et al. 2015). In turn, nuclear microsatellite sequences are highly polymorphic and codominant markers, exhibit a mutational rate of 10^{-2}–10^{-6} per locus per generation and, when used in combination with mitochondrial markers, are appropriate to detect MUs in fish species (Chistiakov et al. 2006). Previous studies carried out in Siluriformes have proven the suitability of microsatellites for detecting population genetic structure at microgeographic scale (So et al. 2006; Pereira et al. 2009).

In this study, we use a phylogenetic, phylogeographic and genetic population approach to delineate *Rhamdia* species, and to elucidate the pattern of genetic differentiation of *Rhamdia* at different geographic levels within the cis-andean region. To this end, the two molecular markers mentioned above will be analyzed at different geographic scales: main cis-andean basins, LP-PM-AO, and finally, in three coastal lagoons important for artisanal fisheries. The results obtained will be an important contribution to the understanding of *Rhamdia* systematics and of the geological events that determined the pattern of genetic differentiation. Moreover, our results will set the ground for defining conservation units in the three Uruguayan coastal lagoons referred to before.

Material and methods

Sample collection and DNA extraction

All sampling protocols were approved by the CNEA (Comisión Nacional de Experimentación Animal) from Uruguay.

In the LP-PM-AO basin system (Fig. 1a), a total of 188 individuals of silver catfish were obtained from 24 collecting sites (1–24 Fig. 1b, Table 1). The three coastal lagoons where the silver catfish is most exploited by artisanal fisheries were thoroughly sampled: 1-Sauce lagoon (1-SL): 66 samples; 2- Rocha lagoon (2-RL): 24 samples; and 3- Castillos lagoon (3-CL): 39 samples (Fig. 1c). All collecting sites correspond to natural populations of silver catfish, excepting Villa Constitución (site 21, Fig. 1b), which constitutes a *R. quelen* hatchery for aquaculture development and fishery management of the Uruguayan Government. In addition to the collected samples, the following *cyt b* sequences were retrieved from GenBank and included in the *cyt b* data set: *Rhamdia* from cis-andean basin (Fig. 1a; Table 1), trans-andean *Rhamdia* species, and two sequences of *Pimelodella* as outgroup (Table 2). In addition, two *Rhamdella* individuals were sampled and included in the phylogenetic analyses as outgroup taxa (Table 2), since *Rhamdia* and *Rhamdella*, as well as *Pimelodella* are related genera, belonging to the Heptapteridae family (Bockmann and Guazzelli 2003). Total genomic DNA was extracted from adipose fin, liver and/or muscle tissue (preserved in ethanol 95%) using proteinase K digestion, followed by sodium chloride extraction and ethanol precipitation (modified from Medrano et al. 1990). The voucher specimens were stored in ethanol 95% at the Sección Genética Evolutiva, the Colección de Zoología de Vertebrados of Facultad de Ciencias and/or at the Museo de Historia Natural of Uruguay.

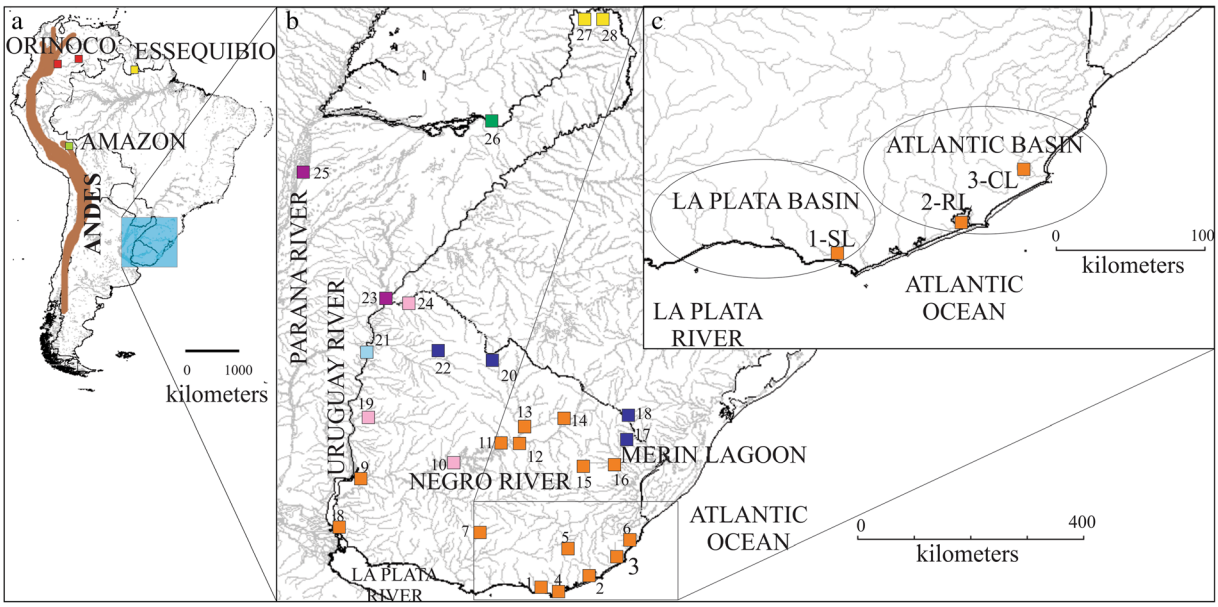

Fig. 1 Collecting sites of all *Rhamdia* samples analyzed. **a** Cis-andean region localities assayed in the phylogeographic analysis. The different basins are identified as: Orinoco (red); Essequibio (yellow); Amazon (green); and La Plata, Patos-Merin and coastal lagoon draining to SW Atlantic Ocean (LP-PM-AO) shaded in light-blue. **b** Distribution map of LP-PM-AO localities included in the population analysis: 1-Sauce lagoon (SL); 2-Rocha lagoon (RL); 3-Castillos lagoon (3-CL); 4-Blanca lagoon; 5-Salamanca caves; 6-Negra lagoon; 7-Talita stream; 8-Colonia department; 9-Abrojal stream; 10-Rincón del Bonete dam; 11-Paso 329; 12-Sauce Grande town; 13-Caraguatá town; 14-Paso Mazangano; 15-Quebrada de los cuervos; 16-Vergara town; 17-Sarandí del quebracho town; 18-Paso Centurión; 19-Queguay river; 20-Lunarejo stream; 21-Villa Constitución hatchery; 22-Arapey river; 23-Franquía lagoon; 24-Cuareim river; 25-Santa Lucía river; 26-Misiones, Cuñapirú; 27-Misiones, Ñandú stream; 28-Yacuy stream. The seven AMOVA groups identified in LP-PM-AO region based on the composition of *cytochrome b* lineages are highlighted as: yellow, green, violet, orange, blue, sky-blue and pink (see Results, Table 5, for details). **c** The three coastal lagoons assayed in the microgeographic analyses: 1-SL (Sauce lagoon), 2-RL (Rocha lagoon) and 3-CL (Castillos lagoon)

Table 1 Geographic distribution of cis-andean *Rhamdia* specimens studied: Major basin, Collecting site, Basin, Catalog sample number, GenBank accession numbers, lineage, *cytochrome b* haplotypes of *Rhamdia* analyzed at a mesogeographical scale. Individuals included in microsatellites analysis (X)

Major basin	Collecting site	Basin	Catalog sample number	GenBank accession number *cyt b*	Lineage	*Cyt b* haplotype	Microsatellite analysis
LP-PM-AO	1-Sauce lagoon	La Plata river	P1683				X
			P1697				X
			P1821				X
			P1699				X
			P1680	KP798791	*Rq6*	H04	
			P1689	KP798783	*Rq6*	H04	
			P1691	KP798781	*Rq6*	H04	
			P1693	KP798779	*Rq6*	H04	X
			P1983	KP798660	*Rq6*	H04	
			P1994	KP798643	*Rq6*	H04	
			P2076	KP798644	*Rq6*	H05	
			P1661	KP798807	*Rq6*	H06	
			P1664	KP798804	*Rq6*	H06	
			P1666	KP798802	*Rq6*	H06	X
			P1667	KP798801	*Rq6*	H06	
			P1668	KP798800	*Rq6*	H06	
			P1669	KP798799	*Rq6*	H06	X
			P1670	KP798798	*Rq6*	H06	X
			P1671	KP798797	*Rq6*	H06	X
			P1672	KP798796	*Rq6*	H06	
			P1673	KP798795	*Rq6*	H06	X
			P1674	KP798794	*Rq6*	H06	
			P1675	KP798793	*Rq6*	H06	
			P1679	KP798792	*Rq6*	H06	X
			P1682	KP798789	*Rq6*	H06	X
			P1687	KP798785	*Rq6*	H06	X
			P1688	KP798784	*Rq6*	H06	
			P1690	KP798782	*Rq6*	H06	
			P1692	KP798780	*Rq6*	H06	X
			P1694	KP798778	*Rq6*	H06	X
			P1696	KP798776	*Rq6*	H06	X
			P1701	KP798774	*Rq6*	H06	X
			P1728	KP798758	*Rq6*	H06	X
			P1729	KP798757	*Rq6*	H06	X
			P1733	KP798754	*Rq6*	H06	X
			P1734	KP798753	*Rq6*	H06	X
			P1735	KP798752	*Rq6*	H06	
			P1736	KP798751	*Rq6*	H06	X
			P1749	KP798750	*Rq6*	H06	
			P1974	KP798668	*Rq6*	H06	
			P1975	KP798667	*Rq6*	H06	

Table 1 (continued)

Major basin	Collecting site	Basin	Catalog sample number	GenBank accession number *cyt b*	Lineage	*Cyt b* haplotype	Microsatellite analysis
			P1976	KP798666	*Rq6*	H06	
			P1977	KP798665	*Rq6*	H06	
			P1979	KP798664	*Rq6*	H06	
			P1982	KP798661	*Rq6*	H06	
			P1985	KP798659	*Rq6*	H06	
			P1986	KP798658	*Rq6*	H06	
			P1988	KP798657	*Rq6*	H06	
			P1989	KP798656	*Rq6*	H06	
			P1990	KP798655	*Rq6*	H06	
			P2077	KP798645	*Rq6*	H06	
			P1981	KP798662	*Rq6*	H13	
			P1973	KP798669	*Rq6*	H15	
			P1731	KP798755	*Rq6*	H31	
			P1730	KP798756	*Rq6*	H32	X
			P1662	KP798806	*Rq6*	H58	
			P1703	KP798773	*Rq6*	H39	
			P1698	KP798775	*Rq6*	H40	X
			P1695	KP798777	*Rq6*	H41	X
			P1686	KP798786	*Rq6*	H42	X
			P1685	KP798787	*Rq6*	H43	X
			P1684	KP798788	*Rq6*	H44	X
			P1681	KP798790	*Rq6*	H45	X
			P1660	KP798808	*Rq6*	H46	
			P1665	KP798803	*Rq6*	H46	
			P1663	KP798805	*Rq6*	H46	X
LP-PM-AO	2-Rocha lagoon	SW Atlantic Ocean	P1837	KP798715	*Rq6*	H06	X
			P1838	KP798714	*Rq6*	H06	X
			P1839	KP798713	*Rq6*	H06	X
			P1840	KP798712	*Rq6*	H06	X
			P1841	KP798711	*Rq6*	H06	X
			P1842	KP798710	*Rq6*	H06	X
			P1843	KP798709	*Rq6*	H06	X
			P1844	KP798708	*Rq6*	H06	X
			P1845	KP798707	*Rq6*	H06	X
			P1846	KP798706	*Rq6*	H06	X
			P1847	KP798705	*Rq6*	H06	X
			P1848	KP798704	*Rq6*	H06	X
			P1892	KP798690	*Rq6*	H06	
			P1893	KP798689	*Rq6*	H06	
			P1894	KP798688	*Rq6*	H06	
			P1895	KP798687	*Rq6*	H06	
			P1896	KP798686	*Rq6*	H06	
			P1897	KP798685	*Rq6*	H06	

Table 1 (continued)

Major basin	Collecting site	Basin	Catalog sample number	GenBank accession number *cyt b*	Lineage	*Cyt b* haplotype	Microsatellite analysis
			P1898	KP798684	*Rq6*	H06	
			P1899	KP798683	*Rq6*	H06	
			P1900	KP798682	*Rq6*	H06	
			P1902	KP798680	*Rq6*	H06	
			P1901	KP798681	*Rq6*	H19	
LP-PM-AO	3-Castillos lagoon	SW Atlantic Ocean	P1824				X
			P1706	KP798770	*Rq6*	H06	X
			P1815	KP798735	*Rq6*	H06	X
			P1816	KP798734	*Rq6*	H06	X
			P1818	KP798732	*Rq6*	H06	X
			P1820	KP798730	*Rq6*	H06	X
			P1822	KP798729	*Rq6*	H06	X
			P1823	KP798728	*Rq6*	H06	X
			P1828	KP798724	*Rq6*	H06	X
			P1829	KP798723	*Rq6*	H06	X
			P1830	KP798722	*Rq6*	H06	
			P1831	KP798721	*Rq6*	H06	
			P1833	KP798719	*Rq6*	H06	
			P1834	KP798718	*Rq6*	H06	
			P1878	KP798702	*Rq6*	H06	
			P1880	KP798700	*Rq6*	H06	
			P1881	KP798699	*Rq6*	H06	
			P1883	KP798697	*Rq6*	H06	
			P1884	KP798696	*Rq6*	H06	
			P1885	KP798695	*Rq6*	H06	
			P1886	KP798694	*Rq6*	H06	
			P1887	KP798693	*Rq6*	H06	
			P1888	KP798692	*Rq6*	H06	
			P1890	KP798691	*Rq6*	H06	
			P1707	KP798769	*Rq6*	H20	X
			P1817	KP798733	*Rq6*	H20	X
			P1819	KP798731	*Rq6*	H20	X
			P1832	KP798720	*Rq6*	H20	
			P1879	KP798701	*Rq6*	H20	
			P1882	KP798698	*Rq6*	H20	
			P1877	KP798703	*Rq6*	H21	
			P1835	KP798717	*Rq6*	H22	
			P1827	KP798725	*Rq6*	H22	X
			P1826	KP798726	*Rq6*	H23	X
			P1825	KP798727	*Rq6*	H24	
			P1709	KP798767	*Rq6*	H35	X
			P1708	KP798768	*Rq6*	H36	X
			P1705	KP798771	*Rq6*	H37	X

Table 1 (continued)

Major basin	Collecting site	Basin	Catalog sample number	GenBank accession number *cyt b*	Lineage	*Cyt b* haplotype	Microsatellite analysis
			P1704	KP798772	*Rq6*	H38	X
LP-PM-AO	4-Blanca lagoon	SW Atlantic Ocean	P1723	KP798760	*Rq6*	H29	
			P1764	KP798746	*Rq6*	H29	
			P1768	KP798745	*Rq6*	H29	
			P1769	KP798744	*Rq6*	H29	
			P1722	KP798761	*Rq6*	H33	
LP-PM-AO	5-Salamanca caves	Merin lagoon	P1836	KP798716	*Rq6*	H06	
	6-Negra lagoon	SW Atlantic Ocean	P1716	KP798766	*Rq6*	H06	
			P1992	KP798654	*Rq6*	H06	
			P1993	KP798653	*Rq6*	H06	
LP-PM-AO	7-Talita stream	La Plata river	P1724	KP798759	*Rq6*	H06	
			P1752	KP798748	*Rq6*	H06	
			P1753	KP798747	*Rq6*	H06	
			P1750	KP798749	*Rq6*	H30	
LP-PM-AO	8-Colonia department	La Plata river	P1717	KP798765	*Rq6*	H06	
			P1718	KP798764	*Rq6*	H06	
LP-PM-AO	9-Abrojal stream	Negro river	P2136	KP798647	*Rq6*	H06	
			P2135	KP798646	*Rq6*	H07	
LP-PM-AO	10-Rincón del Bonete	Negro river	P2141	KP798648	*Rq4*	H08	
			P2143	KP798649	*Rq6*	H09	
			P2144	KP798650	*Rq6*	H10	
			P2142	KP798652	*Rq6*	H12	
			P2154	KX379747	*Rq6*	H47	
			P2158	KX379748	*Rq4*	H48	
LP-PM-AO	11-Paso 329	Negro river	P2160	KX379749	*Rq6*	H06	
LP-PM-AO	12-Sauce Grande town	Negro river	P1771	KP798743	*Rq6*	H06	
			P1773	KP798742	*Rq6*	H06	
LP-PM-AO	13-Caraguatá town	Negro river	P2148	KX379742	*Rq6*	H47	
LP-PM-AO	14-Paso mazangano town	Negro river	P1776	KP798739	*Rq6*	H26	
			P1777	KP798738	*Rq6*	H06	
LP-PM-AO	15-Quebrada de los cuervos	Merin lagoon	P2174	KX379759	*Rq6*	H47	
			P2175	KX379760	*Rq6*	H47	
LP-PM-AO	16-Vergara town	Merin lagoon	P2162	KX379750	*Rq6*	H47	
			P2163	KX379751	*Rq6*	H51	
	17-Sarandí del quebracho town	Merin lagoon	P2150	KX379743	*Rq4*	H48	
LP-PM-AO	18-Paso Centurión	Merin lagoon	P2153	KX379746	*Rq4*	H50	
LP-PM-AO	19-Queguay river	Uruguay river	P1980	KP798663	*Rq4*	H14	
			P2164	KX379752	*Rq4*	H47	
			P2176	KX379761	*Rq6*	H53	
			P2177	KX379762	*Rq4*	H54	
			P2178	KX379763	*Rq4*	H52	
LP-PM-AO	20-Lunarejo stream	Negro river	P1789	KP798737	*Rq4*	H25	
LP-PM-AO	21-Villa Constitución hatchery	Uruguay river	P1960	KP798679	*Rq6*	H06	

Table 1 (continued)

Major basin	Collecting site	Basin	Catalog sample number	GenBank accession number *cyt b*	Lineage	*Cyt b* haplotype	Microsatellite analysis
			P1962	KP798677	*Rq6*	H06	
			P1966	KP798674	*Rq6*	H06	
			P1970	KP798672	*Rq6*	H06	
			P1971	KP798671	*Rq6*	H06	
			P1972	KP798670	*Rq6*	H06	
			P1969	KP798673	*Rq2*	H16	
			P1964	KP798675	*Rq6*	H17	
			P1963	KP798676	*Rq2*	H18	
LP-PM-AO	22-Arapey river	Uruguay river	P2165	KX379753	*Rq4*	H48	
			P2167	KX379755	*Rq4*	H48	
			P2170	KX379758	*Rq4*	H48	
			P2166	KX379754	*Rq4*	H55	
			P2168	KX379756	*Rq4*	H56	
			P2169	KX379757	*Rq4*	H57	
LP-PM-AO	23-Franquia lagoon	Uruguay river	P1721	KP798762	*Rq2*	H34	
LP-PM-AO	24-Cuareim river	Uruguay river	P1720	KP798763	*Rq6*	H06	
			P0485	KP798651	*Rq4*	H11	
			P2152	KX379745	*Rq4*	H48	
			P2151	KX379744	*Rq6*	H49	
LP-PM-AO	25-Santa Lucía river	Parana river		AY036742	*Rq2*	H2	
LP-PM-AO	26-Misiones, Cuñapirú	Parana river	P284	EF564743	*Rq5a*	H3	
LP-PM-AO	27-Misiones, Ñandú stream	Iguazu river		AY036743	*Rq5b*	H1	
LP-PM-AO	28-Yacuy stream	Iguazu river		AY036744	*Rq5b*	H1	
Amazon	Manu river			AY036740	*Rq3*		
				AY036741	*Rq3*		
Esssequibio	Guyana			AY036739	*Rq1*		
	Unknown			DQ119395	*Rq1*		
Orinoco	Venezuela			AY036737	*R. laukidi*		
				AY036738	*R. laukidi*		
Orinoco	Colombia			KM489084	*R. laukidi*		
				KM489083	*R. laukidi*		
				KM489081	*R. laukidi*		

PCR amplification and sequencing of mitochondrial *cyt b* gene

Cyt b gene was amplified using the Gludg-L and CB6-H or CB3-H primers (Palumbi et al. 1991) using the following cycling profile: 10 min at 94 °C min; 35 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1:30 min; and 72 °C for 10 min. The total volume reaction was 20 μl including 1X Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each primer, 1 unit of Taq DNA polymerase (Invitrogen) and 45 ng of template DNA. The amplified fragment was sequenced directly on both strands with a Perkin-Elmer ABI Prism 377 Automated Sequencer (MACROGEN, Seoul, Korea). Sequence alignments were performed using Clustal W 1.8 (Thompson et al. 1994).

Table 2 GenBank accession numbers of sequences retrieved from the trans-andean *Rhamdia* species, *Rhamdella* and *Pimelodella* genera

Accession number	Species/Genera
AY036709	*R. laticauda*
AY036708	*R. laticauda*
AY036672	*R. guatemalensis*
AY036671	*R. guatemalensis*
AY036693	*R.* aff. *wagneri*
AY036694	*R.* aff. *wagneri*
AY036725	*R.* aff. *cabrerae*
AY036726	*R.* aff. *cabrerae*
AY036735	*R. cinerascens*
AY036736	*R. cinerascens*
AY036734	*R.* aff. *rogersi*
AY036733	*R. rogersi*
AY036718	*R. nicaraguensis*
AY036719	*R. nicaraguensis*
KM489075	*R.saijaensis*
KM489076	*R.saijaensis*
KM489077	*R.saijaensis*
KX379764	*Rhamdella*
KX379765	*Rhamdella*
AY036750	*Pimelodella*
AY036751	*Pimelodella*

PCR amplification and genotyping of microsatellite markers

Five microsatellite loci previously assayed in *R. quelen* by Ribolli and Zaniboni-Filho (2009) were used in the present study: *Pcor1* and *Pcor2* (Revaldaves et al. 2005); *Pc17* and *Pc97* (Moeser and Bermingham 2005); and *Rh1* (Ribolli and Zaniboni-Filho 2009). Amplification of these SSR (Simple Sequence Repeats) fragments was carried out using the same mix reaction described above for *cyt b,* and the following cycling profile: 3 min at 94 °C min; 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min; and 72 °C for 10 min. The PCR products were separated using an ABI 377 automated sequencer (MACROGEN, Seoul, Korea). Results were visualized with PEAKSCANNER v.1.0 software (Applied Biosystems). Alleles were scored using GeneScan™ 500 LIZ® Size Standard and Genotyper® software (Applied Biosystems, Inc.).

DNA variation in *cyt b* sequences

DNA polymorphism was measured by estimating the number of variable and phylogenetically informative sites, as well as haplotype diversity (h) and nucleotide diversity (π) using DNAsp v5 (Librado and Rozas 2009).

Phylogeographic analysis

The phylogeographic analysis of cis-andean *Rhamdia* was performed at three hierarchical nested levels: a) at a macrogeographic scale, in the cis-andean region, using the mitochondrial *cyt b* marker (Fig. 1a); b) at a mesogeographic scale, in LP-PM-AO system, also employing *cyt b* data (Fig. 1b); c) at a microgeographic scale, in three important coastal lagoons for artisanal fisheries from La Plata basin and SW Atlantic Ocean, using both mitochondrial and nuclear markers (Fig. 1c).

Phylogenetic analysis in the Neotropical region

A non-model-based method, Maximum parsimony (MP) was performed in PAUP* 4.0b10 (Swofford 2002) using heuristic search with random addition sequence, tree bisection reconnection (TBR), branch swapping and statistical support for 1000 pseudoreplicates. A strict consensus tree was selected from equally parsimonious topologies.

Additionally, two model-based approaches were assayed: Maximum likelihood (ML) and Bayesian Inference (BI), using PhyML 3.1 (Guindon and Gascuel 2003) and MrBayes 3.2 (Ronquist et al. 2012), respectively.

The nucleotide substitution model used for ML analysis was selected in jModelTest v.0.1.10 (Darriba et al. 2012), based on the Bayesian Information Criterion (Schwarz 1978), the corrected Akaike Information Criterion (Sugiura 1978), and Decision Theory criterion (Minin et al. 2003). Among the 88 models tested, the HKY + G (gamma distribution) (Hasegawa et al. 1985) was the nucleotide substitution model that best fit to the *cyt b* data set. A heuristic ML search was performed using NNI (a fast nearest neighbor interchange search) and the robustness of the nodes was determined using Bootstrap analysis (100 pseudoreplicates). The nucleotide substitution model used for BI was GTR + G (Lanave et al. 1984), which was selected as the best model with Mrmodeltest2 (Nylander 2004) applying

Akaike Information Criterion (Akaike 1974). The BI was run using four Markov chains for 10 million generations. The Tracer v1.5 (Rambaut and Drummond 2007a) package was used to verify stationarity reached. The genetic differentiation among the Neotropical region lineages –identified through the phylogenetic analysis- was assessed by computing the pairwise p-distance between *cyt b* sequences, using the program MEGA v.7.0 (Kumar et al. 2016).

Dating *Rhamdia* divergence

Divergence time was estimated with BEAST v.2.3.0 (Bouckaert et al. 2014). The analysis was performed using Reversible-jump Based Substitution (RBS) method, since it fits the appropriate model given a particular data set. The Yule process was assumed as the prior species tree, since it is the most suitable for species-level phylogenies (Drummond and Rambaut 2007). The time of divergence was calculated using a strict clock and calibrated with the Andes uplifting (10 million years ago (MYA), Lundberg et al. 1998), which was responsible for the divergence of the cis- and trans-andean regions. The Tracer v1.5 (Rambaut and Drummond 2007a) package was used to verify stationarity and convergence of all parameters. The mean and the 95% highest posterior density (HPD) of divergence time of each clade were estimated in million years. The same applies for the TMRCA (Time to the Most Recent Common Ancestor). Posterior probabilities and maximum clade credibility trees were calculated using TreeAnnotator 1.5.4 (Rambaut and Drummond 2007b).

Species delimitation in the genus *Rhamdia*

The Generalized Mixed Yule Coalescent (GMYC) method was used to determine putative species applying a likelihood ratio test to assess the fit of the branch lengths to a mixed lineage birth-population coalescence (Pons et al. 2006). For this purpose, an ultrametric tree was employed, which was based on a Yule process and obtained with BEAST v.2.3.0 (Bouckaert et al. 2014). The single-threshold GMYC analysis was conducted in R using APE (Paradis et al. 2004) and SPLIT (Ezard et al. 2009) packages. In order to cross-check the GMYC results, the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012) algorithm was employed. It is a cluster method that enables species delimitation based on a distance matrix. The ABGD analysis was carried out on February 2017 on the web interface (http://wwwabi.snv.jussieu.fr/public/abgd/) using a Jukes-Cantor distance and setting the relative gap width on 1.5; minimum and maximum of prior intraspecific divergence were set 0.001 and 0.100, respectively.

Population structure of *Rhamdia* in LP-PM-AO basins

Departure from neutrality was examined by means of Tajima's (1989) and Fu's (1997) tests. The genetic structure among *R. quelen* populations from LP-PM-AO was investigated using the analysis of molecular variance (AMOVA). Two hypotheses were tested based on different criteria: a geographic criterion, where populations were grouped by basins; and a taxonomic criterion, where the groups were defined by the presence of the different lineages. The most plausible grouping hypothesis was selected maximizing the intergroup variance value (F_{CT} statistics). The significance of variance components was calculated by 1000 pseudoreplicates. Neutrality tests and AMOVA was carried out using ARLEQUIN v.3.5 (Excoffier and Lischer 2010). The haplotypes network was constructed with NETWORK v.5.0.0.0 (Bandelt et al. 1999).

Microsatellite data analyses

MICRO-CHECKER v.2.2.3 (Van Oosterhout et al. 2004) was used to identify genotyping errors and null alleles. Polymorphism at microsatellite *loci* was measured by the number of alleles (A), allelic richness (r_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) using FSTAT v.2.9.3 (Goudet 2001). The existence of significant differences in genetic diversity between coastal lagoons was conducted using the Mann-Whitney test. Deviations from Hardy-Weinberg (H-W) equilibrium and linkage disequilibrium were tested using GENEPOP v.4.0.10 (Rousset 2008). Sequential Bonferroni correction was applied for multiple tests.

Population genetic structure of *R. quelen* at microgeographic scale

The analysis of population subdivision in the three coastal lagoons (1-SL, 2-RL, 3-CL; Fig. 1c) was carried out with microsatellite data. The analysis was performed using STRUCTURE software, applying Bayesian Inference and Markov Chain Monte Carlo (MCMC) estimation (Pritchard et al. 2000). The likelihood of P

(X|K) was calculated for different population numbers (k = 1 to 5), under an admixture model and correlated allele frequencies. A burn-in period of 50,000 steps followed by 100,000 MCMC replicates was set. In order to obtain a reproducible value of the estimate P (X|K), 10 replicates were run for each k. The most probable k value was determined employing both likelihood and Delta k criteria (Δk, Evanno et al. 2005), and calculated using STRUCTURE HARVESTER (Earl and vonHoldt 2012). The inter-population genetic differentiation was calculated through pairwise F_{ST} estimates with ARLEQUIN v.3.5 (Excoffier and Lischer 2010), for mitochondrial and microsatellite data sets.

Results

DNA polymorphism in the *cyt b* gene

The present study included 184 new *cyt b* sequences of *R. quelen* (KP798643-KP798808, KX379742-KX379763; Table 1) and two of the genus *Rhamdella* (KX379764 and KX379764, Table 2). In the total data set of 1043 bp, 324 variable and 268 phylogenetically informative sites were found among sequences of the *Rhamdia* genus. Haplotype diversity in the entire *Rhamdia* genus was high (0.894), whereas nucleotide diversity was moderate (0.02).

Phylogenetic analyses of the genus *Rhamdia* in South American basins

Given that all phylogenetic methods resulted in similar topologies, Fig. 2 shows only the ultrametric tree topology of coalescent analysis, inferred by Bayesian Inference using Beast v.2.3.0 (Bouckaert et al. 2014). The node statistical supports of MP, ML and BI obtained with MrBayes 3.2 analyses were included in the topology mentioned above. The MP analysis resulted in the 28,750 shortest trees of length 878, with a consistence index (CI) of 0.52 and retention index (RI) of 0.84, whereas the ML analysis yielded a final tree with a log likelihood of −5696.8.

All phylogenetic reconstructions confirmed the monophyly of the genus *Rhamdia,* as well as the divergence time (mean: 9.96; 95% HPD: 10.79–14.71 MYA), and revealed the presence of 16 mitochondrial lineages distributed into two major clades (Fig. 2). Eight lineages belong to the cis-andean region (8.28; 7.06–9.44 MYA) and eight to the trans-andean region (7.02; 5.82–8.19 MYA) (Fig. 2). Trans-andean lineages corresponded to previously described species and were divided into four sub-clades (I to IV) composed of different *Rhamdia* species: I- *R. laticauda*, *R. rogersi*, *R. cabrerae*, *R. nicaraguensis*; II- *R. saijaensis*; III- *R. cinerascens;* and IV- *R. guatemalensis* and *R. wagneri*. Cis-andean lineages were also clustered into four sub-clades (A to D), three of them (A-C) including different *R. quelen* lineages (*Rq*) distributed across all geographic locations (Fig. 1; Table 1): A- *Rq1* from Essequibio (0.13; 0.00–0.30 MYA); B- *Rq2* and *Rq3* from LP-PM-AO and Amazon River, respectively (4.07; 3.07–5.02 MYA); C- *Rq4*, *Rq5a, Rq5b* and *Rq6* from LP-PM-AO (4.55; 3.62–5.45 MYA); and D- *R. laukidi* from Orinoco (0.24; 0.05–0.50 MYA). Figure 2 shows the cis-andean groups A and B forming a monophyletic clade (5.39; 4.33–6.39 MYA), and its sister taxa sub-clade C. Finally, the major clade comprising the sub-clades A, B and C is a monophyletic sister group (5.94; 4.90–6.95 MYA) of the sub-clade D (Fig. 2).

On the other hand, the intragroup p-distance calculated for both cis-andean and trans-andean lineages were similar (median = 0.06), whereas the higher differences within *Rhamdia* genus were observed between the cis-andean and trans-andean lineages (average = 0.09, Table 3).

Species delimitation in genus *Rhamdia*

A total of 49 putative species composing the *Rhamdia* genus was estimated using the GMYC algorithm. In this analysis, *R. laticauda* and *R. rogersi* formed a single hypothetical species, whereas *Rq6* was divided into 35 putative species (Fig. 2). GMYC analysis yielded a maximum likelihood value of 683.83. Using ABGD algorithm, 16 delimited species of *Rhamdia* were found (Fig. 2).

Population structure of *Rhamdia* in LP-PM-AO basins

Tajima's test resulted negative and statistically significant in *Rq4* (D = −2.25, P = 0.004) whereas Fu's test showed a value close to zero, but non-significant (Fs = 0.10, P = 0.542). With regard to *Rq6* individuals, both tests were negative and statistically significant (D = −1.85, P = 0.002; Fs = −5.29, P = 0.004).

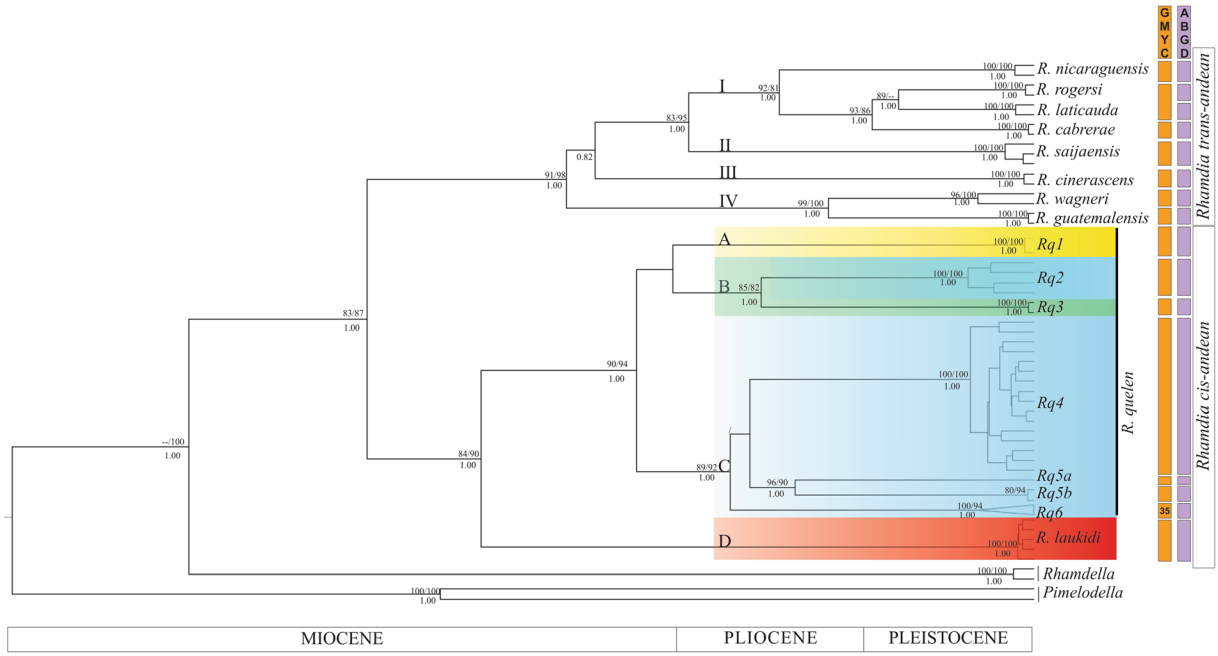

Fig. 2 Phylogenetic analysis of the genus *Rhamdia* in the Neotropical region. Tree topology generated based on 219 sequences of *cytochrome b* gene, using a Reversible-Jump based substitution on BEAST v.2.3.0 (Bouckaert et al. 2014). The triangle clade represents 169 collapsed sequences. Numbers below branches refer to the Bayesian posterior probability of occurrence for clades obtained by MrBayes, while bootstrap support values from Maximum Likelihood/Maximum Parsimony analyses are above branches. The bottom bar outlines the geologic epochs. At right are showed the name of species or lineage and the Andean region. The four trans-andean sub-clades (I–IV) as well as the cis-andean sub-clades (A-D) are shown over the nodes. The eight cis-andean lineages are highlighted according to the basins indicated in Fig. 1a. *Rhamdia quelen* lineages are indicated and numbered (*Rq1–6*). On the right, Generalized Mixed Yule Coalescent (GMYC) and Automatic Barcode Gap Discovery (ABGD) results are indicated with bars, where each bar represents a hypothetical species. GMYC identified 35 putative species within *Rq6* linages

In relation to the geographic-based hypothesis, AMOVA test showed a proportion of genetic diversity found among populations (F_{ST}) of 0.74, among populations within groups (F_{SC}) of 0.70, and among groups (F_{CT}) of 0.15 (Table 4). The second grouping hypothesis, based on the lineage composition of the different populations, resulted in a F_{ST} of 0.87, F_{SC} of 0.06 and F_{CT} of 0.86 (Table 5, Fig. 1b).

The *cyt b* haplotypes arising from the NETWORK analysis are shown in Table 1. The NETWORK topology (Fig. 3) shows three clusters corresponding to the cis-andean lineages *Rq2*, *Rq4* and *Rq6,* and two highly divergent and separated haplotypes (H3 and H1) that correspond to *Rq5a* and *Rq5b* lineages. Within the LP-PM-AO region, most haplotypes were grouped into the *Rq6* clade. A star-like topology was evidenced, with H6 as the central haplotype; and most of the remaining derived haplotypes were separated from H6 by 1 to 10 mutation steps. *Rq2* and *Rq4* clusters showed a smaller number of haplotypes than *Rq6*, and were restricted to a few basins.

Microsatellite diversity in three coastal lagoons

Employing MICRO-CHECKER software, no evidence of genotyping errors due to large drop out or stuttering peaks was detected, but signs of null alleles were found at the *Pc97* locus in the samples from Sauce lagoon (1-SL) (freq. 0.197). Accordingly, *Pc97* was the only locus in this population that showed a significant H-W equilibrium deviation after Bonferroni correction (Table 6). No indication of significant linkage disequilibrium between pairs of loci was found after Bonferroni correction.

Based on five microsatellite loci analysis, moderate to high genetic diversity was observed (A: 8.07; rA: 7.104; H_E: 0.697) in all three lagoons. Through Mann-Whitney test, non-significant differences in genetic diversity were found between pairwise comparisons of the lagoons. *Pcor2* and *Rh1* appeared as the most variable loci (Table 6). Using the statistic (rA) which is less biased working with heterogeneous sample sizes,

Table 3 Pairwise p-distance based on *cytochrome b* sequences of trans- and cis-andean *Rhamdia* lineages, and of the genera *Rhamdella* and *Pimelodella* used as outgoups

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rhamdia trans-andean	*1-R. cabrerae*																	
	2-R. cinerascens	**0.05**																
	3-R. guatemalensis	**0.04**	**0.03**															
	*4-R. nicaraguensis**	0.03	0.05	0.03														
	5-R. rogersi	0.03	0.03	0.03	0.02													
	6-R. wagneri	0.07	0.07	0.06	0.05	0.04												
	7-R. laticauda*	**0.08**	**0.07**	**0.07**	**0.07**	**0.06**	**0.08**											
	8-R. saijensi	**0.06**	**0.06**	**0.07**	**0.07**	**0.06**	**0.07**	**0.03**										
Rhamdia cis-andean	*9-Rq1*	0.11	0.10	0.09	0.10	0.09	0.10	0.10	0.10									
	10-Rq2	0.09	0.09	0.08	0.07	0.06	0.06	0.09	0.09	0.07								
	11-Rq3	0.09	0.09	0.09	0.08	0.07	0.06	0.09	0.09	0.06	0.06							
	12-Rq4	0.09	0.10	0.09	0.09	0.08	0.09	0.09	0.08	0.05	0.07	0.05						
	13-Rq5a	0.10	0.11	0.10	0.10	0.10	0.10	0.13	0.12	0.12	0.10	0.11	0.10					
	14-Rq5b	0.08	0.08	0.07	0.06	0.06	0.08	0.09	0.09	0.05	0.05	0.06	0.04	0.06				
	15-Rq6	0.09	0.08	0.07	0.07	0.06	0.07	0.08	0.09	0.05	0.05	0.05	0.03	0.09	0.03			
	16-R. laukidi*	**0.09**	**0.08**	**0.09**	**0.07**	**0.07**	**0.10**	**0.10**	**0.10**	**0.07**	**0.07**	**0.07**	**0.08**	**0.12**	**0.05**	**0.06**		
	17-Rhamdella	0.12	0.10	0.11	0.10	0.10	0.11	0.10	0.10	0.12	0.09	0.11	0.11	0.12	0.08	0.10	0.09	
	18-Pimelodella	0.10	0.11	0.10	0.09	0.09	0.10	0.10	**0.11**	0.13	0.12	0.12	0.12	0.13	0.12	0.12	0.13	0.12

Valid species are in bold. Asterisk signal species valid sensu Silfvergrip (1996). F_{ST} significant values are in bold ($P < 0.05$)

Table 4 Analysis of molecular variance (AMOVA) based on *cytochrome b* gene in *R. quelen* populations from Parana-Uruguay-Rio de la Plata-Atlantic Ocean basins. The populations coded as in Fig. 1b were grouped in seven basins (**i-vii**): **i) La Plata basin,** 1-Sauce lagoon, 7-Talita stream, 8-Colonia department; **ii) Atlantic Ocean basins**, 2-Rocha lagoon, 3-Castillos lagoon, 4-Blanca lagoon, 6-Negra lagoon; **iii) Negro River**, 10-Rincón del Bonete dam, 11-Paso 329, 12-Sauce grande town, 13-Caraguatá town, 14-Paso Mazangano, 20-Lunarejo stream; **iv) Merin lagoon,** 5-Salamanca caves, 15-Quebrada de los cuervos, 16-Vergara town, 17-Sarandí del quebracho town, 18- Paso Centurión; **v) Uruguay river,** 9-Abrojal stream, 21-Villa Constitución hatchery, 19-Queguay river, 22-Arapey river, 24-Cuareim river; 23-Franquía lagoon; **vi) Parana river,** 25-Santa Lucía-Paraná, 26-Misiones, Cuñapirú; **vii) Iguazu basin**, 27-Misiones, Ñandú stream, 28-Yacuy stream

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation
Among groups	6	184.278	0.457*	14.75
Among population within groups	20	183.479	1.847*	59.69
Within populations	159	125.760	0.791*	25.56

(*) Represents significant values [significance level: $\alpha = 0.05$; *p* values calculated from a random permutation test (1000 replicates)]. $F_{ST} = 0.74$*; $F_{SC} = 0.70$*; $F_{CT} = 0.15$*

Rocha and Castillos lagoons (2-RL and 3-CL) showed the highest and lowest allelic diversity (rA: 8.4 and 5.4), respectively. STRUCTURE analysis yielded a number of population clusters ((PX|K) = −1096.53 and $\Delta k = 88.43$ for k = 3) that represents the most plausible grouping hypothesis and properly matched the three coastal lagoons analyzed (Fig. 4a). Additionally, Fig. 4b shows the second most probable population clustering for k = 2 ((PX|K) = −1160.80, $\Delta k = 25.50$).

The F_{ST} estimate derived from *cyt b* data set analysis, revealed significant divergence of Castillos lagoon (3-CL) with respect to the other two coastal lagoons (Sauce (1-SL) and Rocha (2-RL), F_{ST}: 0.13 and 0.15, respectively; $P < 0.05$; Table 7). Also, low differentiation between 1-SL and 2-RL collecting sites was observed based on *cyt b* ($F_{ST} = 0.02$; $P > 0.05$). F_{ST} analysis based on microsatellites markers showed significant values of differentiation among the three studied coastal lagoons (pairwise F_{ST}: 0.19–0.24; $P < 0.05$; Table 7).

Discussion

Species boundaries delimitation in *Rhamdia* from the Neotropical region

This phylogeographic study constitutes the first *Rhamdia* analysis focused in the cis-andean region, corresponding to the distribution area of the *R. quelen* species complex. The systematics of *Rhamdia* -and particularly that of *R. quelen*- remains largely unresolved, since several studies (Perdices et al. 2002;

Table 5 Analysis of molecular variance (AMOVA) based on *cytochrome b* gene in *R. quelen* populations from Parana-Uruguay-Rio de la Plata-Atlantic Ocean basins. The populations (numerical codes as shown in Fig. 1b) were organized in seven groups (showed with different colors in Fig. 1b) based on the presence of different lineages: ***Rq2***– 23-Franquía lagoon, 25-Santa Lucía river (Fig. 1b: violet); ***Rq4***– 17-Sarandí del quebracho town, 18-Paso Centurión, 20-Lunarejo stream, 22-Arapey river (Fig. 1b: blue); ***Rq5a-*** 26-Misiones, Cuñapirú (Fig. 1b: green); ***Rq5b-*** 27-Misiones, Ñandú stream, 28-Yacuy stream (Fig. 1b: yellow); ***Rq6***– 1-Sauce lagoon, 2-Rocha lagoon, 3-Castillos lagoon, 4-Blanca lagoon, 5-Salamanca caves, 6-Negra lagoon, 7-Talita stream, 8-Colonia department, 9-Abrojal stream, 11-Paso 329, 12-Sauce grande town, 13-Caraguatá town, 14-Paso Mazangano, 15-Quebrada de los Cuervos 16-Vergara town (Fig. 1b: orange); ***Rq4-Rq6***–10-Rincón del Bonete dam, 19-Queguay river, 24-Cuareim river (Fig. 1b: pink); ***Rq2-Rq6***– 21-Villa Constitución hatchery (Fig. 1b: sky-blue)

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation
Among groups	6	345.268	5.124*	85.86
Among population within groups	20	22.489	0.053	0.88
Within populations	159	125.760	0.791*	13.25

(*) Represents significant values [significance level: $\alpha = 0.05$; *p* values calculated from a random permutation test (1000 replicates)]. $F_{ST} = 0.87$*; $F_{SC} = 0.06$; $F_{CT} = 0.86$*

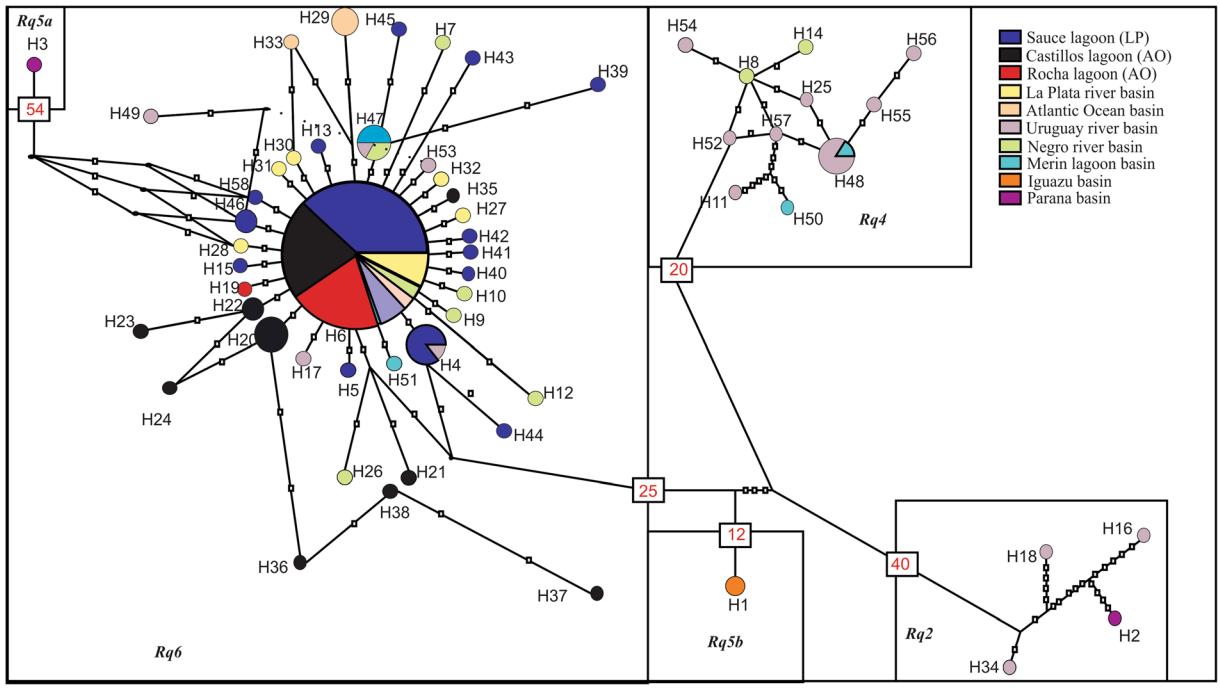

Fig. 3 Haplotype network of *R. quelen* samples inhabiting LP-PM-AO basin. White dots and number over the links represent missing haplotypes and circle size is proportional to haplotype frequency. The filler of each circle indicates the basin described in Table 1. Haplotypes that will be analyzed at microgeographic scale are indicated for each lagoon (basin lagoon is reported as AO: Atlantic Ocean; or LP: La Plata). The black square represents the lineages found in the phylogenetic analysis. The reference for each haplotype recognized by NETWORK v.5.0.0.0 (Bandelt et al. 1999) is detailed on Table 1

Martinez et al. 2011; Hernandez et al. 2015; Garavello and Shibatta 2016) have revealed inconsistencies concerning the last morphological revision of the genus (Silfvergrip 1996). In this sense, the GMYC and ABGD algorithms were here employed to define putative species in this complex. It has been reported that GMYC frequently delivers a higher species count than other methods (Kekkonen et al. 2015). Indeed, GMYC exhibited more splits than ABGD (Fig. 2). Conversely, we followed a more conservative criterion and the species delimitation was conducted taking into account the ABGD results. These results also were in agreement with the well-supported lineages found in present phylogenetic analysis. Furthermore, supporting the ABGD algorithm, genetic divergence among cis-andean species ranged between 3 to 12% (Table 3), which are similar values to those reported for valid species (Perdices et al. 2002; Hernandez et al. 2015) of the trans-andean region (from 3 to 8%). Altogether, the *Rhamdia* genus would be composed of 16 hypothetical species, divided in eight trans-andean and eight cis-andean species (Fig. 2). *Rhamdia quelen* was splitted into seven highly differentiated genetic lineages (*Rq1*, *Rq2*, *Rq3*, *Rq4*, *Rq5a*, *Rq5b*, *Rq6*), and this is consistent with the species complex hypothesis. This suggests the existence of cryptic species, considering the absence of substantial morphological differences observed by Silfvergrip (1996). However, a recent study carried out in *Rhamdia,* that included morphological as well as cytogenetic data, revelaed evidence regarding the reappraisal of two species from Iguazu basin (*R. branneri* and *R. voulezi*) (Garavello and Shibatta 2016). It is worth noting that *Rq5b* inhabits this basin, and hence it could be one of these new revalidated *Rhamdia* species by the aforementioned authors. Based on these results, we strongly advise an exhaustive morphological revision of the divergent lineages, as they are indicative of putative cryptic species. The seven hypotethical species of *R. quelen* complex, together with *R. laukidi,* have an interesting differentiation history associated with particular geological events, as we will discuss below.

On the other hand, in accordance with Hernandez et al. (2015) the present analyses confirmed, *R. laticauda*, *R. guatemalensis*, *R. saijaensis*, *R. laukidi* and *R. cinerascens* as distinct species. Here, we support the

Table 6 Genetic diversity estimates based on five microsatellite loci in *R. quelen* populations from three coastal lagoons (1-Sauce lagoon, 2-Rocha lagoon and 3-Castillos lagoon; see Fig. 1c)

1-SL							
Locus	N	A	r_A	Ho	He	PIC	*P* value
Pcor1	32	8	6.053	0.625	0.663	0.622	0.080
Rh1	27	18	11.123	0.815	0.897	0.870	0.200
Pcor2	30	14	8.818	0.900	0.833	0.803	0.610
Pc97	32	10	6.431	0.563	0.726	0.679	0.000
Pc17	23	6	5.200	0.696	0.688	0.621	0.590
Mean/Total		11.2	7.525	0.720	0.761	0.719	0.000
2-RL							
Pcor1	12	4	3.917	0.750	0.630	0.541	0.740
Rh1	12	5	4.909	0.667	0.656	0.581	0.530
Pcor2	12	11	10.406	0.833	0.790	0.742	0.740
Pc97	12	4	3.913	0.667	0.612	0.515	1.000
Pc17	11	4	4.000	0.636	0.697	0.684	0.450
Mean/Total		5.6	5.429	0.711	0.677	0.613	0.789
3-CL							
Pcor1	20	3	2.770	0.300	0.272	0.247	1.000
Rh1	20	10	10.929	0.800	0.835	0.792	0.710
Pcor2	20	11	12.092	0.800	0.891	0.856	0.110
Pc97	20	9	8.320	0.900	0.771	0.719	0.690
Pc17	20	4	5.270	0.600	0.499	0.409	0.820
Mean/Total		7.4	8.358	0.680	0.654	0.605	0.944

Sample size (N); Number of alleles (A); allelic richness based on minimum sample size of 11 diploid individuals (r_A); observed (H_O) and expected (H_E) heterozygosity; Polymorphic information content (PIC); Hardy-Weinberg equilibrium (*P* value)

validity of *R. nicaraguensis* and *R. wagneri*, which have been previously considered synonyms of *R. latikauda* and *R. guatemalensis,* respectively (Perdices et al. 2002; Hernandez et al. 2015). In our analyses, *R. rogersi* and *R. cabrerae* appear as two putative species, in contrast to those results found by Perdices et al. (2002) in which these taxa were included within the *R. laticauda* species group.

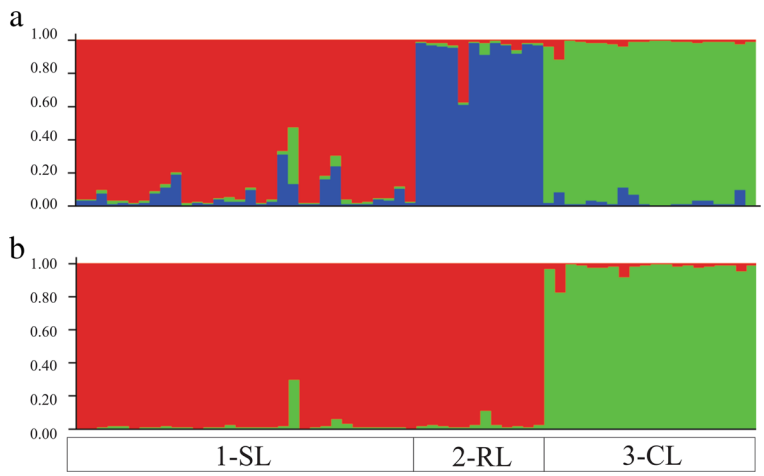

Fig. 4 Estimated population structure of *R. quelen* in three coastal lagoons, based on five nuclear microsatellite loci. Each bin or colored vertical bar represents the estimated membership fraction of an individual into the major population clusters. Codes of three sampling sites are indicated in Fig. 1c (Sauce, Rocha and Castillos lagoons, 1-SL, 2-RL, and 3-CL, respectively). The histograms (**a**) and (**b**) correspond to the clustering analysis of population structure using K = 3 and K = 2, respectively

Table 7 Pairwise F_{ST} values based on *cytochrome b* (lower left corner) and microsatellite data (upper right corner) between all pairs of coastal lagoon populations studied: Sauce lagoon (1-SL); Rocha lagoon (2-RL); Castillos lagoon (3-CL)

Localities	1	2	3
1- SL	-	**0.208**	**0.237**
2- RL	0.020	-	**0.191**
3- CL	**0.129**	**0.153**	-

F_{ST} significant values are in bold ($P < 0.05$)

Biogeographic history can explain the divergence of the cis-andean species complex

The phylogenetic analysis supported the monophyly of the genus *Rhamdia*, composed of two reciprocally monophyletic clades (Fig. 2). These major groups were in agreement with the cis- and trans-andean *Rhamdia* clades suggested by Perdices et al. (2002) in an evolutionary study centered on trans-andean *Rhamdia*. The distribution of these two major clades is consistent with the uplift of the Andes –the longest continental mountain range-, separating Magdalena and Orinoco river basins. Thus, these clades would have experimented vicariance divergence in the late Miocene (10 MYA) (Lundberg et al. 1998). When considering a macrogeographic scale, *R. laukidi* from Orinoco diverged to the most basal cis-andean node (8.28; 7.06–9.44 MYA). In addition, the fact that this area is the closest one to the trans-andean region would suggest that Orinoco basin represents a possible ancestral area. According to Fig. 2, the most plausible hypothesis of divergence of the cis-andean clade is consistent with successive allopatric fragmentation events corresponding to major South American basins, as follows: Essequibo lineage (*Rq1*; sub-clade A); Amazon (*Rq2* and *Rq3*; sub-clade B); La Plata basin, Patos-Merin basin system, and the coastal lagoons draining to SW Atlantic Ocean (LP-PM-AO) (*Rq4, Rq5a, Rq5b* and *Rq6*; sub-clade C); and Orinoco (sub-clade D). However, the sub-clade B also inhabits the Parana basin, suggesting a possible secondary connection event between Amazon and Parana basins during the Pliocene (4.07; 3.07–5.02 MYA). The Essequibo lineage from Guyana region, is phylogenetically related to Amazon and LP-PM-AO basin systems (sub-clade B; Fig. 2). Interestingly, a molecular study investigating the evolutionary history of another catfish species (Cardoso and Montoya-Burgos 2009) also indicated a relationship between biota from Amazon basin and Guyana region. Another phylogeographic analysis based on the ribosomal gene 16S and the mitochondrial control region, conducted in *Serrasalmus* and *Pygocentrus* genera (neotropical piranhas), further supported the occurrence of a biota exchange by dispersal between Amazon and LP-PM-AO in the last 1.7 MYA (Hubert et al. 2007). The sub-clades B and C, inhabiting LP-PM-AO region, would not have originated in the same basin. Whereas the divergence whithin the sub-clade B is probably due to the connection between Amazon and LP-PM-AO basins, the divergence within the sub-clade C could be explained by vicariance events. Such events are possibly to the outcome of marine transgressions, that had limited river connectivity, thus increasing the genetic differentation among them (Lundberg et al. 1998). Therefore, the evolution within the sub-clades B and C could be explained by dispersal and vicariance events, respectively.

Population analysis of *R. quelen* from LP-PM-AO basin system

Interestingly, at the mesogeographic scale, the pattern of genetic differentiation is better described in terms of lineage distribution (Table 5, Fig. 1b) than of the geographic structure of basins (Table 4). The haplotype network displayed a similar topology to that of MP, ML and BI analyses, excepting for the haplotypes H1 (*Rq5b*) and H3 (*Rq5a*), which are distantly located and show a high number of mutation-steps between them (Fig. 3). The five lineages inhabiting the LP-PM-AO basin system (*Rq2*, *Rq4*, *Rq5a, Rq5b* and *Rq6*) showed significant genetic differentiation and restricted gene flow among them, with F_{ST} values greater than 0.21 (data not shown). Particularly, *Rq6* lineage shows a star-like topology with a central and more frequent haplotype (H6), indicating that this is probably the ancestral haplotype. In addition, the low number of mutation–steps between haplotypes and the large number of derivative haplotypes from H6 support rapid population expansion events, as the most plausible hypothesis of differentiation. The results of both neutrality tests were consistent with this expansion scenario; and, alternatively, can also account for the presence of selective pressures. It is quite possible that *Rq6* lineage would have dispersed during periods of flooding among basins, due to sea level fluctuations (Cavallotto et al. 2005).

Evidences in favor of this hypothesis are the wide geographic distribution of *Rq6* in the star-like topology, and the presence of several endemic and rare haplotypes from different basins or lagoons. The current basin structure explains the divergence of these endemic haplotypes.

Microgeographic population analysis in coastal lagoons

There are few studies evaluating genetic diversity of *R. quelen,* and no knowledge regarding the structure of wild populations in LP-PM-AO. This study is the first approach to *R. quelen* genetic structure, using both nuclear and mitochondrial markers, in three coastal lagoons where this species represent an important fishery resource. Previous microsatellite data analyses were reported by Ribolli and Zaniboni-Filho (2009), in a study performed on a large *R. quelen* broodstock caught from wild populations in the upper course of Uruguay River basin, in Brazil. The authors found a high genetic diversity per locus in *Pcor1*, whereas *Pc97* showed lower values than the ones obtained in our analyses. Genetic diversity was similar among the three lagoons, and it also resembled the value of the broodstock analyzed (He = 0.73) by Ribolli and Zaniboni-Filho (2009). In the present study, we found signs of null alleles in the *Pc97* locus at moderate frequencies in Sauce lagoon (1-SL) individuals. This finding suggests that *Pc97* should be use with caution in further paternity assays within hatcheries that include individuals of such population.

Microsatellite data analyses support the geographic structure of coastal lagoons, suggesting that each lagoon represents an isolated system (Fig. 4a; Table 7). The high microsatellite pairwise F_{ST} values between lagoons are in agreement with the haplotype network obtained using *cyt b* data, which showed several endemic haplotypes in each coastal lagoon (Fig. 3). The second most plausible number of clusters, identified through STRUCTURE analysis, showed the close relationship between Sauce lagoon (1-SL) and Rocha lagoon (2-RL) (Fig. 4b). This result qualifies the historical evolutionary scenario suggesting that an ancestral relationship between these two lagoon populations would have existed, and in turn, it is consistent with the F_{ST} values observed from *cyt b* analyses (Table 7).

Conservation units based on microsatellites and *cyt b* markers

Based on *cyt b* analyses, we propose that new seven potential cryptic species compose the *R. quelen* species complex belonging to the cis-andean major clade, a discovery that should be taken into account for conservation legislation and fishery management. Microsatellite analyses revealed that the populations found in the three coastal lagoons show strong genetic structure and significant isolation among them (Fig. 4a). Therefore, these populations could represent three different genetic pools that should be considered as independent MUs. Moreover, the potential connection between the three analyzed coastal lagoons and SW Atlantic Ocean are historically different. Castillos lagoon (3-CL) is frequently connected to the Ocean, whereas Rocha lagoon (2-RL) has an intermittent and variable connection, both representing different estuarine environments. Finally, Sauce lagoon (1-SL) has currently no connection to the Ocean at all. These varying types of connections with the SW Atlantic Ocean have an impact on the salinity, creating a significant salinity gradient within the affected lagoons (Bonilla et al. 2006). Therefore, the three detected MUs should be conserved, in order to preserve local adaptations and the total genetic diversity of *R. quelen* found in the study area.

Final remarks

Our work represents a significant step in the understanding of *Rhamdia* systematics, since 16 putative species have been recognized to form the *Rhamdia* genus. Moreover, we provide evidence of seven novel putative cryptic species. *Rhamdia* has shown to be a useful model to test biogeographic hypotheses, given that the divergence between the different species of the genus is closely related to important geological events, such as the Andes uplift and the evolution of South America's most important river basins. Thus, the population structure within and among river basins is determined by these events. However, phenomena related to sea level fluctuation, like marine transgressions/regressions, may also play an important role in isolating and connecting populations. At a microgeographic scale, analyses based on nuclear and mitochondrial markers, revealed that populations of *R. quelen* species complex that inhabit the studied coastal lagoons, remain isolated from each other, despite some lagoons are connected with other freshwater bodies. Finally, comparison of the genetic

structure at different geographic scales enables us to identify different phenomena affecting the evolution of *R. quelen* species complex.

Acknowledgements We thank Fondo Maria Viñas-Agencia Nacional de Investigación e Innovación (FMV_2009_2793) for the financial support. We thank the following colleagues for kindly providing *R. quelen* specimens from different basins of Uruguay: F. Texeira, M. Loureiro, WS. Serra, A. Duarte, W. López and C. Clavijo. The authors are also grateful to the Japanese government for the donation of laboratory equipment. The research of G.G. and V.G. was also supported by Sistema Nacional de Investigadores-Agencia Nacional de Investigación e Innovación (SNI-ANII).

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Capítulo II

Desarrollo de marcadores de tipo microsatélite mediante tecnologías de secuenciación masiva

MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2013) **13**, 546–549 doi: 10.1111/1755-0998.12095

PERMANENT GENETIC RESOURCES NOTE

Permanent Genetic Resources added to Molecular Ecology Resources Database 1 December 2012–31 January 2013

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Abstract

This article documents the addition of 268 microsatellite marker loci to the Molecular Ecology Resources Database. Loci were developed for the following species: *Alburnoides bipunctatus, Chamaerops humilis, Chlidonias hybrida, Cyperus papyrus, Fusarium graminearum, Loxigilla barbadensis, Macrobrachium rosenbergii, Odontesthes bonariensis, Pelteobagrus vachelli, Posidonia oceanica, Potamotrygon motoro, Rhamdia quelen, Sarotherodon melanotheron heudelotii, Sibiraea angustata, Takifugu rubripes, Tarentola mauritanica, Trimmatostroma* sp. and *Wallago attu*. These loci were cross-tested on the following species: *Alburnoides fasciatus*, *Alburnoides kubanicus*, *Alburnoides maculatus*, *Alburnoides ohridanus*, *Alburnoides prespensis*, *Alburnoides rossicus*, *Alburnoides strymonicus*, *Alburnoides thessalicus*, *Alburnoides tzanevi*, *Carassius carassius*, *Fusarium asiaticum*, *Leucaspius delineatus*, *Loxigilla noctis dominica*, *Pelecus cultratus*, *Phoenix canariensis*, *Potamotrygon falkneri*, *Trachycarpus fortune* and *Vimba vimba*.

Table 1 Information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Alburnoides bipunctatus	11	*A. fasciatus*, *A. kubanicus*, *A. maculatus*, *A. ohridanus*, *A. prespensis*, *A. rossicus* *A. strymonicus*, *A. thessalicus*, *A. tzanevi*, *Carassius carassius*, *Leucaspius delineates*, *Pelecus cultratus*, *Vimba vimba*	50534–50544	See ms for details.	Urbánková, Soňa; Mendel, Jan; Vyskočilová, Martina

Table 1 (Continued)

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Chamaerops humilis	28	*Phoenix canariensis*, *Trachycarpus fortunei*	50469–50496	GF112058–GF112085	Guarino, Carmine; Giovino, Antonio; Scibetta, Silvia; Cipriani, Guido
Chlidonias hybrida	11	n/a	50400–50410	AY083596–AY083599, AY083601, AY091847, AY091851, AY091853, AY597041, AY597042, AY597044	Minias, Piotr; Minias, Alina; Dziadek, Jarosław
Cyperus papyrus	9	n/a	50578–50586	KC460657–KC460663, KC460665, KC460666	Triest, Ludwig; Sierens, Tim; Terer, Taita
Fusarium graminearum	9	*F. asiaticum*	50569–50577	JX871447–JX871462 See ms for details.	Lee, Seung-Ho; Lee, Jeonghwa; Lee, Soohyung; Kim, Mija; Yun, Jong-Chul; Oh, Kyeong-Suk; Lee, Theresa
Loxigilla barbadensis	26	*L. noctis dominica*	50508–50533	GF112030–GF112055	Cezilly, Frank; Leclercq, Pierre; Daniel, Carla; Wattier, Rémi A.
Macrobrachium rosenbergii	9	n/a	50419–50427	JF319151–JF319155, KC191673–KC191676	Dharaneedharan, Subramanian; Balasundaram, Chellam; Heo, Moon-Soo
Odontesthes bonariensis	20	n/a	50587–50607	KC188637–KC188656	Villanova, Gabriela V.; Vera, Manuel; Díaz, Juan; Martinez, Paulino; Calcaterra, Nora B.; Arranz, Silvia E.
Pelteobagrus vachelli	8	n/a	50411–50418	GU338368–GU338375	Wang, Hongying; Du, Man
Posidonia oceanica	14	n/a	50608–50633	See ms for details.	D'Esposito, D.; Orsini, L.; Procaccini, G.
Potamotrygon motoro	10	*P. falkneri*	50296–50305	See ms for details.	Cruz, V. P.; Mendes, N. J.; Mendonça, F. F.; Pardo, B. G.; Vera, M.; Martinez, P.; Oliveira, C.; Foresti, F.
Rhamdia quelen	10	n/a	50347–50356	KC117543–KC117552	Ríos, Néstor; Bouza, Carmen; Pardo, Belén G.; Guerra-Varela, Jorge; Gutiérrez, Verónica; Martinez, Paulino; García, Graciela

Table 1 (Continued)

Species	No. of primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Sarotherodon melanotheron heudelotii	8	n/a	50339–50346	KC256806–KC256813	Gauffre-Autelin, Pauline; Durand, Jean-Dominique; Avarre, Jean-Christophe
Sibiraea angustata	21	n/a	50548–50568	JX560436, JX560437, JX560440–JX560445, JX560447, JX560450, JX560451, JX560454–JX560457, JX661245–JX661247, JX661249, JX661251, JX661252	Fu, Peng-cheng; Gao, Qing-bo; Zhang, Fa-qi; Li, Yin-hu; Xing, Rui; Khan, Gulzar; Zhang, Jing-hua; Wang, Jiu-li; Chen, Shi-long
Takifugu rubripes	26	n/a	50442–50458, 50460–50468	JX448593–JX448618	Liu, Shufang; Yin, Guibo; Yuan, Yanjiao; Liu, Hongbo; Zhuang, Zhimeng
Tarentola mauritanica	11	n/a	50497–50507	KC470197–KC470204, KC489794–KC489796	Rato, C.; Harris, D. J.; Perera, A.
Trimmatostroma sp.	11	n/a	50373–50383	JX515524-JX515534	Lauth, Jérémie; Malé, Pierre-Jean G.; Voglmayr, Hermann; Mayer, Veronika E.; Dejean, Alain; Orivel, Jérôme
Wallago attu	14	n/a	50357–50370	JX971059, JX971060, JX971063, JX971064, JX971071, JX971075, JX971076, JX971086, JX971092, JX971094, JX971097, JX971099, JX971112, JX971115	Singh, Akhilesh; Lakra, Wazir S.; Mandhan, Rishi Pal; Goswami, Mukunda; Yadav, Kamalendra; Sharma, Bhagwati S.

This article documents the addition of 268 microsatellite marker loci to the Molecular Ecology Resources Database. Table 1 contains information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column. The MER database and GenBank accession numbers and the authors responsible are also listed. A full description of the development protocol for the loci presented here can be found on the Molecular Ecology Resources Database (http://tomato.biol.trinity.edu/).

Pyrosequencing for microsatellite discovery and validation of markers for population analysis in the non-model Neotropical catfish *Rhamdia quelen.*

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Keywords: Rhamdia quelen, Neotropical catfish, pyrosequencing, microsatellites.

Running title: Microsatellite loci for *Rhamdia quelen*

Abstract

The technology 454 GS-FLX pyrosequencing shotgun run allowed to discover more than 13,000 putative microsatellite loci from 0.02% of the genome of the non-model teleost

Rhamdia quelen. The AC/GT was the most frequent repeat motif detected. Ten resolutive and polymorphic loci were selected for validation on a wild lagoon population from Uruguay. A mean number of 8 alleles per locus and 0.673 mean expected heterozygosity were observed. A single locus deviated from Hardy–Weinberg equilibrium, likely due to null alleles. The loci panel showed high exclusion probabilities, usefulness for population and parentage analysis in captive programs for this species.

The silver catfish *Rhamdia quelen* (Siluriformes, Heptapteridae) inhabits rivers and freshwater lakes of the Neotropical region (da Silva et al. 2011). It has commercial relevance as an aquatic resource for hatchery exploitation and aquaculture, especially in Argentina, Brazil and Uruguay (Vaz et al. 2010), as well as by artisanal hatcheries and fisheries along South America. This promising species for intensive aquaculture is generally produced below their potential due to the scarcity of biological knowledge on rearing and breeding (Parra et al. 2008). Moreover, few studies have been carried out for evaluating genetic structure of wild populations to select aquaculture broodstocks (Vaz et al. 2010). In this context, microsatellites or single sequence repeats (SSRs) are an invaluable tool as genetic markers for a wide range of applications in genetics, ecology and evolution (Jarne & Lagoda 1996). The scarce microsatellite-based analyses in *R. quelen* were mostly based on cross-amplification from other catfish groups with only a single marker being specifically isolated in *R. quelen* (Ribolli & Zaniboni-Filho 2009). For this purpose the next-generation sequencing (NGS) techniques becomes in a important tools allowing to researchers to obtain large numbers of microsatellite markers through a much more cost-effective procedure compared to traditional approaches (Perry & Rowe 2010).

The aim of this work was to develop the first panel of microsatellites in *R. quelen* by a shotgun pyrosequencing technique, in order to validate a handful microsatellite tool for population studies and broodstock evaluations, to provide genetic support on fishery and aquaculture management for this species.

Samples and 454 GS-FLX pyrosequencing

The study was conducted on 24 samples of *R. quelen* from Laguna del Sauce, Uruguay. Total genomic DNA was extracted from adipose fin or muscle (conserved in ethanol 95%) using proteinase K digestion followed by sodium chloride extraction and ethanol precipitation (modified from Medrano et al. 1990). DNA quality and concentration were determined monitored by spectrophotometer measurement in a Nanodrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc). Approximately, 1500 ng of genomic DNA from two individuals were pooled and resuspended in 15 ml 1xTE buffer to implement 1/16 plate of a Roche 454 GS FLX sequencer Titanium run at the "Genome sequencer 20 System" platform (Instituto Agrobiotecnológico de Rosario – INDEAR, Argentina). Contig assemblage of the raw data was performed in MIRA program V3.2.0 (Chevreux et al. 2004).

Identification of SSR motifs

The identification and statistics of microsatellites were performed in MISA (MIcro SAtellite identification tool) software (Thiel 2003) using raw sequence data. The search criterion was set to a minimum of 8 repeats for dinucleotide or trinucleotide motifs and 5 repeats for tetra-penta- and hexanucleotide motifs. Primers were finally designed for amplification using Primer3 v. 0.4.0 software (Rozen & Skaletsky 2000) under the following criteria: GC content of 60% (min= 40%, max= 80%), product size of 80-450 pb, primer size of 20 bp (min= 18,

max= 22 bp), primer Tm of 60ºC (min= 50ºC, max= 63ºC), max self complementarity of 5, max 3' self complementarity of 2 and max Poly-X of 3. These last three criteria were took into account to select an optimized random sample of 30 primer pairs from all microsatellite-containing sequences, mostly with dinucleotide motifs (22) to maximize polymorphism, in order to develop a final panel of approximately 10-12 loci to be validated for further population analysis. To avoid twice designing primers for the same locus, all microsatellite-containing sequences and their reverse and complement were clustered to identify redundant sequences using Clustal X (Thompson et al. 1997). BLAST searches of the SSR-containing sequences were performed against the GenBank for gene annotation, using a significance threshold of E-values< 10^{-10}.

Testing polymorphism in microsatellite markers

Technical validation of the 30 selected loci was initially tested using a subset of DNA samples from 6 individuals belonging to different populations using unlabelled primer pairs. The polymerase chain reaction (PCR) cycling profile was as follows: an initial denaturing for 10 min at 94°C; 35 cycles of 94°C for 45 s, the primer-specific annealing temperature (Table 1) for 50 s, and 72°C for 50 s; and a 10 minute final extension at 72°C. The annealing temperature was calculated taking into account 2°C less than the average Tm (melting temperature) of the corresponding primer pair. The total reaction volume was 15 µl including 1x Buffer 10X (Applied Biosystems), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), 3 pmoles of each primer and 30 ng of DNA. The annealing temperature was adjusted for microsatellites which generated multiple bands or did not amplify in the samples used. PCR products were visualized by electrophoresis on a 2% agarose gel for preliminary checking of polymorphism. Those loci

which produced bands of expected size and signs of polymorphism were selected and further analyzed for polymorphism screening on an ABI 3730 xl DNA sequencer at the Sequencing and Fragment analysis Unit of the University of Santiago de Compostela (Campus Lugo, Spain), using the forward primer labeled with a fluorescent dye (FAM, NED, PET, or VIC) (Table 1). Fluorochromes were defined for multiplexing according with product size ranges of microsatellites, so that loci of different length could be labelled with the same dye. Allele sizes were determined by using the LIZ 500 (Applied Biosystems) as internal standard and the peak scoring software GeneMapper® v3.7 (Applied Biosystems®).

CERVUS v3.0 software (Kalinowski et al. 2007) was used to estimate the number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E) as well as theoretical exclusion probabilities for each locus (EXC) and global over loci on a sample of 24 individuals from Laguna del Sauce. The MICRO-CHECKER V. 2.2.3 (van Oosterhout et al. 2004) was used to check evidence of genotyping errors and null alleles. GENEPOP 4.0.10 (Rousset 2008) was used to test deviations from Hardy-Weinberg (H-W) equilibrium in each locus, combined locus through Fisher's method and linkage disequilibrium between loci. Bonferroni correction was applied for significance of multiple tests.

The sixteenth-plate 454 run of *R. quelen* genome resulted in 73.8 Mb (megabases) spread over 54,694 individual sequence reads with an average read length of 324 bp. MIRA program successfully assembled 7,935 sequences (14.51%), resulting in 1,097 contigs with an average length of 407 bp. The combined contigs had a length of 0.2 Mb and represented about 0.02% of the *R. quelen* genome, which could be estimated to be ~1,000 Mb (C value: 1.04 pg; Fenerich et al. 2004).

MISA program identified 13,552 microsatellite repeats with the selection criteria applied to the 54,694 sequence reads. This data suggests a density of microsatellite repeats in the genome of the catfish *R. quelen* around one microsatellite repeat per 5,446 bp. Of the total microsatellites, 3,141 were compound ones. Among the different perfect repeat motifs, the most abundant ones were dinucleotides (9,870) as expected, which agrees with the estimate of microsatellite motifs in other catfish genomes (Serapion et al. 2004; Liu et al. 1999), followed by tetranucleotides (1,853), trinucleotides (1,507), pentanucleotides (278), and hexanucleotide (44) repeats (note that compound microsatellites may be represented in several repeat classes and multiple times; Figure 1a-c). Similar to other vertebrates genomes and according to similar findings in *Ictalurus punctatus (*Channel catfish) (Serapion et al. 2004), the most common motif was AC/GT containing 3742 microsatellites. However, while CAT and GTGA were the most represented in tri- and tetranucleotides, TTA and TTTG were the most common in Channel catfish. PRIMER3 was able to design primers for 3,233 microsatellite loci discovered here with a design success rate of 34%, ranging from 64.54% (dinucleotide loci) to 0.15% (pentanucleotide loci), previous to the technical validation.

Twenty-four out of thirty selected SSR were properly amplified in most of the 6 individuals and they showed fragments of the expected size in 2% agarose gel. In addition, twelve of these twenty-four loci revealed preliminary signs of polymorphism and were selected for amplification using fluorescent labelled-primers and genotyped on an automatic sequencer. Two microsatellites were finally eliminated due to genotyping difficulties, yielding to a panel set of ten variable loci for population analysis.

The panel of ten polymorphic microsatellite loci was tested for genetic diversity estimation on 24 wild individuals from Laguna del Sauce. The average numbers of alleles per locus was 7.9 (ranging from 4 at Rhq8 to 12 at Rhq16); expected heterozygosity ranged from 0.545 at Rhq7

to 0.871 at Rhq28 (average= 0.673); and observed heterozygosity ranged from 0.125 at Rhq8 to 0.917 at Rhq2 with an average of 0.614 (Table 1). The highest exclusion probabilities were exhibited by the locus Rhq28 (EXC1= 0.540; EXC2= 0.704) and the lowest ones by Rhq7 (EXC1= 0.152; EXC2= 0.311). The combined probability of exclusion of a false parent for this set of loci when the other parent is unknown or known was 0.987 and 0.999, respectively (Table 1). All these data support the usefulness of this set of loci for population genetic studies and parentage analysis. Pedigree tracing is an essential factor to be monitored to preserve genetic diversity and avoid inbreeding in aquaculture, especially in species under natural spawning, such as *R. quelen* (Salhi and Bessonart 2011).

There was no evidence of genotyping errors due to large drop out or stuttering peaks. Only one locus (Rhq8) showed homozygote excess deviation for most allele size classes strongly suggesting the presence of null alleles by MICRO-CHECKER program analysis. In accordance with these data, this locus also exhibited significant deviation from H-W equilibrium after Bonferroni correction ($P<0.005$). The Fisher's test was significant for all loci combined ($P<0.001$) but there was no evidence of deviations of H-W equilibrium when Rhq8 was excluded ($P= 0.617$). Even so, this locus may be helpful for further parentage analysis of captive offspring given its exclusionary power when one parent is known (Table 1), especially if null allele frequency for candidate parents can be estimated using population or family data (López et al. 2012). Evidence of linkage disequilibrium was found between Rhq2-Rhq7 loci ($P<0.05$), although none of them resulted significant after Bonferroni correction for multiple tests.

Two microsatellite-containing sequences were functionally annotated, suggesting their consideration as putative type I markers: Rhq16, *Danio rerio* TRAF2 and NCK interacting kinase b (NM_001037676.2; e-12) and Rhq20, *Danio rerio* lysocardiolipin acyltransferase

mRNA; (NM_213270.1; 4e-10). It should be taken into account for further population studies, since the comparison between gene-linked and anonymous microsatellites has been useful for searching adaptative variation in populations (Vilas et al. 2010).

The 1/16 plate pyrosequencing 454 GS-FLX run implemented in *R. quelen* samples reinforce that this technique is a cost-effective tool to develop microsatellites markers. The number of reads and percentage of reads containing microsatellites obtained were similar to that of Perry & Rowe (2010) in *Gerris incognitus*. All microsatellite-containing sequences identified in this study constitute valuable resources to select genetic markers for further studies in this species under different purposes. Indeed, the ten *R. quelen*-specific microsatellite markers validated in this work comprise a useful loci panel for evaluating genetic diversity of wild and captive populations, as well as to implement genealogical traceability in breeding programs. Also, the inclusion in further population analyses of different genetic lineages of *R. quelen* species complex (Garcia et al. 2010) could clarify the taxonomic status of these different groups for conservation and production purposes.

Acknowledgements

Financial support from FMV_2009_1_2793_-Project_ ANII of Uruguay allowed this collaborative research with the ACUIGEN Laboratory in the Universidad de Santiago de Compostela (Lugo, Spain). We thank Susana Sanchez, Lucia Insua, Vanessa Perez, María López and Maria Portela for technical support.

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Figure legends

276 Figure 1. Distribution frequencies of microsatellites motifs in *R. quelen*. (a) dinucleotide; (b)

277 trinucleotide and (c) tetranucleotide.

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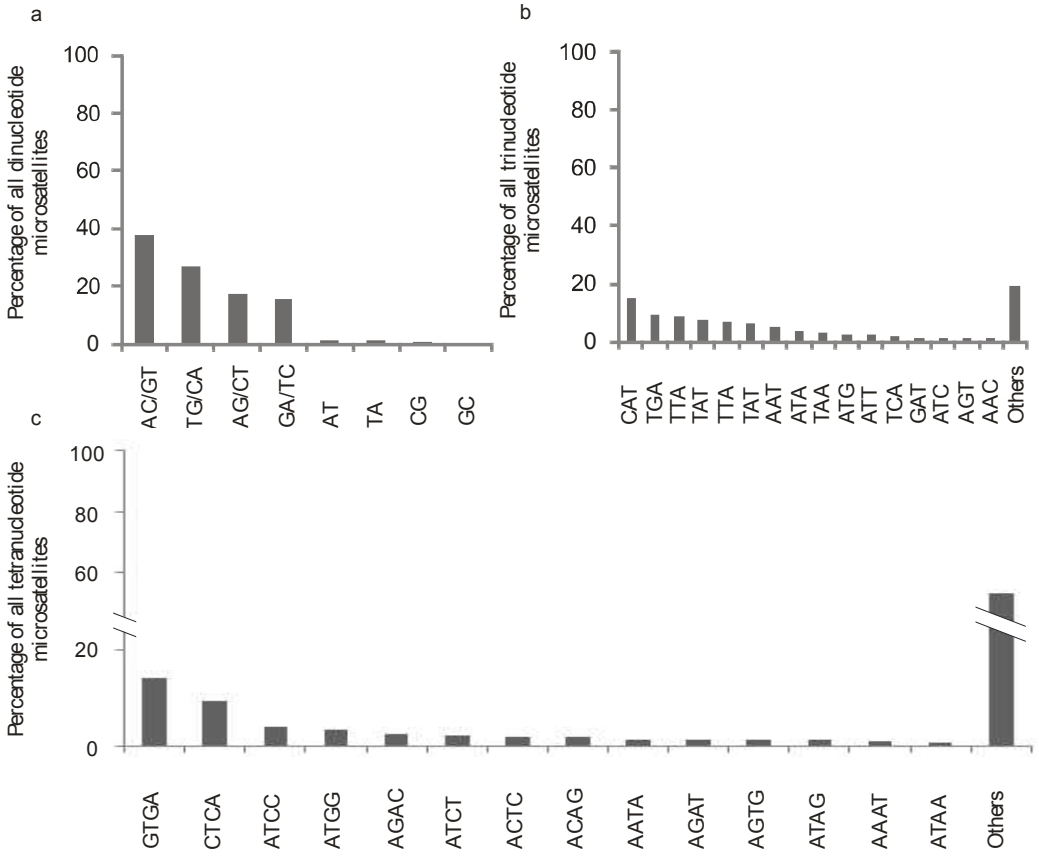

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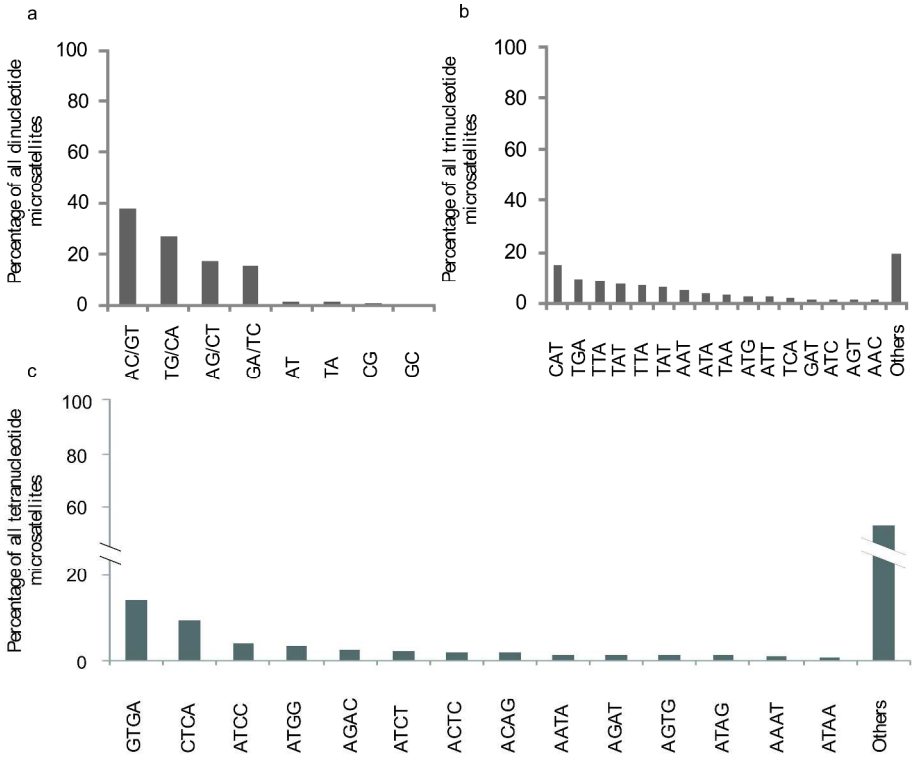
Table 1. Characteristics of the 10 microsatellite loci validated on a test panel of 24 individuals of *R. quelen.*

Locus	Accession n°	Primer sequence (5'-3')	Dye	Locus repeat	T_m (° C)	N	Na	Allele size (bp)	H_O	H_E	P_{HWE}	EXC1	EXC2
Rhq2	KC117543	F: CCTCTTTCTCCTTCCCGTTT; R: CGCACTTGTTCTGTCTGTCC	VIC	$(GA)_{15}$	58	24	10	243-285	0.917	0.870	0.570	0.539	0.703
Rhq7	KC117544	F: CTGCGGGAAGGTCTCTGTC; R: GTCTCCGGCTCCTGGTATC	NED	$(GT)_{14}$	58	20	5	253-277	0.450	0.545	0.672	0.152	0.311
Rhq8	KC117545	F: AGTGCATGGATGGTCAATAGG; R: TCGTGATTGGAGGTGAGATG	PET	$(AC)_8$	58	24	4	243-253	0.125	0.600	0.000	0.183	0.330
Rhq13	KC117546	F: CCAGCTCCCAACAGTGAAAT; R: CGTGGAGAGGGTTCGTATGT	PET	$(CA)_{13}$	58	24	8	315-348	0.625	0.775	0.101	0.366	0.543
Rhq15	KC117547	F: AGTCTAATGTCTTAACCGCTGA; R: CGGTATCCTCTTTGGTATGC	VIC	$(AC)_{14}$	54	24	10	134-179	0.792	0.754	0.898	0.358	0.540
Rhq16	KC117548	F: CGAGGAAGTGATGTTCTATTGT; R: ACACGGAGGATTGGTGATT	NED	$(AC)_{11}$	58	23	12	299-339	0.696	0.766	0.120	0.369	0.548
Rhq20	KC117549	F: TCGTGAGCAATGTGGTTATGT; R: GCCGCGAGATTAGTTTGTGT	PET	$(AC)_{13}$	58	22	8	203-253	0.591	0.644	0.427	0.236	0.417
Rhq26	KC117550	F: AACTACGCAGCCATTTACGG; R: CGGTCGTGGGATGTCTATTC	VIC	$(GCT)_7$	58	23	9	231-288	0.739	0.753	0.751	0.345	0.525
Rhq28	KC117551	F: CCTCAGTGGTGGTAAGAGCAG; R: TCGAGCTTCCATGATACAAA	FAM	$(TATTA)_{13}$	58	24	8	165-210	0.875	0.871	0.233	0.540	0.704
Rhq29	KC117552	F: TATACCTGCGCGTACCTGCT; R: TGCTAGGTGAATTACTGTGTCG	FAM	$(TAT)_9$	58	21	5	350-365	0.714	0.713	0.684	0.285	0.459
mean/global							7.9		0.614	0.673		0.987	0.999
SD									0.230	0.107			

Primer annealing temperature (T_m); number of individuals analyzed (N); number of alleles (Na); observed (H_O) and expected (H_E) heterozygosity; significant departure from Hardy-Weinberg equilibrium (P_{HWE}) ($P< 0.05$); exclusion probability when no parent is known (EXC1) and when one parent is known (EXC2).



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Capítulo III

Hibridación histórica entre linajes mitocondriales y patrones de diversidad genética de *R. quelen* en cuencas de Uruguay

Hydrobiologia
https://doi.org/10.1007/s10750-018-3861-z

PRIMARY RESEARCH PAPER

Past hybridisation and introgression erased traces of mitochondrial lineages evolution in the Neotropical silver catfish *Rhamdia quelen* (Siluriformes: Heptapteridae)

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Received: 9 August 2018 / Revised: 11 December 2018 / Accepted: 13 December 2018

Abstract The Neotropical species complex *Rhamdia quelen* is composed of at least six mitochondrial lineages. Three of these occur in sympatry in several regional basins, which encompass La Plata basin, Patos-Merin basin system and the coastal lagoons draining to SW Atlantic Ocean. Based on both mitochondrial cytochrome b gene and 10 nuclear microsatellite loci markers, this study aims to investigate the genetic diversity pattern and the reproductive isolation among *R. quelen* mitochondrial lineages. Past hybridisation and introgression were evidenced among at least two mitochondrial lineages. The ancestral structure recovered in this study was divided into two groups, which could have diverged in the last marine regression. The recent population structure of *R. quelen* species complex is mostly recovered following the geographic distribution pattern. We delimited seven management units, three inhabiting riverine environments and four associated to different coastal lagoons. Lagoon populations, unlike the riverine ones, would have diverged in a scenario with null or restricted gene flow and small size population, possibly related to bottleneck events. Population genetic structure should be considered for conservation legislation, fishery management and aquaculture regulation. Additionally, the present genetic structure could aggravate the impact of specimen translocation and escapees from aquaculture farms.

Keywords Population genetics · *Rhamdia quelen* · Neotropical · Hybridisation · Microsatellite loci

Handling editor: Diego Fontaneto

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10750-018-3861-z) contains supplementary material, which is available to authorized users.

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Introduction

Rhamdia quelen (Quoy & Gaimard, 1824) is widely distributed along South America, specifically from the extreme northeast of Andes to the centre of Argentina (Perdices et al., 2002). This species is an important resource exploited across its geographical distribution in aquaculture and fishery (mainly artisanal and sport fishery) (Vaz et al., 2010; Ittzés et al., 2015; Menezes et al., 2015; Scaranto et al., 2018). Silver catfish, as *R. quelen* is commonly known, is considered a priority

Published online: 18 December 2018

for conservation in Uruguay because of its cultural and economic value (Loureiro et al., 2013). Despite its potential and relevance, life history characteristics of *R. quelen*, as well as of several other South American fish species, remain largely unknown (Schulz & Leuchtenberger, 2006). The genus *Rhamdia,* and particularly *R. quelen,* has a controversial taxonomic history. In this sense, several authors have attempted to solve this systematic problem by morphological, cytogenetic or phylogenetic approaches (Silfvergrip, 1996; Perdices et al., 2002; Garcia et al., 2003, 2010; Martinez et al., 2011; Hernández et al., 2015; Garavello & Shibatta, 2016; Ribolli et al., 2017; Ríos et al., 2017, Angrizani & Malabarba, 2018). These studies have synonymised, reappraised and evidenced populations and mitochondrial lineages that are highly divergent. In this regard, six mitochondrial lineages (Rq1–Rq6) have been characterised within the species complex *R. quelen,* based on cytochrome b (cyt b) gene (Ríos et al., 2017). These mitochondrial lineages have diverged deeply in allopatry, since the date of the most recent common ancestor of this species complex was estimated to be 5.94 million years ago (MYA) (Ríos et al., 2017). Moreover, systematic reappraisal of species and lineages were proposed mainly based on mitochondrial gene markers (Hernández et al., 2015; Garavello & Shibatta, 2016; Ribolli et al., 2017). However, species delimitation should be based not only on a deep phylogenetic structure using mitochondrial sequences, but also confirmed by multi-locus nuclear data analyses (Singhal & Moritz, 2013). Scaranto et al. (2018) found evidence of presence in native *R. quelen* populations of individuals originated from hatcheries. These authors highlighted the need to regulate aquaculture activities, due to the presence of two mitochondrial lineages of the *R. quelen* species complex, other two species of *Rhamdia* and possible hybrids in fish farms. Thus, the study of reproductive isolation among mitochondrial lineages in water bodies where these lineages are in sympatry should be a priority for discriminating whether they are different cryptic species or behave as a single population.

The basin system inhabited by the four most Southern mitochondrial lineages of *R. quelen* (Rq2, Rq4, Rq5 and Rq6) encompasses La Plata basin (LP), Patos-Merin basin system (PM) and the coastal lagoons draining to SW Atlantic Ocean (AO) (altogether, LP-PM-AO). These mitochondrial lineages have an overlapping distribution, even inhabiting the same basin, which offers a great advantage to study the evolution of *R. quelen*, specifically the reproductive isolation among these lineages in sympatry. However, such areas inhabited by more than one mitochondrial lineage were scarcely represented in previous field surveys to date (Ríos et al., 2017).

Mitochondrial DNA sequences have often been used to resolve phylogenetic relationships (e.g. Yang et al., 2006; Hernández et al., 2015). Nevertheless, mitochondrial gene trees could differ from species tree phylogenies, due to introgression, paralogy and differential lineage sorting of polymorphisms (Wallis et al., 2017). Freshwater species group divergence is favoured by a highly structured environment (e.g. river, tributaries, lake and ponds), causing a high phylogeographic structure in the group (Wallis et al., 2017). However, secondary contact due to geomorphological evolution (e.g. river capture and extreme flooding) or climatic change can promote the contact between allopatrically distributed populations and hybridisation (Wallis et al., 2017). Hybridisation is defined as the interbreeding among individuals that belong to genetically distinct populations, regardless of the taxonomic status of these populations (Rhymer & Simberloff 1996; Allendorf et al., 2013). On the other hand, microsatellite nuclear markers are codominant, have a mendelian inheritance, high mutational rate and are highly polymorphic (Chistiakov et al., 2006). Microsatellites are powerful tools for understanding genetic and demographic processes, specially over recent time periods (Gordeeva et al., 2010; De Barba et al., 2017). These markers are useful for detecting phenomena related with population dynamics, such as bottleneck events, effective population size and population size fluctuations (Chistiakov et al., 2006). Genetic analyses based on microsatellites have identified hybridisation in different fish groups (e.g. Hänfling et al., 2005), and particularly between catfish species (e.g. Zhong et al., 2015; do Prado et al., 2017). Specific microsatellite loci have been isolated in *R. quelen* (Molecular Ecology Resources Primer Development Consortium et al., 2013; Danyelle et al., 2015). However, microsatellite population genetic diversity studies of *R. quelen* have been scarce and based on few (5) and mostly cross-species transferable loci (Ribolli & Zaniboni-Filho, 2009; Ríos et al., 2017).

Delineation of conservation units (CUs) is the starting point for the wildlife managers to protect biodiversity (Moritz, 1994; Funk et al., 2012). CUs can be divided into evolutionarily significant units (ESUs) and management units (MUs) (Moritz, 1994). The ESUs are defined as a set of populations historically isolated. Thus, they are distinguished as monophyletic groups in phylogenies and show significative divergence of allele frequencies at nuclear loci. Populations recently diverged are usually classified as MUs. In this case, units are identified by multi-locus genetic differentiation, usually based on microsatellite loci, but they are paraphyletic in mitochondrial phylogenies (Moritz, 1994; Chistiakov et al., 2006; Funk et al., 2012; Rutkowski et al., 2017). In a previous phylogeographic survey, six mitochondrial-based ESUs within *R. quelen* complex species have been identified; however, the MUs analysis based on microsatellite and mitochondrial loci was only focused in three coastal lagoons which are inhabited by a single mitochondrial lineage (Rq6) (Ríos et al., 2017).

This study aims to understand the genetic structure of *R. quelen* mitochondrial lineages from the LP-PM-AO basin system, including environments where they are in sympatry. For this purpose, we used the mitochondrial cyt b locus and described a *R. quelen*-specific set of 10 microsatellite loci to analyse the genetic diversity pattern and to investigate the reproductive isolation among mitochondrial lineages in sympatry, as a step towards defining conservation units. Secondarily, the present study compares riverine and lagoon populations of *R. quelen*. This study expanded the sampling survey in river basins inhabited by more than one mitochondrial lineage of this species.

Materials and methods

Sample collection and DNA extraction

All sampling protocols were approved by the CNEA (Comisión Nacional de Experimentación Animal) from Uruguay.

A total of 194 *R. quelen* specimens were analysed from 14 localities belonging to five major basins (Fig. 1; Supplemental file I): Uruguay River basin (UR): Cuareim River (1-UR-CR), Arapey River (2-UR-AR), Queguay River (3-UR-QR); Negro River basin (NR): Abrojal Stream (4-NR-A), Rincón del Bonete dam (5-NR-RB), Tacuarembó River (6-NR-T), Paso Mazangano town (7-NR-PM); La Plata River basin (LP): Talita Stream (8-LP-T), Sauce Lagoon (9-LP-SL); coastal SW Atlantic Ocean basin (AO): Blanca Lagoon (10-AO-BL), Rocha Lagoon (11-AO-RL), Castillos Lagoon (12-AO-CL); Merin Lagoon basin (ML): Negra Lagoon (13-ML-NL), Quebrada de los Cuervos (14-ML-QC). The 14 localities are grouped in two kinds of environment: riverine (1-UR-CR, 2-UR-AR, 3-UR-QR, 4-NR-A, 5-NR-RB, 6-NR-T, 7-NR-PM, 8-LP-T, 14-ML-QC) and lagoon (9-LP-SL, 10-AO-BL, 11-AO-RL, 12-AO-CL, 13-ML-NL). This basin division is in accordance with geographical evidence presented by Achkar et al. (2013), except for Uruguay and Negro rivers, which were considered altogether as Uruguay River basin. However, we distinguished between Negro River basin and Uruguay River basin based on ichthyofaunal criteria presented by Serra et al. (2014). Sampling was designed to complete previous surveys in the LP-PM-AO basin system by Ríos et al. (2017), in order to expand the poorly represented basins inhabited by more than one lineage. A total of 47 new specimens were collected in the current study. Additionally, we analysed a sample from a hatchery Villa Constitución (VC) that is the main Uruguayan producer of *R. quelen* fish seed for aquaculture. The 4-NR-A, 6-RN-TR, 8-RN-SL and 13-ML-NL localities are poorly represented due to sampling difficulties, but were included in order to extend the sampling area in the genetic structure analyses. All samples were captured between 2010 and 2017. The voucher specimens were stored in ethanol 95% at Sección Genética Evolutiva, Colección de Zoología de Vertebrados de Facultad de Ciencias or at Museo de Historia Natural of Uruguay. Some specimens were stored in formol 1%, but a tissue sample was always previously stored in ethanol 95%. Total DNA was extracted from muscle, liver or adipose fin (preserved in ethanol 95%) using proteinase K digestion, followed by sodium chloride extraction and ethanol precipitation (modified from Medrano et al., 1990).

Mitochondrial marker amplifications and sequencing

The cyt b gene was partially amplified in the new 47 individuals collected in this study, using the following

Fig. 1 Collecting sites of *Rhamdia quelen* samples analysed from La Plata basin (LP), Patos-Merin basin system (PM) and the coastal lagoons draining to SW Atlantic Ocean (AO) (LP-PM-AO). The different localities are included in geographical basins: Uruguay River basin (UR: green): Cuareim River (1-UR-CR), Arapey River (2-UR-AR), Queguay River (3-UR-QR); Negro River basin (NR: red): Abrojal Stream (4-NR-A), Rincón del Bonete dam (5-NR-RB), Tacuarembó River (6-NR-T), Paso Mazangano town (7-NR-PM); La Plata River basin (LP: violet): Talita Stream (8-LP-T), Sauce Lagoon (9-LP-SL); coastal SW Atlantic Ocean basin (AO: yellow): Blanca Lagoon (10-AO-BL), Rocha Lagoon (11-AO-RL), Castillos Lagoon (12-AO-CL); Merin Lagoon (ML: orange): Negra Lagoon (13-ML-NL), Quebrada de los Cuervos (14-ML-QC). Additionally, Villa Constitución hatchery (15-VC) individuals were analysed. Pie charts at each population display the mitochondrial lineage frequency in each population (Rq2: grey; Rq4: black and Rq6: white)

universal primers for fish: Gludg-L and CB3-H primers (Palumbi et al., 1991). The amplification conditions were carried out as follows: 10 min at 94°C; 35 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 1:30 min and 72°C for 10 min. The total volume reaction was 20 µl including 1 × Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each primer, 1 unit of Taq DNA polymerase (Invitrogen) and 45 ng of template DNA. The amplified fragment was sequenced directly on both strands with a Perkin-Elmer ABI Prism 377 Automated Sequencer (MACROGEN, Seoul, Korea). Sequence alignments were performed using Clustal W 1.8 (Thompson et al., 1994).

Mitochondrial lineage identification using partial sequence of cyt b

A total of 194 individuals were analysed in the cyt b dataset, which comprised 147 sequences retrieved from GenBank (Ríos et al., 2017) in addition to the new samples collected in this study (Supplemental file I). Individuals were classified based on phylogenetic analyses. The phylogeny was built with PhyML 3.1, using Maximum likelihood (ML) (Guindon & Gascuel, 2003). The nucleotide substitution model used for the ML analysis was selected in jModelTest v.2.1.10 (Darriba et al., 2012), based on the Bayesian Information Criterion (BIC) (Schwarz, 1978) and

Table 1 Description of 10 microsatellite loci and amplification characteristics

Loci	Genbank accession number	Primer sequence (5′-3′)	Fluorescent dye	Annealing temperature	Locus motif
Rhq2	KC117543	F: CCTCTTTCTCCTTCCCGTTT	VIC	58	GA
		R: CGCACTTGTTCTGTCTGTCC			
Rhq7	KC117544	F: CTGCGGGAAGGTCTCTGTC	NED	58	GT
		R: GTCTCCGGCTCCTGGTATC			
Rhq8	KC117545	F: AGTGCATGGATGGTCAATAGG	PET	58	AC
		R: TCGTGATTGGAGGTGAGATG			
Rhq13	KC117546	F: CCAGCTCCCAACAGTGAAAT	PET	58	CA
		R: CGTGGAGAGGGTTCGTATGT			
Rhq15	KC117547	F: AGTCTAATGTCTTAACCGCTGA	VIC	58	AC
		R: CGGTATCCTCTTTGGTATGC			
Rhq16	KC117548	F: CGAGGAAGTGATGTTCTATTGT	NED	58	AC
		R: ACACGGAGGATTGGTGATT			
Rhq20	KC117549	F: TCGTGAGCAATGTGGTTATGT	PET	58	AC
		R: GCCGCGAGATTAGTTTGTGT			
Rhq26	KC117550	F: AACTACGCAGCCATTTACGG	VIC	58	GCT
		R: CGGTCGTGGGATGTCTATTC			
Rhq28	KC117551	F: CCTCAGTGGTGGTAAGAGCAG	FAM	58	TATTA
		R: TCGAGCTTCCATGATACAAA			
Rhq29	KC117552	F: TATACCTGCGCGTACCTGCT	FAM	60	TAT
		R: TGCTAGGTGAATTACTGTGTCG			

Decision Theory criterion (DT) (Minin et al., 2003). A heuristic ML search was performed using NNI (Nearest Neighbour Interchange search) and the robustness of the nodes was determined using Bootstrap analysis (100 pseudoreplicates).

Nuclear loci amplification and genotyping

All 194 specimens were genotyped in the current study for the following species-specific microsatellite loci: Rhq2, Rhq7, Rhq8, Rhq13, Rhq15, Rhq16, Rhq20, Rhq26, Rhq28, Rhq29 (Molecular Ecology Resources Primer Development Consortium et al., 2013). The loci sequences are public in the following GenBank accession numbers: KC117543–KC117552. Here, we present the description of primer sequences and specifications for amplification of these microsatellite loci (Table 1). The 10 loci were amplified using the following cycling profile: 10 min at 94°C; 35 cycles of 45 s at 94°C, 50 s at primer-specific annealing temperature (Table 1) and 50 s at 72°C and finally, 10 min at 72°C. The total reaction volume was 15 µl including 1× Buffer 10X, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), 3 pmol of each primer and 30 ng of template DNA. Allele sizes screening was analysed on an ABI 3730 xl DNA sequencer using LIZ 500 (Applied Biosystems) as internal standard and the peak scoring software GeneMapper® v3.7 (Applied Biosystems®).

Microsatellite data analyses

Presence of null alleles and genotyping errors, such as stuttering and allelic dropouts, were evaluated using Micro-Checker v. 2.2.3. (Van Oosterhout et al., 2004). Hardy–Weinberg equilibrium (HWE) exact test was calculated for each locus. The magnitude and sign of the departure of HWE at each locus were analysed using intrapopulation fixation index (F_{IS}) (Weir & Cockerham, 1984). HWE and F_{IS} were calculated using Genepop v. 4.2 (Raymond & Rousset, 1995). Gametic disequilibrium test (D) between pairs of loci was analysed within each population. D was carried out in the programme LinkDos (Garnier-Gere & Dillmann, 1992). Sequential Bonferroni correction

was applied for multiple comparisons within each population. In addition, the relatedness coefficient between individuals (r_{xy}) was estimated according to Wang (2002) and performed on SPAGEDI (Hardy & Vekemans, 2002). Polymorphism at microsatellite loci was measured by the number of alleles (A), allelic richness (r_A), observed heterozygosity (H_{OBS}) and expected heterozygosity (H_{EXP}). Allelic richness is suitable to compare samples with different sampling sizes, because it is an estimate of allelic diversity adjusted by the smallest sample size. Allelic richness was calculated on locality samples consisting of more than 10 individuals. Number of alleles and r_A were calculated using FSTAT v.2.9.3 (Goudet, 2001), whereas H_{OBS} and H_{EXP} were calculated on Cervus v. 3.7. (Kalinowski et al., 2007). Additionally, private alleles with frequency greater or equal to 5% were counted for each locus.

NewHybrids v.1.1. (Anderson & Thompson, 2002) was used to evidence hybridisation between mitochondrial lineages of *R. quelen* (Rq4 and Rq6). Individuals that belong to Rq2 mitochondrial lineage were excluded from this analysis given the low sample size available (six fish, Supplemental file I). Six ancestry classes were explored: groups of first (Rq6) or second (Rq4) parental mitochondrial lineages, F1 (First filial generation), F2 (Second filial generation), Backcross (BC) with Rq6 and BC with Rq4. Individuals from 9-LP-SL, a well-represented Rq6-fixed population cluster in the Structure analysis (see Results), were used to set the prior allele frequency information of this mitochondrial lineage. Since all collection sites of Rq4 were represented by a low number of fishes that coexist in sympatry with Rq6 specimens (Fig. 1; Supplemental file I), prior allele frequency for Rq4 mitochondrial lineage was not set. The sensitivity of NewHybrids results were examined with both Uniform and Jeffrey prior distributions. Posterior probabilities were evaluated after 100,000 iterations of the Monte Carlo Markov Chains (MCMC), with an initial burn-in of 50,000 steps. A posterior probability threshold of 0.50 was used to assign individuals into ancestry classes. In order to evaluate the power of the 10 microsatellite loci assayed to discriminate among ancestry classes due to hybridisation between differentiated parental pools in *R. quelen*, a simulation analysis was also performed based on available sampling data. In the absence of pure Rq4-fixed populations, as explained above, the best represented and genetically differentiated samples in the studied area (9-LP-SL and 11-AO-CL) were selected as parentals to perform the analysis. Given that both samples are composed only of Rq6 specimens, we can expect even higher power to discriminate between different mitochondrial lineages, due to their high genetic divergence. For that purpose, we simulated the following classes using HYBRIDLAB (Nielsen et al., 2006): F1, F2, BC with 9-LP-SL and BC with 11-AO-CL. BC was generated using an equal number of parental and F1 individuals. A total of 100 individuals of each class were generated. NewHybrids parameter settings were equal to the real population data analysis, using both Uniform and Jeffrey prior distributions, and the same posterior probability threshold.

The genetic structure of *R. quelen* populations from LP-PM-AO was investigated using molecular variance analysis (AMOVA). This is a hierarchal analysis of variance at different levels: among populations (F_{ST}), among populations within groups (F_{SC}) and among groups (F_{CT}). We tested the following hypotheses: (a) *R. quelen* populations are structured by both mitochondrial lineage (Rq2, Rq4 and Rq6; Ríos et al., 2017; this study: See "Results"; Supplemental file I) and basins (Uruguay River, Negro River, La Plata River, Atlantic Ocean drainages and Merin Lagoon; Fig. 1); (b) genetic structure is explained only by basin structure (Fig. 1); (c) the populations are structured only by mitochondrial lineage; (d) finally, the close ancestral relationship between 9-LP-SL and 11-AO-RL proposed by Ríos et al. (2017) was also considered to group populations by basin, 11-AO-RL being grouped with populations from La Plata basin. For the hypothesis (a), the populations were grouped as follows (Supplemental file I): (i) Rq2 Uruguay River (individuals belonging to Rq2 mitochondrial lineage, from Uruguay River basin); (ii) Rq4 Uruguay River; (iii) Rq6 Uruguay River; (iv) Rq4 Negro River; (v) Rq6 Negro River; (vi) Rq6 La Plata River; (vii) Rq6 from coastal lagoon draining to Atlantic Ocean; (viii) Rq4 Merin Lagoon; (ix) Rq6 Merin Lagoon. The hypothesis (b) was established irrespective of mitochondrial lineages as follows (Supplemental file I): (i) Uruguay River basin (Rq2, Rq4 and Rq6); (ii) Negro River (Rq4 and Rq6); (iii) La Plata River (Rq6); (iv) coastal lagoon draining to Atlantic Ocean (Rq6); (v) Merin Lagoon (Rq6). For hypothesis (c), we grouped the populations as follows (Supplemental file

I): (i) Rq2; (ii) Rq4 and (iii) Rq6. Finally, hypothesis (d) was similar to hypothesis (b), except for 11-AO-RL, which was grouped with La Plata basin (Supplemental file I). The most plausible genetic structure is the one that showed the highest F_{CT} value. AMOVA was carried out using ARLEQUIN v.3.5 (Excoffier & Lischer, 2010). Furthermore, inter-sample genetic differentiation was calculated through pairwise F_{ST} estimates with ARLEQUIN v.3.5 (Excoffier & Lischer, 2010).

Discriminant analysis of principal components (DAPC) scatter plots was performed using the R package *adegenet* (Jombart, 2008), to unravel the population structure of *R. quelen*. For this purpose, the number of principal components to be retained was 100, containing 95% of the cumulative variation of the data. Also, Bayesian analysis of population structure was implemented on STRUCTURE v. 2.3 (Pritchard et al., 2000; Falush et al., 2007) to cluster individuals into populations on the basis of the microsatellite dataset. This software uses the MCMC algorithm. Population numbers (K) were analysed from 1 to the number localities (15) plus 3 (K = 1 to K = 18). Ten independent runs for each K were implemented with a burn-in period length of 100,000 iterations, followed by 200,000 MCMC replicates. Admixture model, correlated allele frequencies and recessive alleles for null allele presence were considered. The consensus result for each K was obtained from the independent runs by means of CLUMPAK (Kopelman et al., 2015). The most probable K value was determined using both likelihood and Delta k criteria (Δk, Evanno et al., 2005), and calculated using STRUCTURE HARVESTER (Earl & vonHoldt, 2012).

We tested recent bottlenecks in the *R. quelen* populations from all localities, particularly including Blanca lagoon (10-AO-BL), because this site faced a strong reduction of its fishery volume in 1997, due to a severe drought between 1995 and 1997 related to El Niño events, and to water consumption by the state-owned water supply company (Mazzeo et al., 2003). This critical environmental change reduced or extinguished several species in Blanca lagoon (de Mello et al., 2007). For this purpose, we used the Wilcoxon test for heterozygosity excess implemented in Bottleneck v.1.2. (Luikart & Cornuet, 1999), which is powerful and appropriate for dataset of less than 20 loci assuming TPM model with 95% single-step mutations and 5% multiple-step mutations (variance among multiple steps of approximately 12) (Luikart & Cornuet, 1999). Additionally, short-term/contemporary effective population size (Ne) using the linkage disequilibrium method was performed on NeEstimator v.2.1. for each locality (Do et al., 2014).

Results

Mitochondrial lineages identification

A total of 47 new partial sequences of mitochondrial cytochrome b gene (cyt b) were generated in this study (GenBank accession number: MH669076-MH669122, Supplemental file I), and analysed together with 147 *R. quelen* sequences retrieved from GenBank (Supplemental file I) across the main basins in Uruguay from a previous study (Ríos et al., 2017). In a cyt b dataset of 758 bp, 99 were variable sites and 70 were parsimony informative sites. The 194 individual cyt b sequences of *R. quelen* from LP-PM-AO and the hatchery broodstock (15-VC) are divided into 31 haplotypes. According to the BIC and DT methods, HKY + G (gamma distribution) (Hasegawa et al., 1985) was the nucleotide substitution model that best fitted to the cyt b dataset. Gene tree topology was consistent with Ríos et al. (2017), and accordingly, each individual of *R. quelen* was assigned to one of the three different mitochondrial lineages identified in the studied basins: Rq2, Rq4 or Rq6 (Supplemental file I; Fig. 1).

Genetic variation of nuclear microsatellite loci in *R. quelen*

All 194 individuals were genotyped for a species-specific set of 10 microsatellite loci. The population genetic diversity in rivers (Riv) was higher than that of lagoons (Lag) environments (Table 2). Differences in H_{EXP} and r_A resulted significant (H_{EXP} means: 0.835 and 0.694, test Mann–Whitney P = 0.003; r_A means: 8.347 and 5.136; based on minimum sample size of nine diploid individuals, P = 0.037; Table 2). The locus Rhq26 showed on average the highest r_A (mean = 8.231), while Rhq29 exhibited the lowest r_A value (mean = 3.966) (Supplemental file II). Riv populations showed more private alleles (Ap mean: 4) on average than Lag ones (Ap mean: 2), but differences were not significant (test Mann–Whitney

$P = 0.342$). The hatchery sample (15-VC) showed intermediate genetic diversity value (Table 2). Null alleles per locus were scattered across populations, except in the case of the locus Rhq8, for which signs of null alleles were found in almost all localities analysed (Supplemental file II). For this reason, this locus should be used with caution. MICRO-CHECKER revealed lines of evidence of stuttering errors in Rhq8 and Rhq26 in 11-AO-RL, however, we revised the electropherograms and stuttering was not detected. These could be due to false positives caused by genotypes heterozygosity deficiency with alleles differing in one repeat unit as consequence to small sample size. Significative departures of HWE observed across loci were mostly associated to null allele evidence (except for Rhq16 on 9-LP-SL) and heterozygote deficiency (F_{IS} mean 0.191 ± S.E. 0.095). All localities analysed were possibly in HWE with some loci showing signs of a null alleles (Supplemental file II). No sign of significant gametic disequilibrium linkage between pairs of loci in each population was evidenced after Bonferroni correction.

NewHybrids analyses of hybridisation between Rq4 and Rq6 mitochondrial lineages in LP-PM-AO based on Uniform prior distributions (Fig. 2a, c) identified two individuals as F2 hybrids, and eight were indeterminate (unassigned to parental or hybrid classes due to low posterior probability below the set threshold 0.5). In this analysis, 80 specimens were assigned to pure Rq4, despite the real mitochondrial lineage was Rq6 (Fig. 2c), suggesting a hybrid nuclear genetic component for a total of 82 fish (41.4%) distributed across different localities (Fig. 2 *a*): 1-UR-CR, 2-UR-AR, 3-UR-CR, 4-NR-A, 5-NR-RB, 6-NR-TR, 7-NR-PM, 8-LP-T, 10-AO-BL, 12-AO-CL, 13-ML-NL, 14-ML-QC, 15-VC. NewHybrids ran with Jeffrey priors (Fig. 2b, d) evidenced 35 F2 hybrids, 37 backcrosses with Rq4 and 10 indeterminate. In addition, 26 individuals were misassigned to Rq4 mitochondrial lineage (Fig. 2d), suggesting hybridisation signals in a total of 98 individuals (50.5%) across all localities analysed, except in 9-LP-SL (used as prior allele frequencies for Rq6 lineage).

NewHybrids sensitivity analysis of the simulated populations using Uniform prior distribution correctly identified 58% of individuals (parental 9-LP-SL: 93%; parental 11-AO-CL: 87%; F1: 87%; F2: 9%; BC with 9-LP-SL: 61%; BC with 11-AO-CL: 51%) (Supplemental file III). Using Jeffrey prior distribution, 66% of individuals were successfully classified (9-LP-SL: 91%; 11-AO-CL: 83%; F1: 79%; F2: 43%; BC with 9-LP-SL: 70%; BC with 11-AO-CL: 54%) (Supplemental file III). While 94% of individuals were correctly identified as parental individuals using Uniform prior distribution, 87% were correctly identified using Jeffrey prior distribution. A total of 77% and 83% of the individuals were correctly identified as admixed individuals using Uniform and Jeffrey, respectively (Supplemental file III).

AMOVA results for hypothesis *a* (i.e. populations of *R. quelen* are structured by mitochondrial lineage and basin) showed a proportion of genetic diversity among populations (F_{ST}) of 0.136 ($P = 0.000$), among populations within groups (F_{SC}) of 0.126 ($P = 0.000$), and among groups (F_{CT}) of 0.011 ($P = 0.230$). The second grouping, hypothesis (b), where localities were grouped only by basin, resulted in an F_{ST} of 0.137 ($P = 0.000$), F_{SC} of 0.123 ($P = 0.000$) and F_{CT} of 0.016 ($P = 0.077$). Then, when the populations were grouped by mitochondrial lineages (hypothesis (c)), the AMOVA results showed an F_{ST} of 0.149 ($P = 0.000$), F_{SC} of 0.128 ($P = 0.000$) and F_{CT} of 0.025 ($P = 0.011$). Finally, hypothesis (d), which grouped populations by basin, with exception to 11-AO-RL that was grouped together La Plata basin, resulted in F_{ST} of 0.140 ($P = 0.000$), F_{SC} of 0.103 ($P = 0.000$) and F_{CT} of 0.041 ($P = 0.000$).

DAPC scatter plots of genetic variation in multivariate space revealed a pattern that corresponds to the geographical distribution of localities, following a pattern North–South and East–West (Fig. 3). As exceptions to this general pattern, 1-UR-CR was found next to 4-NR-A and 10-AO-BL was placed between Merin Lagoon (ML: 13-ML-NL and 14-ML-QC) group and 12-AO-CL. Structure analyses led to an estimation of two clusters, based on the Delta (K) criterion (ΔK) in STRUCTURE HARVESTER (Fig. 4a; $\Delta K = 61.84$; Mean Ln(PX|K) = − 8745.02). Additionally, the most probable hypothesis according Ln(PX|K) was $K = 7$ (Fig. 4b; Mean Ln(PX|K) = − 7940.74). Figure 4c also shows the histogram for hypothesis $K = 7$, but here individuals are sorted by mitochondrial lineages. DAPC and STRUCTURE analyses were carried out excluding Rhq8 locus, due to the presence of null alleles across populations, and similar genetic structuration was found (data no shown).

Table 2 Genetic diversity estimates based on 10 microsatellite loci in *R. quelen* collecting sites from (LP-PM-AO)

Locality	Envir.	*N*	L	L freq.	Bottlen.	Significant HWE		D	F_{IS}	r_A	H_{OBS}	H_{EXP}	Mean r	Ne(CI)	Ap
				%	*P* val	NA	WO NA								
01-UR-CR	Riv	7	Rq2	29	0.248	3	0	ND	0.333	ND	0.632	0.858	− 0.110	Infinite (Inf.–Inf.)	6
			Rq4	43											
			Rq6	29											
02-UR-AR	Riv	11	Rq4	91	0.188	4	0	1	0.240	8.977	0.722	0.859	− 0.056	605.7 (14.2–Inf.)	2
			Rq6	9											
03-UR-QR	Riv	6	Rq4	67	0.246	1	1	ND	0.284	ND	0.660	0.860	− 0.010	Inf. (Inf.–Inf.)	8
			Rq6	33											
04-NR-A	Riv	3	Rq6	100	ND	0	0	ND	0.217	ND	0.634	0.773	− 0.004	Inf. (0.7–Inf.)	9
05-NR-RB	Riv	23	Rq4	17	0.009	2	1	14	0.165	8.533	0.695	0.850	− 0.027	130.7 (43.6–Inf.)	0
			Rq6	83											
06-NR-TR	Riv	3	Rq4	67	ND	0	0	ND	0.269	ND	0.700	0.800	− 0.107	Inf. (Inf.–Inf.)	1
			Rq6	33											
07-NR-PM	Riv	7	Rq4	14	0.780	1	0	ND	0.207	ND	0.700	0.900	− 0.074	Inf. (362.1–Inf.)	0
			Rq6	86											
08-LP-T	Riv	4	Rq6	100	0.216	0	0	ND	0.128	ND	0.742	0.832	− 0.017	Inf. (Inf.–Inf.)	5
09-LP-SL	Lag	46	Rq6	100	0.002	1	1	3	0.126	5.715	0.622	0.714	0.209	128.2 (36.2–Inf.)	1
10-AO-BL	Lag	10	Rq6	100	0.001	1	0	0	− 0.002	4.647	0.733	0.731	0.249	55.1 (10.6–Inf.)	1
11-AO-RL	Lag	16	Rq6	100	0.003	2	0	0	0.207	4.791	0.490	0.614	0.269	148.3 (16.6–Inf.)	0
12-AO-CL	Lag	30	Rq6	100	0.002	1	0	4	0.117	5.389	0.585	0.662	0.219	149.4 (24.8–Inf.)	1
13-ML-NL	Lag	3	Rq6	100	ND	0	0	ND	0.077	ND	0.700	0.747	0.044	Inf. (3.3–Inf.)	6
14-ML-QC	Riv	13	Rq4	23	0.042	5	0	5	0.343	7.530	0.490	0.779	− 0.053	13.6 (5.2–85.8)	4
			Rq6	77											
15-UR-VC		12	Rq2	33	0.007	1	2	2	0.163	7.190	0.652	0.771	0.052	6.4 (2.4–20.6)	3
			Rq6	67											

Kind of environmental (Envir): riverine (Riv) or lagoon (Lag); sample size (N); lineage (L); lineage frequency relative (L freq. %); significance of Wilcoxon test for H excess (Bottlen. p-val); number of significative exact test departure of Hardy–Weinberg equilibrium (HWE). Significative HWE test in locus with null allele evidence (Signif NA); significative HWE test without null allele presence (Signif. WO NA); gametic disequilibrium test (D); mean intrapopulation fixation index over loci (F_{IS}); allelic richness based on minimum sample size of 9 diploid individuals (r_A); observed heterozygosity (H_{OBS}); expected heterozygosity (H_{EXP}); mean intrapopulation relatedness coefficient (r); long-term effective size (Ne), confidence interval (CI), infinite value (Inf.); total private alleles (Ap). Unperformed analyses due to insufficient data (ND)

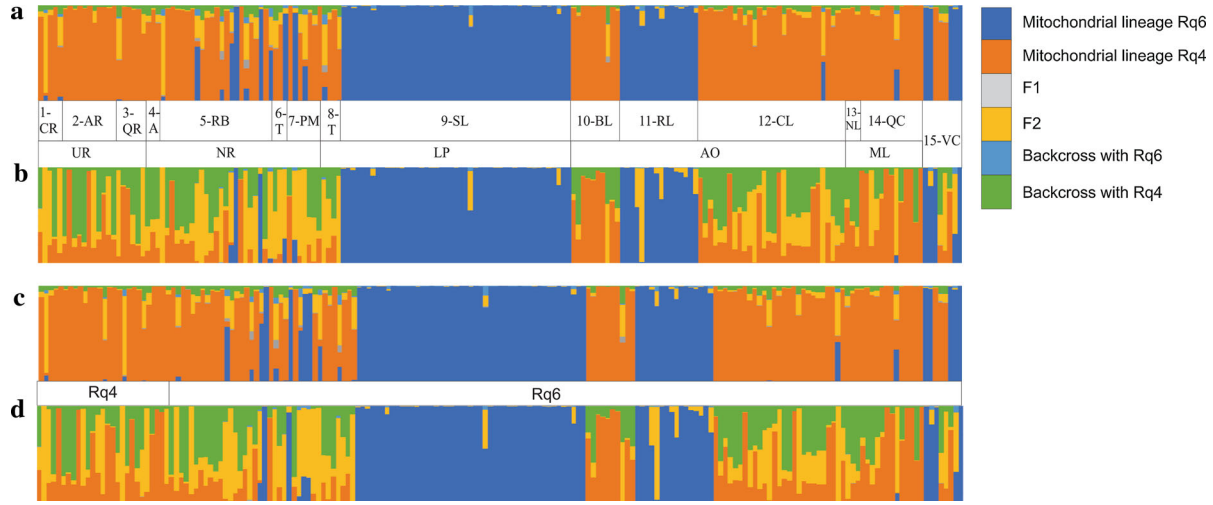

Fig. 2 Histograms of NewHybrids admixture analyses using real data from LP-PM-AO. Each bin or coloured vertical bar represents the posterior probability of belonging to each ancestry class. **a**, **c** Analysis using uniform prior distribution; **b**, **d** analysis using Jeffrey prior distribution. In (**a**, **b**), individuals are arranged according to localities and basin origin, while in (**c**, **d**) individuals are sorted by mitochondrial lineage. The colour reference of each ancestry class is displayed on the right top box

Pairwise genetic differentiation results were mostly significant (Table 3). Mean pairwise F_{ST} values were lower among river localities (mean Riv: 0.071; range − 0.003 to 0.192) than among lagoons (mean Lag: 0.161; range 0.113–0.216); the latter values were higher than those observed between lagoons and rivers (mean Riv vs. Lag: 0.134; range 0.024–0.250). These differences between Riv and Lag samples were significant (Mann–Whitney test; $P < 0.001$). Relatedness coefficients were higher within lagoons (mean Lag: 0.198) than within river localities (mean Riv: − 0.051) (Table 2; Mann–Whitney test ($P < 0.003$).

With regard to the tests for bottleneck events, significant heterozygosity excess was detected in 10-AO-BL, as well as 9-LP-SL, 11-AO-RL, 12-AO-CL, 13-ML-NL, 14-ML-QC and 15-UR-VC (Wilcoxon probability test; $P < 0.050$, Table 2). The sample 15-VC showed the lowest effective size (Ne) (6.4; confidence intervals (CI) 2.4–20.6), whereas 1-UR-CR, 3-UR-QR, 8-LP-T and 13-ML-NL showed the highest Ne (infinite; CI infinite–infinite). Point estimates of short-term Ne in Riv and Lag localities ranged from 130.7 (CI 43.6–infinite) to infinite (CI infinite–infinite) and from 55.1 (CI 10.6–infinite) to infinite (CI infinite–infinite), respectively.

Discussion

Our study constitutes the first analysis of the recent history of *R. quelen* populations, in a region inhabited by various highly divergent mitochondrial lineages of the species. Here, we considered the possible reproductive isolation among these sympatric mitochondrial lineages and explored the consequent population genetic structure.

Natural past admixture among mitochondrial *R. quelen* lineages

Beyond the deep divergence time among mitochondrial lineages (Rq2 vs. Rq4 + Rq6: 5.94 MYA; Rq4 vs. Rq6: 4.55 MYA; Ríos et al., 2017), microsatellite diversity estimates within population appeared mainly concordant with random mating and independent segregation/assortment between pairs of loci, irrespective of whether the localities are inhabited by one or various mitochondrial lineages. HWE could be restored in only one generation, but linkage disequilibrium is expected in hybrid populations or samples under Wahlund effect and the gametic disequilibrium signal of admixture would be retained by several generations (Martínez et al., 2007). NewHybrids analyses of the simulated populations evidenced

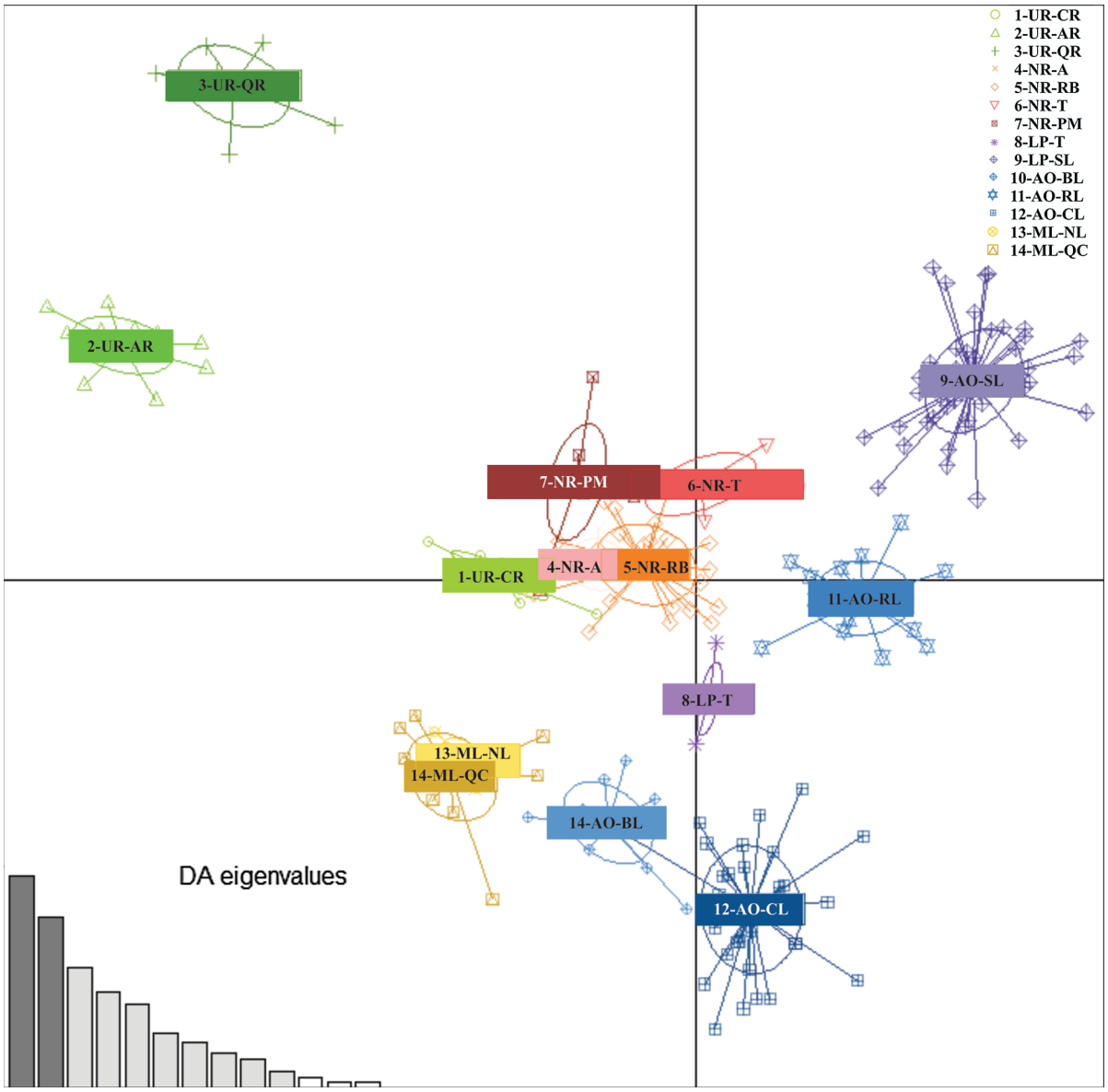

Fig. 3 DAPC scatterplot of *Rhamdia quelen* from La Plata basin (LP), Patos-Merin basin system (PM) and the coastal lagoons draining to SW Atlantic Ocean (AO) (altogether, LP-PM-AO). Individuals are represented for different shapes as are described on right top box. Individuals are grouped for locality (Fig. 1). Eigenvalues containing > 95% of the cumulative variation of the data are displayed on left bottom box

moderate ability to identify different ancestry classes between genetically differentiated parentals, but a higher accuracy in discriminating parental from admixed individuals, suggesting high informativeness of the assayed microsatellite panel for this purpose. Although the results of real data analyses in the studied region LP-MP-AO showed some differences to identify the ancestry classes under two different prior distributions (Fig. 2a, b), both analyses found more than 40% of possible interbred individuals distributed in several localities. The discordances could be associated with the sensitivity of posterior probabilities to prior distributions, especially when the frequency of some ancestral classes is low, or there

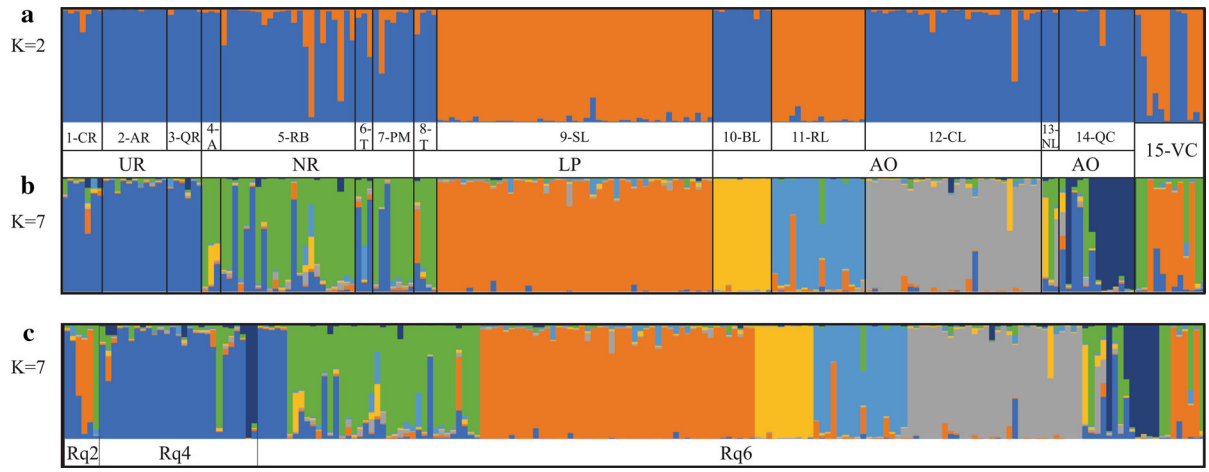

Fig. 4 Estimated population structure in STRUCTURE of *Rhamdia quelen* from La Plata, Merin Lagoon and costal lagoons from SE Atlantic Ocean based on ten microsatellite loci. Each bin or coloured vertical bar represents the estimated membership fraction of an individual into the major population clusters. The histograms (**a**) and (**b**, **c**) correspond to the clustering analysis of population structure using $K = 2$ and $K = 7$, respectively. Individuals are ordered according sampling site on (**a**, **b**). Sampling sites' numbers are indicated in Fig. 1. Individuals on histograms (**c**) are sorted by mitochondrial lineages

Table 3 Pairwise F_{ST} values based on 10 microsatellite data between localities

	1	2	3	4	5	6	7	8	14	9	10	11	12	13
Riverine														
01-UR-CR														
02-UR-AR	0.013													
03-UR-QR	0.015	0.012												
04-NR-A	**0.113**	**0.085**	**0.097**											
05-NR-RB	**0.072**	**0.063**	**0.063**	0.035										
06-NR-T	0.004	0.028	0.039	0.056	0.035									
07-NR-PM	**0.050**	**0.040**	**0.044**	0.016	-0.003	0.011								
08-LP-T	**0.090**	**0.092**	**0.099**	0.056	0.027	0.054	0.013							
14-ML-QC	**0.142**	**0.151**	**0.149**	**0.192**	**0.160**	**0.142**	**0.148**	**0.156**						
Coastal lagoons														
09-LP-SL	**0.116**	**0.147**	**0.131**	**0.131**	**0.090**	**0.089**	**0.090**	**0.096**	**0.229**					
10-AO-BL	**0.145**	**0.148**	**0.139**	**0.101**	**0.096**	**0.092**	**0.091**	**0.123**	**0.214**	**0.183**				
11-AO-RL	**0.170**	**0.176**	**0.172**	**0.177**	**0.114**	**0.148**	**0.134**	**0.155**	**0.250**	**0.125**	**0.210**			
12-AO-CL	**0.172**	**0.148**	**0.163**	0.170	0.084	0.122	**0.099**	**0.158**	**0.241**	**0.157**	**0.165**	**0.216**		
13-ML-NL	**0.141**	**0.112**	**0.123**	0.067	**0.024**	0.101	**0.051**	**0.072**	**0.207**	**0.126**	**0.116**	**0.199**	**0.113**	

Significant F_{ST} values are in bold ($P < 0.05$). Localities are grouped according to the riverine and coastal lagoon environments

are some rare alleles (Anderson & Thompson, 2002). Moreover, this could be due to several generations of interbreeding that would increase the number of ancestral classes. The number of private alleles found in several localities (Supplemental file II) suggests that Jeffrey distribution (Fig. 2b, c) could be a more suitable prior distribution in this scenario, providing lower and more plausible fraction of misclassifications as pure Rq4 mitochondrial lineage. In this sense, analysis of simulated populations using Jeffrey prior distributions also showed higher accuracy to identify admixed individuals. Furthermore, AMOVA results

suggested that the most plausible and significant population structure could be explained according to their basin origin, but considering 11-AO-RL as having originated in La Plata basin (hypothesis (d): $F_{CT} = 0.041$, $P = 0.000$). Conversely, lower component of genetic structure was evidenced considering together both mitochondrial lineages and basin origin ($F_{CT} = 0.011$, $P = 0.230$) and only the mitochondrial lineage (and $F_{CT} = 0.025$, $P = 0.011$). Previous AMOVA results based on cyt b data indicated that the pattern of genetic differentiation is better described in terms of lineage distribution (Ríos et al., 2017). The present study reinforces the importance of combining nuclear and mitochondrial information to get a complete view of the evolutionary history of *R. quelen* populations. The presence of admixed individuals in all basins (Fig. 2a, b), even when Rq4, at the present, is not found in La Plata basin nor in coastal lagoons draining to Atlantic Ocean (Fig. 1), suggests past admixture among lineages (at least Rq4 and Rq6) along the geological history of basin structure associated with low and high sea-level periods (Ochoa et al., 2015). Thus, according to AMOVA results, the recent evolutionary history of basin structure would be causing the differentiation among the populations that inhabit them. The relationship between 9-LP-SL and 11-AO-RL is discussed in more detail below. Similar scenarios of hybridisation have been reported frequently in different freshwater fish taxa (e.g. do Prado et al., 2017; Sefc et al., 2017). In brown trout from Western Mediterranean streams, lines of evidence exist of past introgression between ancestral Mediterranean and Adriatic mitochondrial lineages originated by parapatric divergence, associated with glacial periods that favoured extensive intergradation in most native Mediterranean basins (Cortey et al., 2004). In addition, recent hybridisation between native Mediterranean and allochthonous Atlantic lineages due to stocking practices with fish of hatchery origin was also reported as a threat for the conservation of locally adapted genomic resources (Vera et al., 2013). Ancestral introgression and hybridisation phenomena could explain the complex and controversial systematics between morphology and molecular data that is observed in *Rhamdia* (e.g. Silfvergrip, 1996; Perdices et al., 2002; Ríos et al., 2017; Angrizani & Malabarba, 2018). In this sense, species delimitation studies in this genus require not only taxonomic and phylogenetic analyses, but also population genetic analyses with both nuclear and mitochondrial markers in order to define and evaluate admixture among different mitochondrial lineages, which could share peculiar local adaptations.

Genetic structure of *R. quelen* from LP-PM-AO basin system

The genetic patterns observed in DAPC scatterplot is arranged according to their geographic distribution (Fig. 3). However, Cuareim river basin (1-UR-CR) is closely related to Abrojal stream (4-NR-A) (even more than to other populations from the same basin, 2-UR-AR and 3-UR-QR). This could suggest a recent past connection between these basins through Uruguay and Negro rivers. Interestingly, Blanca lagoon (11-AO-BL) appeared genetically related to Merin Lagoon basin (13-ML-NL and 14-ML-QC), providing evidence of a possible recent past connection between these currently geographically separated basins. The genetic structure of *R. quelen* divided into two groups (Fig. 4a) could support an ancestral relationship among the different populations, associated with the admixture among mitochondrial lineages. One group is mainly composed by two lagoons draining to La Plata River and SW Atlantic Ocean, Sauce Lagoon (9-LP-SL) and Rocha Lagoon (11-AO-RL), respectively. The close ancestral relationship between these lagoons had already been described using five other microsatellite loci (Ríos et al., 2017). Currently, these two lacustrine water bodies, 9-LP-SL and 11-AO-RL, are not connected and belong to different basins. However, these lagoons could have been connected 14,000–6000 years ago during the last marine regression events. At the time, La Plata River and the Atlantic Ocean coastal region consisted of an extensive coastal plain with a system of several rivers (Laborde, 1997). On the other hand, recent population structure could be mostly recovered following the pattern of geographic distribution (Fig. 4b). Thus, La Plata and Atlantic coastal lagoons 9-LP-SL, 10-AO-BL, 11-AO-RL and 12-AO-CL represent distinct population clusters. In addition, the low genetic differentiation among localities from Uruguay River (1-UR-CR, 2-UR-AR and 3-UR-QR) and the evidence of panmixia in this basin could suggest that this population cluster could be originated in this basin. Furthermore, the analysis detected an endemic divergent population cluster inhabiting Merin Lagoon basin

(14-ML-QC) (Fig. 4b). Another differentiated population cluster was observed, which includes Negro River (NR) and 8-LP-T localities (Fig. 4b). Populations inhabiting two and three basins suggest the occurrence of connections among these basins in the recent past. The connectivity among UR, NR, 8-LP-T and ML observed in structure analyses is consistent with the low genetic differentiation observed among these basins, as evidenced by pairwise F_{ST} values (Table 3) and F_{CT} (AMOVA results). On the other hand, for the hatchery samples 15-VC, the existence of an admixed cluster pointed to foundation events from different basins (1-UR-CR, 4-7-NR or 8-LP-T and 9-LP-SL). This is consistent with the information about the origin of Villa Constitución hatchery, regarding the areas where founder breeders were collected (Ríos, personal communication). The patterns of ancestral (Fig. 2a–d, 4a) and recent (Fig. 4b) genetic structure of *R. quelen* are concordant with an admixture scenario among the three mitochondrial lineages.

Patterns of genetic diversity between lagoon and river environments

Based on the present genetic structure patterns, *R. quelen* from each coastal lagoon appeared as different isolated population clusters. In this sense, Angrizani & Malabarba (2018) have recently described two new species which have diverged in coastal lagoon systems from southern Brazil. Moreover, the lower genetic diversity (H_{EXP} and r_A) and the higher average kinship between individuals within populations of coastal lagoons than riverine basins could support a scenario of isolation of small populations in absence of gene flow. In addition, recent bottleneck events in 9-LP-SL, 10-AO-BL, 11-AO-RL, 12-AO-CL supported by Wilcoxon test (Table 2) could become important in driving the interpopulation differentiation observed among coastal lagoons (Table 3). Some riverine localities presented low local effective population size (5-NR-RB and 14-ML-QC; Table 2) but higher connectivity and wider distribution than the ones of lagoons, which might explain the higher diversity and lower relatedness observed in such environments. The populations clusters, inhabiting in sympatry in various localities of Negro River (5-NR-RB, 6-NR-T and 7-NR-PM), could remain isolated by homing behaviour in a metapopulation scenario. Homing behaviour is defined by the return of individuals to their birthplace to reproduce. This behaviour could lead to the differentiation of fish groups that were born in different places, and hence, reproduce in different areas. Homing behaviour has been described for several species of catfish (Pereira et al., 2009; Ochoa et al., 2015). Additionally, these population clusters could remain isolated due to divergent selection. In this sense, genome-wide population analysis based on RADseq (restriction site-associated DNA sequencing) of a catfish species (*Creteuchiloglanis macropterus* (Ng, 2004)) evidenced genetic footprints of natural selection among highly differentiated populations from Nujiang river basin (Kang et al., 2017). Adaptive genetic variability should be investigated in *R. quelen* by means of advanced population genomic approaches using genotyping by sequencing methods (Robledo et al., 2017). Additionally, further studies should be addressed to confirm the homing behaviour in order to delimit homing distribution as well as nurseries areas in *R. quelen*.

Conservation remarks

A strong genetic structure in *R. quelen* populations was detected in the coastal lagoons that drain into the Southeastern Atlantic Ocean. For this reason, the evolutionary potential of these areas is relevant to conserve possible local adaptations and to support the intraspecific diversity of these isolated environments. We also found pieces of evidence of three genetically differentiated populations inhabiting the riverine environments. We propose putative homing areas in the Uruguay River and the Merin Lagoon basin, although another differentiated area could be found in La Plata basin (8-LP-T) or in Negro River basin. The present results confirm the three Management Units previously characterised (9-LP-SL, 11-AO-RL and 12-AO-CL) by Ríos et al. (2017) and allowed identifying four new population clusters that should all be considered for conservation and management purposes. The recent genetic patterns suggest that translocation of non-native MUs could cause a loss of genetic diversity and possible local adaptations (Pavlova et al., 2017). Additionally, we draw attention to the possible impact of aquaculture on wild populations, either due to escapees and/or potential restocking programs, which should be carefully monitored (Vera et al., 2013; Scaranto et al., 2018).

Conclusions

Our results reveal that the pattern of *R. quelen* evolution in LP-PM-AO basin system would be the product of a complex scenario of past admixture by secondary contact among mitochondrial lineages previously differenced in allopatry. In this sense, recent genetic structure allowed identifying panmictic populations or MUs that could be composed by more than one mitochondrial lineage. For this reason, the combination of nuclear and mitochondrial markers is recommended to detect genetic admixture and/or hybridisation among mitochondrial lineages within *R. quelen* species complex across the geographic area of distribution. The genetic diversity pattern of this species is mainly explained by the recent history of basin structure that would have erased an ancestral genetic pattern represented by ancient mitochondrial lineage distribution. Our study underlines the importance of riverine and costal lagoon environments determining population genetic structure putatively associated with adaptive variation in *R. quelen*. The present work adds significant contribution for the definition of management units, supporting conservation and the management of genetic resources in wild fisheries and aquaculture populations.

Acknowledgements We would like to thank Fondo Maria Viñas-Agencia Nacional de Investigación e Innovación (FMV_2009_2793 and FMV_2014_104718) for the financial support, as well as the colleagues involved in these projects: V. Gutiérrez, B. Gomez-Pardo and P. Martínez. We thank the following colleagues for kindly providing *R. quelen* specimens from different basins of Uruguay: F. Texeira, M. Loureiro, WS. Serra, A. Duarte, W. López and C. Clavijo. We are grateful to M. Vera and R. Fernández for their help with DAPC and SPAGEDI programs, respectively. We thank S. Sanchez, L. Insúa, V. Pérez, M. Portela, M. López and J. Guerra for technical support. The authors are also grateful to the Japanese government for the donation of laboratory equipment. The research of N.R. and G.G. was also supported by Sistema Nacional de Investigadores-Agencia Nacional de Investigación e Innovación (SNI-ANII).

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Supplemental file I. Geographic distribution of specimens analyzed: Collecting site, Catalogue sample number, mitochondrial lineage, Genbank accession number for cyt b sequences, population division for AMOVA analyses and groups used for each AMOVA hypothesis (See Results). For AMOVA hypothesis *c*, AMOVA population were grouped according the mitochondrial lineage (Cyt b lineages). Genbank accession numbers of novel sequences are marked with an asterisk. All individuals were genotyped for 10 microsatellite loci. 15-VC samples from hatchery origin were excluded in AMOVA analyses of wild populations.

Locality	Individuals	Cyt b lineages	Genbank accession number cyt b	AMOVA Population	AMOVA Groups Hypothesis *a*	AMOVA Groups Hypothesis *b*	AMOVA Groups Hypothesis *d*
01-UR-CR	P1721	Rq2	KP798762	01-UR-CR-Rq2	Rq2 Uruguay	Uruguay	Uruguay
01-UR-CR	P2197	Rq2	MH669076*	01-UR-CR-Rq2	Rq2 Uruguay	Uruguay	Uruguay
01-UR-CR	P0485	Rq4	KP798651	01-UR-CR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
01-UR-CR	P2152	Rq4	KX379745	01-UR-CR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
01-UR-CR	P2217	Rq4	MH669077*	01-UR-CR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
01-UR-CR	P1720	Rq6	KP798763	01-UR-CR-Rq6	Rq6 Uruguay	Uruguay	Uruguay
01-UR-CR	P2151	Rq6	KX379744	01-UR-CR-Rq6	Rq6 Uruguay	Uruguay	Uruguay
02-UR-AR	P2165	Rq4	KX379753	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2166	Rq4	KX379754	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2167	Rq4	KX379755	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2168	Rq4	KX379756	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2169	Rq4	KX379757	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2170	Rq4	KX379758	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2192	Rq4	MH669078*	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2193	Rq4	MH669079*	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2194	Rq4	MH669080*	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2196	Rq4	MH669081*	02-UR-AR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
02-UR-AR	P2195	Rq6	MH669082*	02-UR-AR-Rq6	Rq6 Uruguay	Uruguay	Uruguay
03-UR-QR	P1980	Rq4	KP798663	03-UR-QR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
03-UR-QR	P2177	Rq4	KX379762	03-UR-QR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
03-UR-QR	P2178	Rq4	KX379763	03-UR-QR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
03-UR-QR	P2190	Rq4	MH669083*	03-UR-QR-Rq4	Rq4 Uruguay	Uruguay	Uruguay
03-UR-QR	P2164	Rq6	KX379752	03-UR-QR-Rq6	Rq6 Uruguay	Uruguay	Uruguay
03-UR-QR	P2176	Rq6	KX379761	03-UR-QR-Rq6	Rq6 Uruguay	Uruguay	Uruguay
04-NR-A	P2135	Rq6	KP798646	04-NR-A-Rq6	Rq6 Negro River	Negro River	Negro River
04-NR-A	P2136	Rq6	KP798647	04-NR-A-Rq6	Rq6 Negro River	Negro River	Negro River
04-NR-A	P2137	Rq6	MH669084*	04-NR-A-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2139	Rq4	MH669085*	05-NR-RB-Rq4	Rq4 Negro River	Negro River	Negro River
05-NR-RB	P2141	Rq4	MH669122*	05-NR-RB-Rq4	Rq4 Negro River	Negro River	Negro River
05-NR-RB	P2158	Rq4	KX379748	05-NR-RB-Rq4	Rq4 Negro River	Negro River	Negro River
05-NR-RB	P2288	Rq4	MH669086*	05-NR-RB-Rq4	Rq4 Negro River	Negro River	Negro River
05-NR-RB	P1770	Rq6	MH669087*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P1771	Rq6	KP798743	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P1772	Rq6	MH669088*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River

05-NR-RB	P1773	Rq6	KP798742	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P1774	Rq6	MH669089*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P1775	Rq6	MH669090*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2140	Rq6	MH669091*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2142	Rq6	KP798652	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2143	Rq6	KP798649	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2144	Rq6	KP798650	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2145	Rq6	MH669092*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2146	Rq6	MH669093*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2147	Rq6	MH669094*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2154	Rq6	KX379747	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2156	Rq6	MH669095*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2157	Rq6	MH669096*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2159	Rq6	MH669097*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2160	Rq6	KX379749	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
05-NR-RB	P2289	Rq6	MH669098*	05-NR-RB-Rq6	Rq6 Negro River	Negro River	Negro River
06-NR-T	P1789	Rq4	KP798737	06-NR-TR-Rq4	Rq4 Negro River	Negro River	Negro River
06-NR-T	P2290	Rq4	MH669099*	06-NR-TR-Rq4	Rq4 Negro River	Negro River	Negro River
06-NR-T	P2148	Rq6	KX379742	06-NR-TR-Rq6	Rq6 Negro River	Negro River	Negro River
07-NR-PM	P2295	Rq4	MH669100*	07-NR-PM-Rq4	Rq4 Negro River	Negro River	Negro River
07-NR-PM	P1776	Rq6	KP798739	07-NR-PM-Rq6	Rq6 Negro River	Negro River	Negro River
07-NR-PM	P1777	Rq6	KP798738	07-NR-PM-Rq6	Rq6 Negro River	Negro River	Negro River
07-NR-PM	P2291	Rq6	MH669101*	07-NR-PM-Rq6	Rq6 Negro River	Negro River	Negro River
07-NR-PM	P2292	Rq6	MH669102*	07-NR-PM-Rq6	Rq6 Negro River	Negro River	Negro River
07-NR-PM	P2293	Rq6	MH669103*	07-NR-PM-Rq6	Rq6 Negro River	Negro River	Negro River
07-NR-PM	P2294	Rq6	MH669104*	07-NR-PM-Rq6	Rq6 Negro River	Negro River	Negro River
08-LP-T	P1724	Rq6	KP798759	08-LP-T-Rq6	Rq6 La Plata	La Plata	La Plata
08-LP-T	P1749	Rq6	MH669105*	08-LP-T-Rq6	Rq6 La Plata	La Plata	La Plata
08-LP-T	P1750	Rq6	KP798749	08-LP-T-Rq6	Rq6 La Plata	La Plata	La Plata
08-LP-T	P1752	Rq6	KP798748	08-LP-T-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1660	Rq6	KP798808	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1661	Rq6	KP798807	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1662	Rq6	KP798806	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1663	Rq6	KP798805	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1664	Rq6	KP798804	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1665	Rq6	KP798803	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1666	Rq6	KP798802	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1667	Rq6	KP798801	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1668	Rq6	KP798800	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1669	Rq6	KP798799	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1670	Rq6	KP798798	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1671	Rq6	KP798797	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1672	Rq6	KP798796	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata

09-LP-SL	P1673	Rq6	KP798795	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1674	Rq6	KP798794	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1679	Rq6	KP798792	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1680	Rq6	KP798791	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1681	Rq6	KP798790	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1682	Rq6	KP798789	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1684	Rq6	KP798788	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1685	Rq6	KP798787	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1686	Rq6	KP798786	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1687	Rq6	KP798785	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1688	Rq6	KP798784	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1689	Rq6	KP798783	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1690	Rq6	KP798782	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1691	Rq6	KP798781	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1692	Rq6	KP798780	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1693	Rq6	KP798779	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1694	Rq6	KP798778	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1695	Rq6	KP798777	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1698	Rq6	KP798775	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1701	Rq6	KP798774	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1973	Rq6	KP798669	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1974	Rq6	KP798668	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1975	Rq6	KP798667	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1976	Rq6	KP798666	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1977	Rq6	KP798665	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1979	Rq6	KP798664	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1985	Rq6	KP798659	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1986	Rq6	KP798658	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1988	Rq6	KP798657	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1989	Rq6	KP798656	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1990	Rq6	KP798655	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P1994	Rq6	KP798643	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
09-LP-SL	P2076	Rq6	KP798644	09-LP-SL-Rq6	Rq6 La Plata	La Plata	La Plata
10-AO-BL	P1722	Rq6	KP798760	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1723	Rq6	KP798761	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1759	Rq6	MH669106*	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1760	Rq6	MH669108*	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1761	Rq6	MH669109*	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1762	Rq6	MH669107*	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1763	Rq6	MH669110*	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1764	Rq6	KP798746	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1768	Rq6	KP798745	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
10-AO-BL	P1769	Rq6	KP798744	10-AO-BL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean

11-AO-RL	P1837	Rq6	KP798715	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1838	Rq6	KP798714	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1839	Rq6	KP798713	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1840	Rq6	KP798712	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1841	Rq6	KP798711	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1842	Rq6	KP798710	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1843	Rq6	KP798709	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1844	Rq6	KP798708	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1845	Rq6	KP798707	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1846	Rq6	KP798706	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1847	Rq6	KP798705	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1848	Rq6	KP798704	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1894	Rq6	KP798688	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1897	Rq6	KP798685	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1899	Rq6	KP798683	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
11-AO-RL	P1901	Rq6	KP798681	11-AO-RL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	La Plata
12-AO-CL	P1704	Rq6	KP798772	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1705	Rq6	KP798771	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1706	Rq6	KP798770	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1707	Rq6	KP798769	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1708	Rq6	KP798768	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1709	Rq6	KP798767	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1815	Rq6	KP798735	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1816	Rq6	KP798734	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1817	Rq6	KP798733	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1818	Rq6	KP798732	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1819	Rq6	KP798731	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1820	Rq6	KP798730	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1822	Rq6	KP798729	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1823	Rq6	KP798728	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1825	Rq6	KP798727	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1826	Rq6	KP798726	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1827	Rq6	KP798725	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1828	Rq6	KP798724	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1829	Rq6	KP798723	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1830	Rq6	KP798722	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1833	Rq6	KP798719	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1834	Rq6	KP798718	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1835	Rq6	KP798717	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1877	Rq6	KP798703	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1879	Rq6	KP798701	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1882	Rq6	KP798698	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1883	Rq6	KP798697	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean

12-AO-CL	P1884	Rq6	KP798696	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1887	Rq6	KP798693	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
12-AO-CL	P1890	Rq6	KP798691	12-AO-CL-Rq6	Rq6 Atlantic Ocean	Atlantic Ocean	Atlantic Ocean
13-ML-NL	P1716	Rq6	KP798766	13-ML-NL-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
13-ML-NL	P1992	Rq6	KP798654	13-ML-NL-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
13-ML-NL	P1993	Rq6	KP798653	13-ML-NL-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2153	Rq4	KX379746	14-ML-QC-Rq4	Rq4 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2234	Rq4	MH669114*	14-ML-QC-Rq4	Rq4 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2237	Rq4	MH669117*	14-ML-QC-Rq4	Rq4 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2162	Rq6	KX379750	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2163	Rq6	KX379751	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2174	Rq6	KX379759	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2175	Rq6	KX379760	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2231	Rq6	MH669111*	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2232	Rq6	MH669112*	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2233	Rq6	MH669113*	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2235	Rq6	MH669115*	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2236	Rq6	MH669116*	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
14-ML-QC	P2238	Rq6	MH669118*	14-ML-QC-Rq6	Rq6 Merin lagoon	Merin lagoon	Merin lagoon
15-UR-VC	P1963	Rq2	KP798676				
15-UR-VC	P1969	Rq2	KP798673				
15-UR-VC	P2228	Rq2	MH669120*				
15-UR-VC	P2261	Rq2	MH669121*				
15-UR-VC	P1960	Rq6	KP798679				
15-UR-VC	P1961	Rq6	MH669119*				
15-UR-VC	P1962	Rq6	KP798677				
15-UR-VC	P1964	Rq6	KP798675				
15-UR-VC	P1966	Rq6	KP798674				
15-UR-VC	P1967	Rq6	KX379755				
15-UR-VC	P1971	Rq6	KP798671				
15-UR-VC	P1972	Rq6	KP798670				

Supplemental file II. Genetic diversity estimates based on 10 microsatellite loci in *R. quelen* collecting sites from LP-PM-AO.

	N		Rhq2	Rhq7	Rhq8	Rhq13	Rhq15	Rhq16	Rhq20	Rhq26	Rhq28	Rhq29	Mean/Total
1- UR-CR	7	A	6	5	4	5	9	5	5	10	4	3	5.6
		H_{OBS}	0.600	0.000	0.250	0.800	1.000	1.000	0.250	1.000	0.750	0.667	0.632
		H_{EXP}	0.889	0.889	0.821	0.756	0.978	0.867	0.893	1.000	0.750	0.733	0.858
		HWE	0.032	0.0000*	0.0013*	0.588	1.000	0.769	0.0000*	0.042	0.921	0.619	
		Sign NA	Yes	Yes	Yes	No	No	No	Yes	Yes	No	No	
		F_{IS}	0.407	0.857	0.808	0.063	-0.012	0.040	0.667	0.250	0.020	0.077	0.333
		Apriv	0	0	1	0	0	0	0	1	0	1	3
2-UR-AR	11	A	7	9	4	6	7	7	6	10	5	5	6.6
		r_A	10.184	11.509	3.971	11.335	8.062	7.400	10.623	11.289	8.395	7.000	8.977
		H_{OBS}	0.833	0.667	0.000	0.833	1.000	1.000	0.500	0.833	0.800	0.750	0.722
		H_{EXP}	0.909	0.939	0.727	0.864	0.879	0.894	0.758	0.970	0.756	0.893	0.859
		HWE	0.025	0.0001*	0.0000*	0.323	0.713	0.804	0.0000*	0.247	0.163	0.0029*	
		Sign NA	Yes	Yes	Yes	No	No	No	Yes	No	No	Yes	
		F_{IS}	0.330	0.336	0.875	0.024	-0.075	0.037	0.296	0.158	0.046	0.475	0.240
		Apriv	0	2	0	0	0	0	2	0	0	2	6
3-UR-QR	6	A	8	7	5	6	5	5	6	5	4	4	5.5
		H_{OBS}	1.000	0.600	0.200	1.000	1.000	0.800	0.600	0.200	0.800	0.400	0.660
		H_{EXP}	0.956	0.911	0.867	0.867	0.933	0.756	0.889	0.867	0.778	0.778	0.860
		HWE	1.000	0.166	0.0000*	0.852	1.000	1.000	0.023	0.003	0.208	0.013	
		Sign NA	No	No	Yes	No	No	No	Yes	Yes	No	No	
		F_{IS}	-0.071	0.286	0.828	-0.176	-0.091	-0.042	0.474	0.643	0.216	0.574	0.284
		Apriv	0	0	0	0	0	0	2	0	1	1	4
4-NR-A	3	A	4	5	2	2	5	4	4	5	3	4	3.8
		H_{OBS}	0.667	0.667	0.000	0.333	0.667	1.000	1.000	0.667	0.667	0.667	0.634
		H_{EXP}	0.800	0.933	0.533	0.333	0.933	0.867	0.800	0.933	0.800	0.800	0.773
		HWE	0.609	0.199	0.202	ND	0.193	0.475	1.000	0.190	0.474	0.614	
		Sign NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
		F_{IS}	0.200	0.333	1.000	ND	0.333	-0.200	-0.333	0.333	0.200	0.200	0.217
		Apriv	1	1	0	0	1	2	0	3	1	0	9
5-NR-RB	23	A	14	19	8	14	24	16	18	24	12	7	15.6
		r_A	8.780	10.411	3.953	7.953	9.901	10.785	9.154	11.725	7.954	4.709	8.533
		H_{OBS}	0.771	0.611	0.167	0.676	0.750	0.944	0.778	0.778	0.943	0.528	0.695
		H_{EXP}	0.896	0.917	0.608	0.830	0.910	0.910	0.838	0.945	0.877	0.771	0.850
		HWE	0.442	0.0000*	0.0000*	0.030	0.035	0.913	0.219	0.0000*	0.424	0.016	
		Sign NA	No	Yes	Yes	No	No	No	No	No	No	No	
		F_{IS}	0.141	0.337	0.729	0.187	0.178	-0.038	0.072	0.179	-0.076	0.318	0.165
		Apriv	0	0	0	0	0	0	0	0	0	0	0

6-NR-T	3	A	3	5	3	5	5	4	4	4	5	3	4.1
		H_{OBS}	0.500	0.667	0.000	0.667	0.667	0.667	1.000	0.667	1.000	0.667	0.7
		H_{EXP}	0.833	0.933	0.800	0.933	0.933	0.800	0.867	0.800	0.933	0.600	0.8
		HWE	0.339	0.206	0.068	0.207	0.211	0.600	1.000	0.597	1.000	1.000	
		Sign NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
		F_{IS}	0.500	0.333	1.000	0.333	0.333	0.200	-0.200	0.200	-0.091	-0.143	0.269
		Apriv	0	0	0	0	0	0	1	0	0	0	1
7-NR-PM	7	A	8	9	5	7	9	8	8	11	9	5	7.9
		H_{OBS}	0.857	0.571	0.429	0.714	0.857	1.000	0.714	0.857	0.857	0.143	0.7
		H_{EXP}	0.912	0.945	0.703	0.857	0.912	0.934	0.769	0.967	0.901	0.780	0.9
		HWE	0.679	0.005	0.073	0.537	0.620	1.000	0.619	0.252	0.756	0.000*	
		Sign NA	No	Yes	No	No	No	No	No	No	No	Yes	
		F_{IS}	0.065	0.415	0.410	0.178	0.065	-0.077	0.077	0.122	0.053	0.829	0.207
		Apriv	0	0	0	0	0	0	0	0	0	0	0
8-LP-T	4	A	4	8	3	6	3	6	6	6	5	4	5.1
		H_{OBS}	0.750	1.000	0.000	0.750	0.667	0.750	0.750	0.750	1.000	1.000	0.742
		H_{EXP}	0.750	1.000	0.714	0.893	0.600	0.929	0.893	0.929	0.857	0.750	0.832
		HWE	1.000	1.000	0.029	0.439	1.000	0.290	0.393	0.318	0.643	1.000	
		Sign NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
		F_{IS}	0.000	0.000	1.000	0.182	-0.143	0.217	0.182	0.217	-0.200	-0.412	0.128
		Apriv	0	0	0	0	0	0	0	2	0	0	2
9-LP-SL	46	A	12	8	6	9	12	14	9	10	9	5	9.4
		r_A	7.258	5.261	4.290	5.353	6.499	6.641	5.174	5.511	7.071	4.092	5.715
		H_{OBS}	0.851	.0.643	0.182	0.617	0.674	0.628	0.605	0.696	0.787	0.558	0.622
		H_{EXP}	0.854	0.641	0.596	0.760	0.730	0.749	0.618	0.718	0.846	0.624	0.714
		HWE	0.529	0.960	0.0000*	0.061	0.238	0.0000*	0.919	0.813	0.008	0.281	
		Sign NA	No	No	Yes	Yes	No	No	No	No	No	No	
		F_{IS}	0.003	-0.003	0.697	0.190	0.078	0.163	0.022	0.031	0.070	0.107	0.126
		Apriv	1	0	0	0	0	3	0	0	0	0	4
10-AO-BL	10	A	5	3	4	4	5	5	6	8	4	3	4.7
		r_A	4.800	3.000	4.000	4.000	4.995	4.895	6.000	7.784	3.995	3.000	4.647
		H_{OBS}	1.000	0.700	0.400	0.900	0.700	0.800	0.778	0.800	0.700	0.556	0.733
		H_{EXP}	0.747	0.674	0.747	0.774	0.805	0.737	0.627	0.884	0.742	0.569	0.731
		HWE	0.051	0.728	0.0031*	0.929	0.214	0.707	1.000	0.038	0.072	0.724	
		Sign NA	No	No	Yes	No	No	No	No	No	No	No	
		F_{IS}	-0.364	-0.041	0.478	-0.174	0.137	-0.091	-0.258	0.100	0.060	0.024	-0.002
		Apriv	0	0	0	0	0	0	0	0	0	0	0
11	16	A	7	9	4	5	7	3	4	6	9	3	5.7

		r_A	5.531	7.281	3.830	4.286	5.702	2.598	3.789	4.976	7.534	2.385	4.791
		H_{OBS}	0.688	0.438	0.133	0.714	0.625	0.467	0.625	0.200	0.933	0.077	0.490
		H_{EXP}	0.659	0.817	0.644	0.725	0.663	0.384	0.653	0.584	0.864	0.151	0.614
		HWE	0.767	0.0000*	0.0000*	0.932	0.783	1.000	0.791	0.001	0.693	0.040	
		Sign NA	No	Yes	Yes	No	No	No	No	Yes	No	No	
		F_{IS}	-0.044	0.472	0.799	0.015	0.060	-0.225	0.045	0.665	-0.083	0.500	0.207
		Apriv	0	0	0	0	0	0	0	0	0	0	0
12-AO-CL	30	A	8	13	3	7	10	4	7	11	11	5	7.9
		r_A	6.193	8.385	2.272	4.607	6.474	3.564	5.425	6.254	7.249	3.463	5.389
		H_{OBS}	0.733	0.767	0.037	0.633	0.767	0.464	0.767	0.552	0.607	0.519	0.585
		H_{EXP}	0.810	0.879	0.175	0.703	0.805	0.631	0.781	0.588	0.794	0.453	0.662
		HWE	0.668	0.106	0.0011*	0.863	0.296	0.007	0.493	0.343	0.025	1.000	
		Sign NA	No	No	Yes	No	No	No	No	No	Yes	No	
		F_{IS}	0.096	0.129	0.792	0.101	0.049	0.267	0.019	0.063	0.238	-0.148	0.117
		Apriv	0	0	0	0	0	0	0	0	0	0	0
13-ML-NL	3	A	2	3	1	5	5	6	4	5	3	4	3.8
		H_{OBS}	0.667	0.333	0.000	1.000	1.000	1.000	0.667	0.667	0.667	1.000	0.700
		H_{EXP}	0.533	0.733	0.000	0.933	0.933	1.000	0.800	0.933	0.733	0.867	0.747
		HWE	1.000	0.200	No infor.	1.000	1.000	1.000	0.600	0.213	1.000	1.000	
		Sign NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
		F_{IS}	-0.333	0.600	ND	-0.091	-0.091	0.000	0.200	0.333	0.111	-0.200	0.077
		Apriv	0	0	0	0	0	1	0	0	0	0	1
14-ML-QC	13	A	4	5	3	5	5	7	8	5	7	3	5.2
		r_A	7.168	7.571	5.391	6.577	9.354	8.348	9.837	9.587	8.771	2.691	7.530
		H_{OBS}	0.400	0.200	0.250	0.250	0.600	0.800	1.000	0.400	0.800	0.200	0.490
		H_{EXP}	0.711	0.822	0.607	0.893	0.844	0.911	1.000	0.756	0.867	0.378	0.779
		HWE	0.0000*	0.0000*	0.0012*	0.015	0.414	0.0025*	0.056	0.0172*	0.881	0.076	
		Sign NA	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No	No	
		F_{IS}	0.549	0.624	0.662	0.290	0.158	0.394	0.096	0.394	-0.040	0.410	0.343
		Apriv	2	1	0	0	0	3	2	0	2	0	10
15-VC	12	A	10	9	7	9	9	9	5	10	7	5	8
		r_A	8.640	8.397	6.780	7.881	7.881	8.121	4.273	8.719	6.817	4.391	7.190
		H_{OBS}	0.833	0.909	0.333	0.917	0.833	0.583	0.364	0.750	0.583	0.417	0.652
		H_{EXP}	0.880	0.896	0.841	0.851	0.844	0.880	0.338	0.891	0.855	0.438	0.771
		HWE	0.578	0.354	0.0000*	0.834	0.584	0.006	1.000	0.001	0.010	0.197	
		Sign NA	No	No	Yes*	No	No	Yes	No	No	Yes	No	
		F_{IS}	0.056	-0.015	0.614	-0.080	0.013	0.347	-0.081	0.165	0.328	0.052	0.163
		Apriv	0	0	0	1	0	1	1	0	0	1	4

Number of alleles (A); allelic richness based on minimum sample size of 9 diploid individuals (r_A); Observed heterozygosity (H_{OBS}); Expected heterozygosity (H_{EXP}); null alleles evidences evidenced in MICROCHECKER (Sign NA); Hardy-Weinberg equilibrium (P value). significative value then Bonferroni correction is indicated by *; mean intrapopulation fixation index over loci (F_{IS}); private alleles (Apriv).

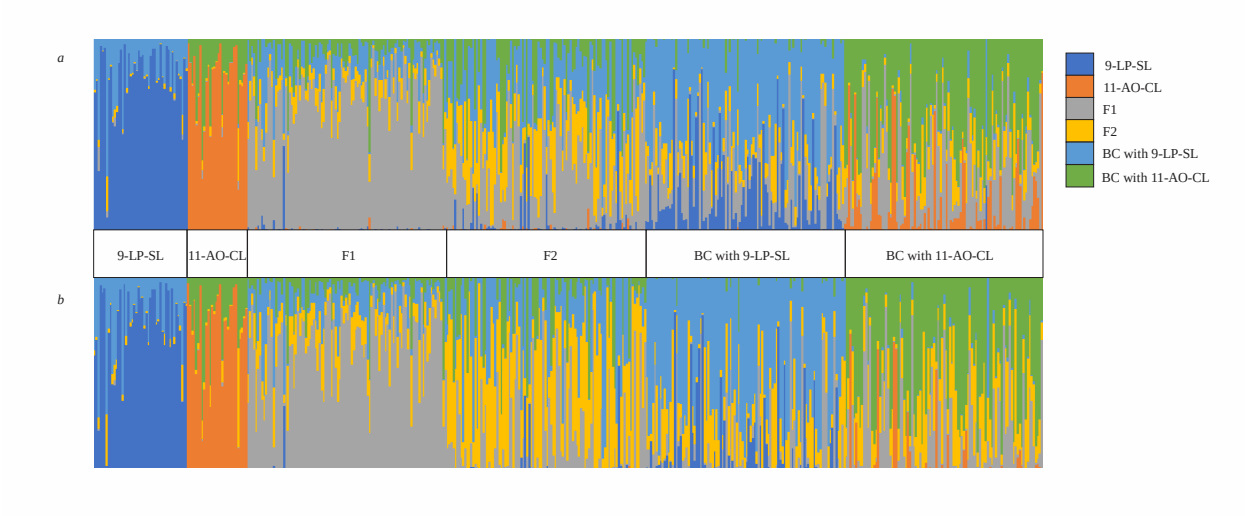

Figure S1. Histograms of admixture analyses of simulated populations using NewHybrids v.1.1. (Anderson & Thompson, 2002). Sauce lagoon (9-LP-SL) and Castillos lagoon (11-AO-LC) individuals were selected as parental populations and simulated an F1 (First filial generation), an F2 (Second filial generation), a Backcross (BC) with 9-LP-SL and a BC with 11-AO-CL, using HYBRIDLAB (Nielsen et al., 2006). Each bin or coloured vertical bar represents the posterior probability of belonging to each ancestry class. *a*) analysis using Uniform prior distribution; *b*) analysis using Jeffrey prior distribution. The colour reference of each ancestry class is displayed on the right top box.

Capítulo IV

Evidencias de selección e incipiente especiación en *R. quelen*

Population genomics in *Rhamdia quelen* (Heptapteridae, Siluriformes) reveals deep divergence and adaptation to coastal lagoons in the most southern basin system of the Neotropical region

Abstract

Rhamdia quelen is an interesting evolution model, in which hybridization between highly divergent mitochondrial lineages was evidenced. Previous studies have suggested demographic differences between coastal lagoons and riverine environments; as well as divergent populations of *R. quelen* that could be reproductively isolated. Thus, based on genomic population analyses using 2bRAD sequencing, this study aimed to investigate the genetic diversity pattern in the southern Neotropical basin system that encompasses La Plata basin, Patos-Merin basin and the coastal lagoons draining to SW Atlantic Ocean. Also, genomic scan analysis was performed in order to evidence selection footprints associated to divergence and local environmental adaptation. Mitochondrial lineage analyses revealed a wider lineage distribution in the southern Neotropical basin system that the one previously described. Two genomic clusters (North Cluster and South Cluster) latitudinally differenced were evidenced, as well as footprints of divergent selection between them. North Cluster inhabits Uruguay river, Negro river and Merin Lagoon basins, while South Cluster inhabits coastal lagoons as well as Negro river and Merin Lagoon. Analyses of population structure based on presumptive neutral loci and loci under selection suggest that the deep divergence between both major genomic clusters may be associated with an incipient speciation process, due to divergent selection in allopatry or to divergent selection with gene flow. Moreover, signs of coastal lagoon adaptation mediated by purifying selection were found. Population substructure within genomic clusters was associated with geographic isolation and genetic drift. Annotation of outlier SNPs identified candidate genes related to osmoregulation and reproduction. Sodium channel protein type 8 subunit alpha-like and sperm acrosome membrane-associated protein 6 were particularly suggestive of association with salinity adaptation and reproductive isolation among clusters, although further studies are needed. These new insights are useful for a better understanding of the complex evolutionary history of *R. quelen* in the most southern basin system of the Neotropical region.

Introduction

Among Neotropical silver catfish, *Rhamdia quelen* (Quoy & Gaimard, 1824) (Heptapteridae) is distributed from the extreme northeast of Andes to the center of Argentina (Perdices et al., 2002), and constitutes a valuable species exploited in artisanal fisheries and regional aquaculture (Ribolli & Zaniboni-Filho, 2009; Ribolli et al., 2017; Scaranto et al., 2018). Moreover, *R. quelen* was proposed as a priority species for conservation in the southernmost distribution because of its cultural and economic value (Loureiro et al., 2013). This species of catfish represents a systematic challenge due to its complex taxonomic history, nevertheless has become an interesting evolution model in the fish fauna of Neotropical region. In the first systematic revision of *Rhamdia* genus more than 100 species were synonymized and 46 were synonymized to *R. quelen* (Silfvergrip et al., 1996). Previous *R. quelen* studies based on mitochondrial marker data have suggested evidence of several taxonomic units (different mitochondrial lineages) included into this species (Ríos et al., 2017; Scaranto et al., 2018; Usso et al., 2018), while others authors have supported morphological evidences that validate sister species previously included in *R. quelen* (Ribolli et al., 2017; Angrizani & Malabarba, 2018). These phylogenetic antecedents have been based on two different mitochondrial markers, cytochrome b (cytb) and cyotochrome c oxidase subunit I (COI), although the mitochondrial lineages identified by each marker have not been compared to date. Thus, integrative analyses of studies at different geographical scales based on both markers have been precluded. In the basin system encompassed by La Plata basin, Patos-Merin lagoon basin, as well as rivers and lagoons draining to SW Atlantic Ocean (altogether LP-PM-AO), five *R. quelen* mitochondrial lineages (*Rq2*, *Rq4*, *Rq5a*, *Rq5b* and *Rq6*) based on cytb marker have been identified by Ríos et al. (2017). *Rhamdia quelen* population genetics based on microsatellite loci provided evidence of hybridization and introgression between mitochondrial lineages of the species (*Rq4* and *Rq6*) in several basins of this region (Uruguay river, Negro river and Merin Lagoon basins, Ríos et al. 2019). In addition, *R. quelen* population genetic structure unraveled differentiated population clusters that inhabit LP-PM-AO, even within a single basin (Ríos et al., 2019). Homing behavior and divergent selection were proposed as possible explanations for these observations. Unlike the riverine populations, *R. quelen* populations from southwestern Atlantic costal lagoon environments appeared geographically isolated and genetically differentiated, and showed small effective sizes, probably associated to bottleneck events. In such evolutionary context, it was hypothesized that genomic footprints of selection may be associated to local adaptations to

different environments, which could be recovered using massive genome scans across populations (Ríos et al. 2019).

Restriction site-associated DNA sequencing (RADseq) is a high-throughput technique that enables to genotype thousands of SNP (Single Nucleotide Polymorphism), indels and microsatellite markers in non-model organisms (Guo et al., 2015; Andrews et al., 2016; Robledo et al., 2017). Genome-wide SNP loci analyses at the population level allows to assess genetic diversity, demographic structure, population structure, adaptive evolution and candidate genes associated with traits of interest (Zhao et al., 2018). Neutral and adaptive genetic variation accounts for the demographic history and adaptive evolution of populations (Funk et al., 2012; do Prado et al., 2018). Adaptive genetic markers may identify populations that experience different environmental conditions, such as temperature or salinity (Batista et al., 2016). Genomic population structure could be defined using both neutral and adaptive SNPs. The use of both types of markers may support more effectively the conservation and management of populations (Batista et al., 2016). Moreover, wild populations adapted to different environmental conditions could be useful as founders for aquaculture broodstock (do Prado et al., 2018). Adaptive genes under divergent selection display higher differentiation than the rest of genome (Yeaman, 2013). Also, loci physically near to genes under divergent selection also could show lower gene flow between populations, which is known as "divergence hitchhiking" in the context of the genomic speciation models (Feder & Nosil et al., 2010; Feder et al., 2012a; Via 2012). Genome hitchhiking is defined as genomic divergence across the genome, even in loci unlinked to those under selection, due to gene flow restriction (Feder et al., 2012b). Moreover, Feder et al. (2012a) proposed a model of four phases that describes speciation processes with gene flow, where divergence and genome hitchhiking are involved. By contrast, in allopatry divergent selection and gene flow are expected to be coupled (Feder et al., 2013). Thus, population genome scans, such as RAD-seq analysis, could provide better understanding of the genome-wide pattern of genetic differentiation (Gagnaire et al., 2013).

This work aimed to evaluate the genetic structure patterns involving three different mitochondrial lineages of *R. quelen* in the LP-PM-AO basin system based on thousands of RADseq-derived SNPs. It was also devoted to integrate previous phylogenetic and population data, particularly focusing on analyzing the mitochondrial cytb lineages using the COI gene in order to extend the

comparative analyses and to redefine lineage distribution in LP-PM-AO basin system from Neotropical region. Finally, the present study intended to search for genomic footprints of selection in *R. quelen,* which could explain the environmental adaptation and genomic differentiation caused by selection.

Materials and methods

Sample collection and DNA extraction

All sampling protocols were approved by the CNEA (Comisión Nacional de Experimentación Animal) from Uruguay.

Analyses were performed on a total of 71 wild specimens belonging to 9 localities from five major basins, selected according to genetic diversity patterns based on previous microsatellite and mitochondrial data (Ríos et al., 2019) (Fig. 1; Table I): Uruguay River basin (UR): Cuareim River (1-UR-CR), Arapey River (2-UR-AR), Queguay River (3-UR-QR); Negro River basin (NR): Rincón del Bonete dam (4-NR-RB); La Plata River basin (LP): Sauce Lagoon (5-LP-SL); coastal SW Atlantic Ocean basin (AO): Blanca Lagoon (6-AO-BL), Rocha Lagoon (7-AO-RL), Castillos Lagoon (8-AO-CL); Merin Lagoon basin (ML): Quebrada de los Cuervos (9-ML-QC). Additionally, specimens from a *R. quelen* hatchery were also included (10-VC). The voucher specimens were stored in ethanol 95% at Sección Genética Evolutiva, Colección de Zoología de Vertebrados de Facultad de Ciencias or at Museo de Historia Natural of Uruguay. Some specimens were stored in formol 1%, but a tissue sample was always previously stored in ethanol 95%. Total genomic DNA was extracted from liver or muscle tissue using proteinase K digestion, followed by sodium chloride extraction and ethanol precipitation (modified from Medrano et al. 1990). DNA quality was visualized by electrophoresis on 1% agarose and only non-degraded DNA samples were used.

Mitochondrial lineages identification based on partial sequences of cytb and COI

Partial sequences of cytb were amplified using the fish universal DNA primers Gludg-L and CB3-H primers (Palumbi et al. 1991) following the polymerase chain reaction (PCR) protocol described by Ríos et al., 2017. A total of 224 *R. quelen* individuals were analyzed in the cytb dataset, which comprised 22 new samples collected in this study (Table I, Supplemental File I) in addition to the sequences retrieved from GenBank (Perdices et al., 2002; Vergara et al., 2008; Hernandez et al., 2015; Ríos et al., 2017; 2019).

In the present study, the COI gene was analyzed in one specimen of each mitochondrial lineage based on cytb (*Rq2*, *Rq4* and *Rq6*, Ríos et al., 2017), together with 126 other COI sequences retrieved from GenBank (Supplemental File II), in order to integrate the data from both mitochondrial markers that were reported separately in previous *R. quelen* studies (Rosso et al.,

2012; Ribolli et al., 2017; Ríos et al. 2017; Scaranto et al., 2018; Usso et al., 2018). COI gene amplification by PCR was performed using universal DNA primers LCO1490 and HC02198 (Folmer et al. 1994). The PCR cycling profile was the following: an initial denaturation of 3 min at 94 °C, followed by four cycles of 94 °C for 1 min, 42 °C for 1 min, 72 °C for 1 min, followed by 29 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final extension of 7 min at 72 °C. The total reaction volume was 20 µl including 1 x Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each primer, 1 unit of Taq DNA polymerase (Invitrogen) and 45 ng of template DNA.

Figure 1. Collecting sites of *Rhamdia quelen* samples analyzed from La Plata basin (LP), Patos-Merin basin system (PM) and the coastal lagoons draining to SW Atlantic Ocean (AO) (LP-PM-AO) from South America. The different localities are included in geographical basins: Uruguay River basin (UR: green): Cuareim River (1-UR-CR), Arapey River (2-UR-AR), Queguay River (3-URQR); Negro River basin (NR: red): Rincón del Bonete dam (4-NR-RB), Tacuarembó; La Plata River basin (LP: violet): Sauce Lagoon (5-LP-SL); coastal SW Atlantic Ocean basin (AO: yellow): Blanca Lagoon (6-AO-BL), Rocha Lagoon (7-AO-RL), Castillos Lagoon (8-AO-CL); Merin Lagoon (ML: orange): Quebrada de los Cuervos (9-ML-QC). Additionally, Villa Constitución hatchery (10-VC) individuals were analyzed. Pie charts at each population display the mitochondrial lineage frequency in each population (Rq2: grey; Rq4: black and Rq6: white) based on Ríos et al. (2019).

The nucleotide substitution models that best fit the cytb and COI datasets were selected in jModelTest v.2.1.17 (Darriba et al. 2012), based on the Bayesian Information Criterion (Schwarz 1978), the corrected Akaike Information Criterion (Sugiura 1978), and Decision Theory criterion (Minin et al. 2003). A heuristic Maximum Likelihood (ML) search was performed using NNI (a fast-nearest neighbor interchange search) and the robustness of the nodes was determined using Bootstrap analysis (100 pseudoreplicates). ML phylogenetic analyses were performed on PhyML 3.1 (Guindon & Gascuel 2003). Sequences of *Imparfinis mirini* and *Pimelodella chagresi* were used as outgroup in the COI phylogenetic tree.

Table I. Geographic distribution of *Rhamdia quelen* specimens studied: Collecting site, Environment, Locality and Basin, Sample code, Cytochrome b mitochondrial lineage according to Ríos et al. (2017), GenBank accession numbers

Collecting site	Environment	Locality sample division	Basin	Sample code	Mitochondrial lineage	GenBank Accession number
01-UR-CR	Riverine		Uruguay river	UC2197	Rq2	MK511219*
				UC2217	Rq4	MH669077
02-UR-AR	Riverine			UA2165	Rq4	KX379753
				UA2167	Rq4	KX379755
				UA2168	Rq4	KX379756
				UA2192	Rq4	MH669078
				UA2193	Rq4	MH669079
				UA2194	Rq4	MH669080
				UA2196	Rq4	MH669081
03-UR-UQ	Riverine			UQ2164	Rq6	KX379752
				UQ2176	Rq6	KX379761
				UQ2178	Rq4	KX379763
				UQ2190	Rq4	MH669083
04-NR-RB	Riverine	04-NR-RB-N	Negro river	NSG1789	Rq4	KP798737
		04-NR-RB-N		NSG2288	Rq4	MH669086
		04-NR-RB-N		NSG2290	Rq4	MH669099
		04-NR-RB-N		NSG2294	Rq6	MH669104
		04-NR-RB-N		NSG2295	Rq4	MH669100
		04-NR-RB-S		NSG2148	Rq6	KX379742
		04-NR-RB-S		NSG2289	Rq6	MH669098
		04-NR-RB-S		NSG2291	Rq6	MH669101
		04-NR-RB-S		NSG2292	Rq6	MH669102
		04-NR-RB-S		NSG2293	Rq6	MH669103

05-LP-SL	Coastal lagoon		La Plata basin	LPS1686	Rq6	KP798786
				LPS1687	Rq6	KP798785
				LPS1689	Rq6	KP798783
				LPS1694	Rq6	KP798778
				LPS1695	Rq6	KP798777
				LPS1696	Rq6	KP798776
				LPS1697	Rq6	MK511196*
				LPS1994	Rq6	KP798643
				LPS2200	Rq6	MK511197*
				LPS2201	Rq6	MK511198*
06-AO-BL	Coastal lagoon		Atlantic Ocean SW	OLB2268	Rq6	MK511199*
				OLB2269	Rq6	MK511200*
				OLB2271	Rq6	MK511201*
				OLB2272	Rq6	MK511202*
				OLB2273	Rq6	MK511203*
				OLB2274	Rq6	MK511204*
				OLB2275	Rq6	MK511205*
				OLB2276	Rq6	MK511206*
				OLB2277	Rq6	MK511207*
07-AO-RL	Coastal lagoon			OR1837	Rq6	KP798715
				OR1839	Rq6	KP798713
				OR1840	Rq6	KP798712
				OR1841	Rq6	KP798711
				OR1843	Rq6	KP798709
				OR1845	Rq6	KP798707
				OR1847	Rq6	KP798705
				OR1848	Rq6	KP798704
				OR2280	Rq6	MK511214*
08-AO-CL	Coastal lagoon			OC1707	Rq6	KP798769
				OC1818	Rq6	KP798732
				OC1820	Rq6	KP798730
				OC1822	Rq6	KP798729
				OC2281	Rq6	MK511208*
				OC2282	Rq6	MK511209*
				OC2283	Rq6	MK511210*
				OC2284	Rq6	MK511211*
				OC2285	Rq6	MK511212*
				OC2286	Rq6	MK511213*
09-ML-QC	Riverine	09-ML-QC-N	Merin Lagoon	M2175	Rq6	KX379760
		09-ML-QC-N		M2232	Rq6	MH669112
		09-ML-QC-N		M2233	Rq6	MH669113

	09-ML-QC-N		M2234	Rq4	MK511194*
	09-ML-QC-N		M2235	Rq6	MH669115
	09-ML-QC-N		M2236	Rq6	MH669116
	09-ML-QC-N		M2237	Rq4	MK511195*
	09-ML-QC-S		M2162	Rq6	KX379750
	09-ML-QC-S		M2163	Rq6	KX379751
	09-ML-QC-S		M2231	Rq6	MH669111
10-VC		Hatchery	UVC1963	Rq2	KP798676
			UVC1969	Rq2	KP798673
			UVC2261	Rq2	MH669121

GenBank accession numbers of novel sequences are marked with an asterisk.

Library construction and sequencing

Library preparation followed the 2b-RAD protocol (Wang et al. 2012) with slight modifications (Maroso et al., 2018). Individual DNA samples were adjusted to 50 ng/μL. DNA was digested using 1.4 units of AlfI (Thermo Fisher), a IIb type restriction enzyme (RE) which cleaves DNA on both sides of the restriction site. As result of digestion with AlfI, the genome is fragmented in thousands of fragments of 36 bp in length. After RE heat inactivation (65 °C for 20 min), double strand adaptors, 2bRADAdapter_2 Forward (CTACACGACGCTCTTCCGATCTNN) and 2bRADAdapter_3 Reverse (CAGACGTGTGCTCTTCCGATCTNN) were ligated to the digestion product at the 5´ and 3´ extremes, respectively. Ligation was carried out at 16 °C for 3 h, using T4 ligase (SybEnzyme). Ligase was subsequently inactivated through heat shock at 65 °C. Also, 2bRADAdapter1 (AGATCGGAAGAGC (InvdT)) was ligated to the digestion product in both 3´ extremes. The DNA fragments of 74 bp were then amplified. Amplification was carried out using four primers: i) two forward, 2bRAD_F (AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT) and P5 (AATGATACGGCGACCACCGA); and ii) two reverse, Primer barcorde (CAAGCAGAAGACGGCATACGAGAT[barcode sequence of 6 nucleotides] GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC) and P7 (CAAGCAGAAGAC-GGCATACGA). 2bRAD_ampl_F primer and Primer barcode included the P5 and P7 sequences in the 5´ extremes of each primer. The P5 and P7 sequences correspond to Illumina adapters, that allow the library fragments to attach to the cell flow surface. Amplification was carried out according to the following PCR protocol: initial denaturation at 98 °C for 4 min; 13 cycles (98 °C for 5 sec; 60 °C for 20 sec; 72 °C for 5 sec); final extension at 72 °C for 5 min. After checking for

amplification success through 1.8% agarose gel, products were purified with AMPure XP Beads (Beckamn Coutler, Brea, CA, USA). The 74 individual 2bRADseq libraries were quantified using fluorescent dyes (QuBit) and equimolarly pooled with the same amount of each library. The pool of libraries was sequenced on the NextSeq 500 Illumina platform with 50-bp single read strategy at FISABIO Bioinformatic Service (Valencia, Spain).

Data processing and SNP genotyping

We performed a custom script to demultiplex the raw reads, trim reads to 36 bp, and to eliminate reads shorter than 36 bp or reads without AlfI restriction site in the correct position ($(N)_{1-12}G_{13}C_{14}A_{15}(N)_{16-21}T_{22}G_{23}C_{24}$ $(N)_{25-36}$). Reads with indeterminate nucleotides or more than eight nucleotides with average Phred quality score below 30 (base call accuracy of 99.9%) were excluded using process_radtags of Stacks v.1.46 (Catchen et al., 2013). Complementary reads, which represent the two strands of the same locus, were oriented in the same sense using cd-hit-est (CD-HIT, Li et al., 2006) and Bowtie 1.1.2 (Langmead et al., 2009).

Filtered sequencing reads were processed using the software pipeline Stacks v.1.46 and MEYER tools v2.1 (http://people.oregonstate.edu/~meyere/2bRAD_analysis2.1.html) except for SHRiMP (SHort Read Mapping Package, Rumble et al., 2009), which was replaced by Bowtie 1.1.2.

SNP genotyping was performed using the Stacks and MEYER software pipelines without a reference genome. In the first one, Ustacks module was used to merge RAD-tags into loci for each individual. For this module we allowed up to two mismatches among RAD-tags (-M 2) and a minimum depth of three (-m 3). Ustacks was carried out using the bounded model type. Next, a catalogue of loci was created by means of cstacks module using all samples (n=74) and allowing two mismatches (-n 2) among them. The rxstacks module was used to correct the genotype call and to filter confounded loci. The rebuilt STACKS catalogue after rxstacks correction, was checked with cd-hit-est to identify putative spurious loci (i.e. loci that should be one locus but had still different orientation) or loci potentially due to indels. Loci that were present in more than one cluster were eliminated from whitelist for populations STACKS component. Only cd-hit references were included in whitelist for populations STACKS component. Populations module was finally used to obtain the initial SNP dataset; which included only loci with a minimum stack depth of 10 reads (-m 10); genotyped in at least 70% of total individuals; and genotyped in at least 60% of the individuals of a population, in at least seven of ten samples (-r 0.60, -p 7). Finally,

SNPs were filtered by MAF (Minimum Allele Frequency of alternative allele, < 0.05), intrapopulation fixation index F_{IS} (Weir & Cockerham, 1984) (loci with $F_{IS} \geq 0.50$ and ≤ -0.50 were discarded in order to minimize the presence of null alleles and paralog sequences, respectively; do Prado et al., 2018) and RAD-tags with more than 3 SNPs were discarded.

For MEYER tools we applied the same values as in STACKS for all analogous parameters. These had a minimum coverage of 10 reads (-c 10), two mismatches were allowed among global references (-c 0.944, cd-hit-est parameter), and three mismatches were allowed to align reads with reference (-v 3, --best --strata, Bowtie parameters). Reference was built selecting 300,000 reads of each sample (-n 300,000) to produce a combined dataset with more than 10 million reads. To determine genotypes from alignments, an interval frequency for minor allele (0.1-0.2) was established, consistent with mean coverage, defining if a locus is homozygous (minor allele frequency < 10% of total coverage), unknown (10-20%), or heterozygous (20%). As with STACKS pipeline, extreme tag positions from each read (positions 1 and 36) were excluded of analysis. RAD-tags with more than 3 SNPs were discarded (-sn 3). Again, following STACKS, SNPs were filtered by MAF threshold (0.05), by total percentage of individuals genotyped (70%) and by percentage of individuals genotyped per populations (60%) (in at least six populations) and number of SNPs by RAD-tag (2).

A new integrative catalogue was constructed with the SNPs shared between STACKS and MAYER pipeline. For this purpose, RAD-tag sequences with selected SNPs from both pipelines were merged and analyzed with cd-hit-est (-c 0.944) to identify shared RAD-tags. Coincident SNPs position of each RAD-tags was confirmed with both catalogues (STACKS and MAYER). The final catalogue of SNPs was obtained considering a unique SNP by RAD-tag, and eliminating those loci that in at least three locality samples showed low or high F_{IS} values ($F_{IS} \leq -0.50$ or $F_{IS} \geq 0.50$).

Detection of putative loci under selection

Outlier loci as candidate SNPs subjected to selection were identified using BayeScan v.2.1 (Foll, 2012) and Arlequin v.3.5. Although BayeScan and Arlequin have the lowest and highest type I and II error rates, respectively (Narum & Hess, 2011); both software were run to detect a more confident set of outlier loci, because different F_{ST} outliers methods could be effective under certain

genetic structure (Narum & Hess, 2011) and the use of different methods is recommended (De Mita et al. 2013). The BayeScan analysis was carried out for 20 pilot runs, 5,000 iterations, 5,000 burn-in steps, and a sample size of 5,000. BayeScan outliers were obtained applying a False Discovery Rate (FDR) of 0.01 and plotted using the R function provided by the program. Arlequin analyses were performed using a non-hierarchical finite island model, and testing 100 simulations with 100 simulated demes. Loci with a p-value < 0.01 or a p-value > 0.99 in Arlequin analysis were selected as putative outliers. Several structures were evaluated with BayeScan and Arlequin to find the most consistent outlier loci. Among these considered structures, we analyzed outliers of all locality samples (with and without the hatchery sample), among mitochondrial lineages (*Rq2*, *Rq4* and *Rq6*; Table I), and among coastal lagoons and riverine groups of samples. We also performed separate analyses of sample subgroups, such as coastal lagoons or riverine locality samples (Table I). Finally, based on the genetic structure observed using Total, Neutral, Outlier datasets (See Structure results), we considered the most divergent groups of samples from the northern and southern areas of the region LP-PM-AO studied. BayeScan analysis was performed considering the following seven samples as populations: 1-UR-CR; 2-UR-AR; 3-UR-QR; 5-LP-SL; 6-AO-BL; 7-AO-RL; 8-AO-CL. In the same sense, Arlequin analysis was carried out considering one group comprised by the same seven samples.

All SNP-bearing RAD-tags of 36 bp including the outlier loci were annotated against GenBank database using blastn search of BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990). An E-value < e^{-4} was considered as the significance threshold. Hypothetical chromosomes that would include the annotated genes were analyzed through a BLASTn search using the *Ictalurus punctatus* (Rafinesque, 1818) genome (Liu et al., 2016), as a close reference genome among model teleosts related to *R. quelen*.

Genetic diversity and population structure of *R. quelen* in LP-PM-AO

For each locality, the mean number of alleles per locus (Na) and the expected heterozygosity were calculated. Magnitude and signs of departure from Hardy-Weinberg equilibrium (HWE) were analyzed using F_{IS} (Weir & Cockerham,1984). Na, F_{IS} and H_{EXP} were calculated in Arlequin v.3.5. (Excoffier & Lisher 2010).

Population Structure analysis were performed using Total, Neutral and Outlier SNP datasets. The Total dataset comprises all 17,575 SNP loci, whereas the Outlier dataset refers to SNPs recognized by BAYESCAN and Arlequin as outlier loci. Loci which were not detected as consistent outliers using both methods were considered as presumptively neutral loci and they compose the Neutral dataset. Analysis of clusters and graphical representation of genetic structure was performed by Discriminant analysis of principal components (DAPC). Scatter plots were performed using the R package adegenet (Jombart, 2008). For Total, Neutral and Outlier datasets, the number of principal components retained were 40, 20 and 10, containing 80 %, 70 % and 90 % of the cumulative variation of the data, respectively. Five, six and four discriminant functions were retained for Total, Neutral, Outlier datasets, respectively. Bayesian analysis of population structure was performed in STRUCTURE v. 2.3 (Pritchard et al., 2000) to cluster individuals into populations based on the Total, Neutral and Outlier dataset. Population numbers (K) were analyzed from 1 to the number localities (10) plus 3 (K = 1 to K = 13). Ten independent runs for each K were implemented with a burn-in period length of 50,000 iterations, followed by 100,000 Monte Carlo Markov Chains (MCMC) replicates. Admixture model and correlated allele frequencies were considered. The consensus result for each K was obtained from the independent runs by means of CLUMPAK (Kopelman et al., 2015). The most probable K value was determined using both likelihood and Delta k criteria (ΔK) (Evanno et al., 2005), and calculated using STRUCTURE HARVESTER (Earl & vonHoldt, 2012). In addition, other K values were explored in order to be contrasted with the microsatellite structure pattern evidenced in the studied area by Ríos et al. (2019). Furthermore, inter-sample genetic differentiation was calculated through pairwise F_{ST} estimates with Arlequin v.3.5, for Total, Neutral and Outlier loci.

Results

Mitochondrial lineages identification based on cytb and COI sequences

In order to identify the different *R. quelen* mitochondrial lineages present in the dataset, a total of 22 partial sequences of cytb were generated in this study (GenBank Accession numbers: MK511194- MK511219; Table I, Supplemental File I). They were analyzed together with 224 *Rhamdia* cytb sequences retrieved from GenBank (Supplemental File I). In a cytb dataset of 745 bp, 225 were variable sites and 171 were parsimony informative sites. The 246 cytb sequences of *Rhamdia* were grouped into 20 haplotypes. Among the 88 models tested, the HKY + G (gamma distribution) (Hasegawa et al. 1985) was the nucleotide substitution model that best fit to the cytb data set. The cytb topology obtained by PhyML was similar to Ríos et al. (2017) and specimens sequenced in this study were identified as *Rq2*, *Rq4* and *Rq6* (Table I, Supplemental File I).

Partial sequences of COI were generated for each cytb mitochondrial lineage (*Rq2*, *Rq4* and *Rq6*) in this study (GenBank Accession numbers: MK511191- MK511193; Supplemental File II). These sequences were analyzed together with 126 sequences retrieved from GenBank (Supplemental File II). COI alignment of 509 bp showed 108 variable sites and 85 parsimony informative sites. The 129 individual COI sequences of *Rhamdia* were divided into 39 haplotypes (Supplemental File II). According to the BIC, GTR + G (Lanave et al. 1984) was the nucleotide substitution model that best fits the COI dataset. COI topology showed two *Rhamdia* clusters that correspond to trans andean and cis andean regions (Supplemental File III). Seven mitochondrial lineages were distributed into cis andean region (*Rq2*, *Rq4*, *Rq6*, *COI1*, *COI2*, COI3 and *COI4*), as well as several sequences, which collapsed in a basal polytomy. Sequences that belong to each mitochondrial lineage are detailed in Supplemental File II.

Data processing and SNP genotyping

A total of 341,495,357 reads were retrieved from the sequencing platform, a mean of 4,614,802.1 per specimen (Min: 2,228,958 and Max: 8,981,759). After applying sequence quality and restriction site position filters, 223,148,950 reads were retained.

After rxstacks correction, a cstacks catalogue of 160,898 SNP loci was built. Population module results showed 60,332 retrieved loci. After MAF and number of SNPs per RAD-tag filters, 33,376 loci were retained. Once the mentioned filters were performed, MEYER catalogue included 43,882

SNPs. A total of 24,659 loci were shared between STACKS and MEYER. Loci that in at least three locality samples showed high or low F_{IS} values were discarded and the final catalogue resulted in 17,575 loci.

Detection of putative loci under selection

A total of 75 and 26 outlier loci shared between Arlequin and BayeScan were found globally among all locality samples with and without the hatchery sample, respectively. No confident outlier loci were found among mitochondrial lineages (*Rq2*, *Rq4* and *Rq6*; Table I), among coastal lagoons and riverine groups of samples; among coastal lagoons nor among riverine locality samples. Based on the genetic structure results (See Structure results), the most consistent dataset analysis to detect outliers was the one that compared localities from highly differentiated clusters in the northern and southern basins of the sampled area. In this analysis, a total of 178 outlier loci were found by BayeScan, while 482 were detected by Arlequin. Finally, 75 loci were retained because they were shared using both BayeScan and Arlequin analyses, and so they were considered the most confident set of outlier loci. Out of these 75 outlier loci, six were annotated and related to different functions using its RAD-tag by BLAST search (Table II). Putative candidate genes under selection were anchored in different chromosome of *I. punctatus* (Table II).

Table II. Functional annotation and gene mining of the *R. quelen* outlier

SNP	RAD-TAG	Sequence homologue	Gene	Function	E-value	Chromosome *I. punctatus*
66502_32	GCGACATCAATGGCAGCTGTATGCG GCCAAATGGCA	XM_027164671.1	Homeobox D3 (hoxd3)	Morphogenesis	5 e^{-7}	Chr 6
449_34	TCAGGTGATCCAGCAGCTGATTGCG CTGCTGGTCAT	XM_027165565.1	F-box and WD repeat domain containing 2 (fbxw2)	Proteolysis and development	5 e^{-7}	Chr 28
9230_26	GGACTAAGCACTGCAGAGGGATGCT GATGAATGGCT	XM_027135514.1	Cysteine-rich hydrophobic domain-containing protein 2-like (CHIC2)	Exocytosis	7 e^{-5}	Chr 8
33517_8	TGAAGATCAGTTGCAAGCTGCTGCA GACACTTTCAT	XM_026914278.1	Sperm acrosome membrane-associated protein 6 (Spaca6)	Reproduction	2 e^{-4}	Chr 1
67564_7	CATAGATGAAGGGCACGCTTTTGCC CGCTTCCAGGT	XM_026921172.1	Sodium channel protein type 8 subunit alpha-like (scn8a)	Osmoregulation	6 e^{-6}	Chr 15
48262_4	AGAAGGGAAGTAGCACTCCATTGCT GGTGTCCATCT	XM_017496177.1	Coronin-1C-like (CORO1C)	Cellular assembly and organization	5 e^{-7}	Chr 20

Sequence homolog: Accession number of sequence homologue; Chromosome *I. punctatus*: chromosome of *I. punctatus*, which gene were anchored.

Genetic diversity and population structure of *R. quelen* in LP-PM-AO

Genetic diversity showed an average of 1.34 (Na) and 0.333 (H_{EXP}) across all populations. Na ranged from 1.20 (6-AO-BL) to 1.60 (4-NR-RB) (Table III), while expected heterozygosity ranged from 0.249 (5-LP-SL) to 0.527 (1-UR-CR). No significant sign of HWE departure was evidenced, and F_{IS} values were close to cero across all populations except for 4-NR-RB and 9-ML-QC, which show high and significant F_{IS} values (Table III).

DAPC results based on the Total, Neutral and Outlier loci showed similar patterns (Fig. 3*a-c*). Locality samples are arranged following a North to South geographic pattern, except 2-UR-QR and the hatchery sample (10-VC). The three DAPC scatterplots revealed higher differentiation among northern locality samples than among southern ones.

Structure analyses led to an estimation of two clusters, based on the Delta K criterion (ΔK) and Ln(PX|K) (Total loci (Fig. 3a) ΔK= 6,945.3, Ln(PX|K)= -501,909.83; Neutral loci (Fig. 3b) ΔK= 6,204.34, Ln(PX|K)= -499,972.36; (Fig. 3c) Outlier loci ΔK= 10,170.11, Ln(PX|K)= -1,006.78).

Table III. Genetic diversity of *R. quelen* samples based on 17.575 loci

Locality	N	Na	H_{EXP} (s.d.)	F_{IS}
1-UR-CR	2	1.26	0.527 (0.249)	-
2-UR-AR	7	1.47	0.276 (0.366)	-0.018
3-UR-QR	4	1.30	0.367 (0.349)	-
4-NR-RB	10	1.60	0.283 (0.399)	0.285*
5-LP-SL	10	1.36	0.249 (0.385)	0.012
6-AO-BL	9	1.20	0.328 (0.373)	-0.015
7-AO-RL	9	1.22	0.268 (0.392)	0.01
8-AO-CL	10	1.24	0.277 (0.388)	0
9-ML-QC	10	1.36	0.324 (0.392)	0.486*
10-VC	3	1.38	0.433 (0.328)	-

N: sample size; Na: mean number of alleles per locus; H_{EXP}: expected heterozygosity, standard deviation (s.d.); F_{IS}: inbreeding coefficient. * F_{IS} values after sequential Bonferroni correction were not significative ($p < 0.001$).

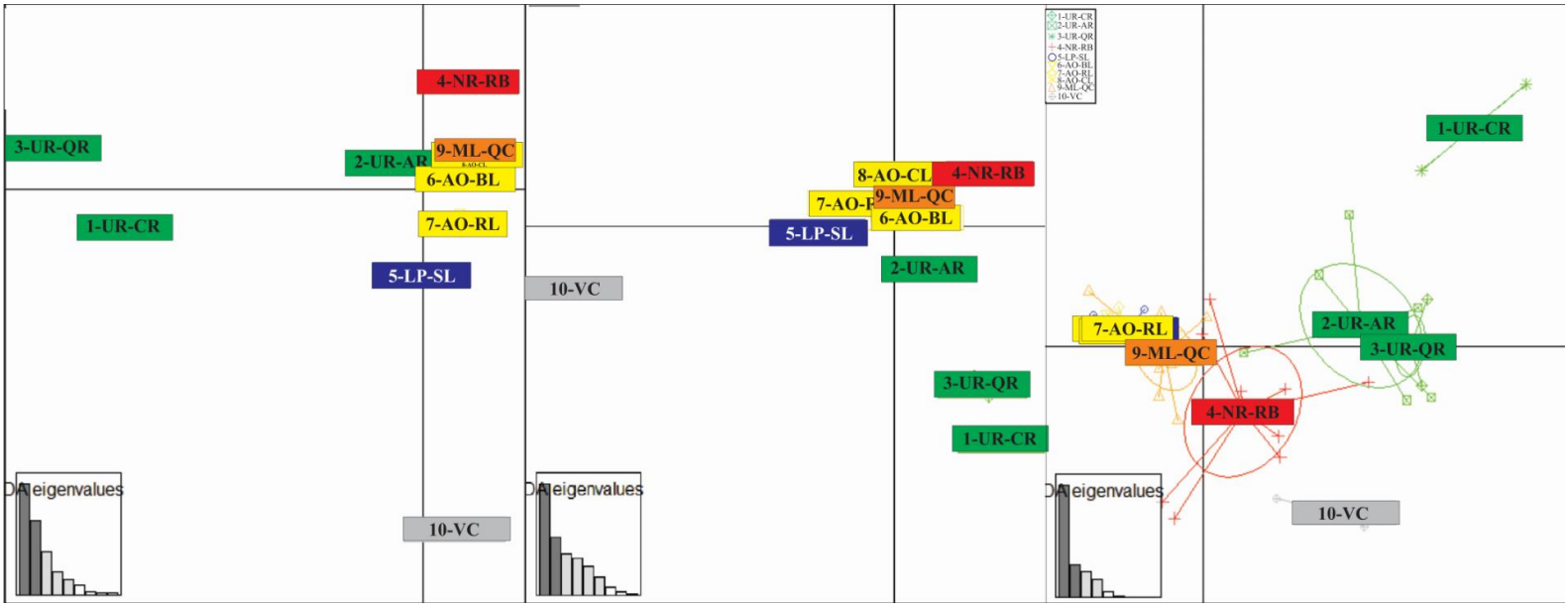

Figure 2. DAPC scatterplot of *Rhamdia quelen* from La Plata basin, Patos-Merin basin system and the coastal lagoons draining to SW Atlantic Ocean based on Total 17575 (*a*), Neutral 17500 (*b*) and Outlier 75 (*c*) loci. Individuals are grouped for locality (Fig. 1). Eigenvalues containing 80 %, 70 %, 90 % of the cumulative variation of the data are displayed on left bottom box. Colors refer to basin as follows: Uruguay river basin (green), Negro river (red), La Plata (blue), Atlantic Ocean (yellow), Merin Lagoon (orange). Hatchery sample is in gray.

Structure histograms based on Total, Neutral and Outlier data were concordant and localities were grouped into two major clusters: Cluster 1: 1-UR-CR, 2-UR-AR, 3-UR-QR, some individuals from 4-NR-RB and 9-ML-QC; and Cluster 2: 5-LP-SL, 6-AO-BL, 7-AO-RL, 8-AO-CL, rest of individuals from 4-NR-RB and 9-ML-QC. Hatchery individuals (10-VC) were not completely assigned to any cluster. Figures 4*a-b* show population substructure across sampled localities (Total loci (Fig. 4*a*) ΔK=1.09, Ln(PX|K)= -45649611.5; Neutral loci (Fig. 4*b*) ΔK=7.40, Ln(PX|K)= -8866263.5), while outlier loci do not evidence substructure (Outlier loci (Fig. 4*c*) ΔK=1.19, Ln(PX|K)=-1026.78).

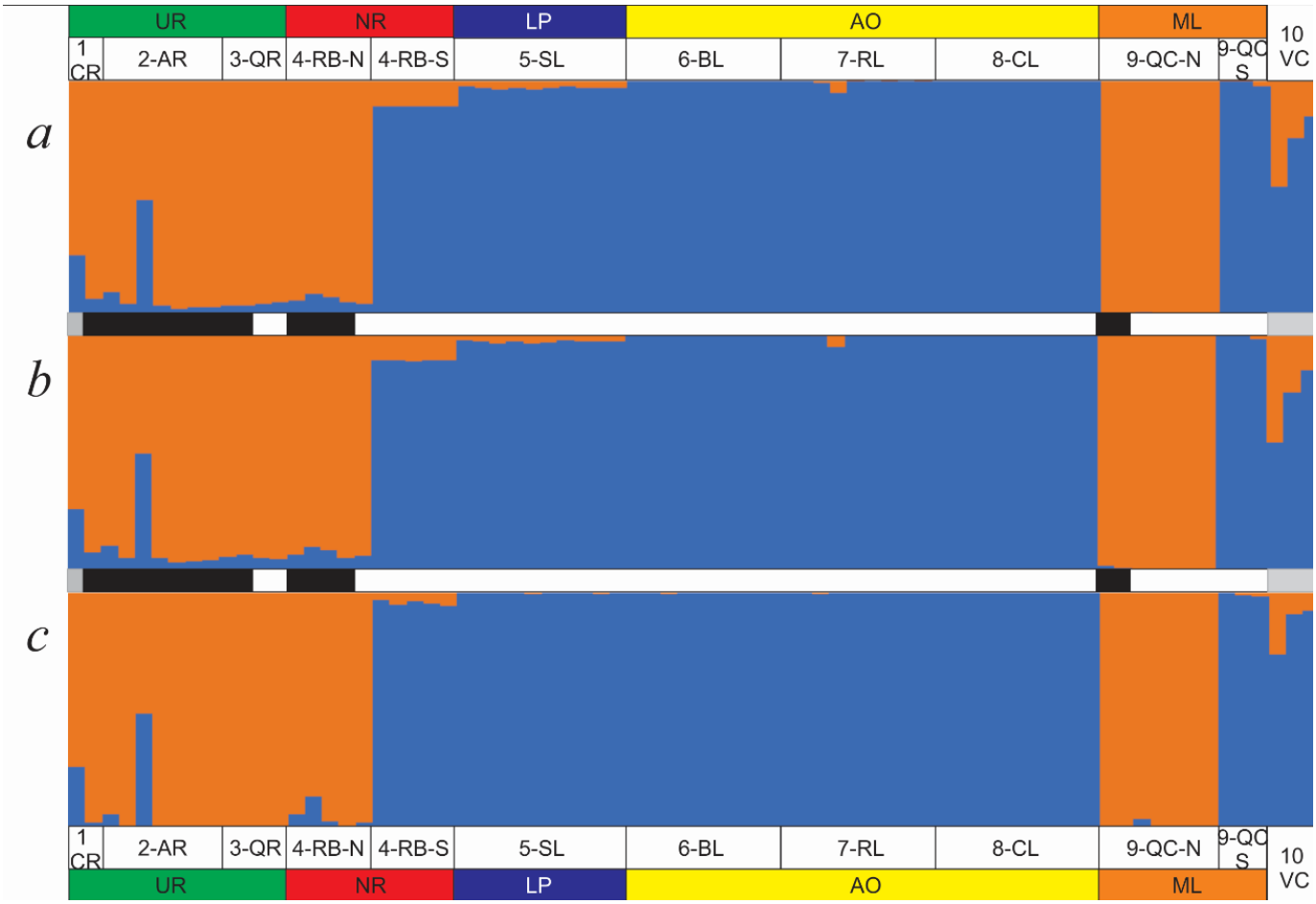

Figure 3. Estimated population structure in STRUCTURE of *Rhamdia quelen* from La Plata, Merin Lagoon and costal lagoons from SE Atlantic Ocean based on Total 17575 (*a*), Neutral 17500 (b), Outliers 75 (c) SNP loci. Each bin or colored vertical bar represents the estimated membership fraction of an individual into the major population clusters. Sampling sites' codes are indicated in Fig. 1. Bar among histograms represent mitochondrial lineage of each individual as following: Rq2: grey; Rq4: black; Rq6: white.

Pairwise genetic differentiation results were significant for most comparisons tested (Table IV*a-c*). Pairwise F_{ST} values were similar between Total and Neutral datasets. Total, Neutral and Outlier analyses showed high differentiation between locality samples from northern (1-UR-CR, 2-UR-AR, 3-UR-QR) and southern (5-LP-SL, 6-AO-BL, 7-AO-RL, 8-AO-CL) basins in the region LP-PM-AO studied. By contrast to the Total and Neutral analyses, Outlier dataset did not evidence genetic differentiation among coastal lagoons (F_{ST}: 0.000), but showed much higher values between locality samples from the North and South Clusters (F_{ST} mean: 0.939).

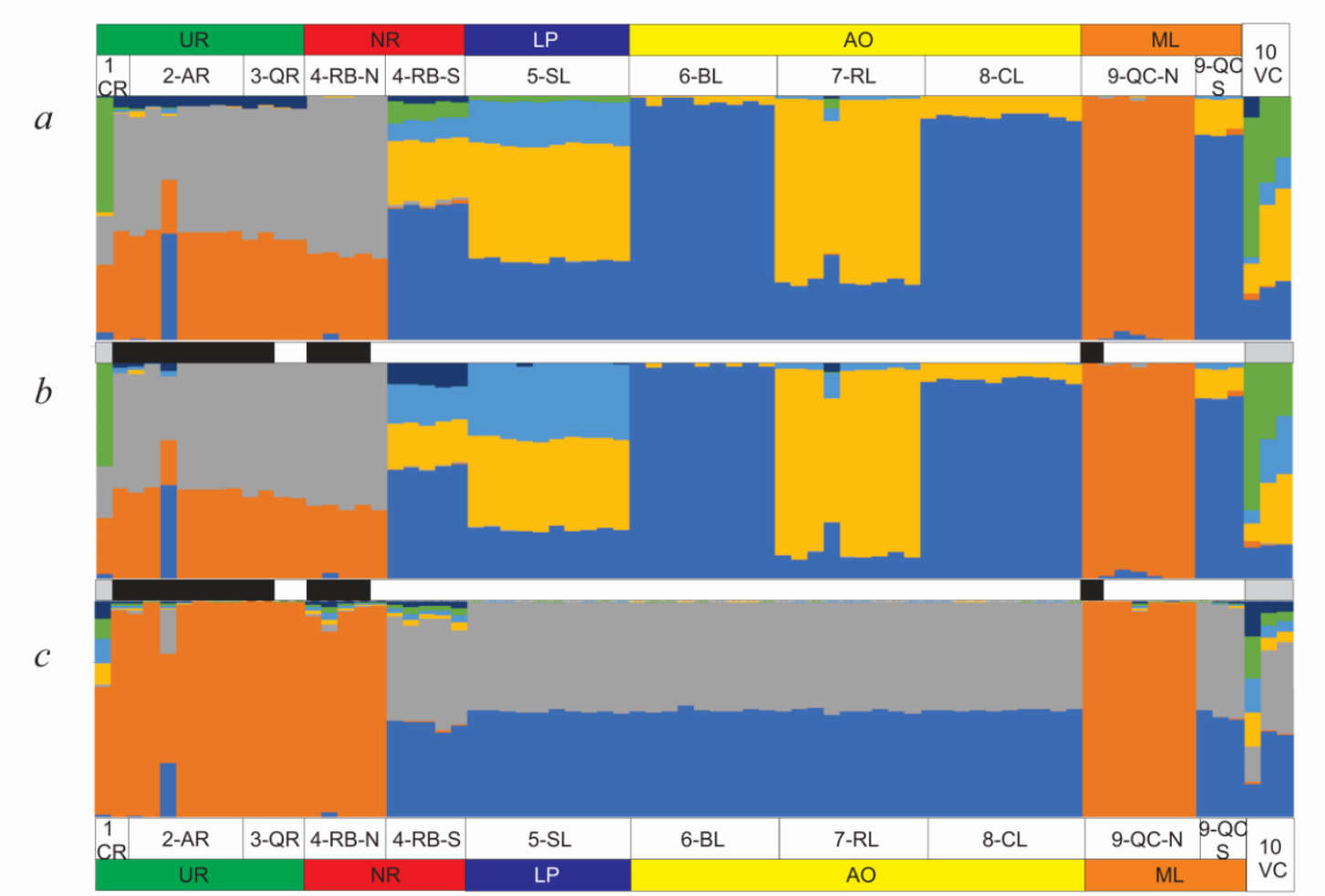

Figure 4. Estimated population substructure (K= 7) in STRUCTURE software of *Rhamdia quelen* from La Plata, Merin Lagoon and costal lagoons from SE Atlantic Ocean based on Total 17575 (*a*), Neutral 17500 (b), Outliers 75 (c) SNP loci. Each bin or colored vertical bar represents the estimated membership fraction of an individual into the major population clusters. Sampling sites' codes are indicated in Fig. 1. Bar among histograms represent mitochondrial lineage of each individual as following: Rq2: grey; Rq4: black; Rq6: white.

Table IV*a*. Pairwise F_{ST} values based on all 17575 SNP loci dataset between localities.

			1-UR-CR	2-UR-AR	3-UR-UQ	4-NR-RB	9-ML-QC	5-LP-SL	6-AO-BL	7-AO-RL	8-AO-CL	10-VC
Riverine	North	1-UR-CR	0.000									
		2-UR-AR	0.037	0.000								
		3-UR-UQ	0.075	**0.040**	0.000							
	Inter.	4-NR-RB	0.077	**0.107**	**0.143**	0.000						
		9-ML-QC	0.121	**0.098**	**0.152**	**0.098**	0.000					
Coastal lagoon	South	5-LP-SL	**0.458**	**0.447**	**0.518**	**0.210**	**0.383**	0.000				
		6-AO-BL	**0.536**	**0.496**	**0.582**	**0.260**	**0.427**	**0.196**	0.000			
		7-AO-RL	**0.536**	**0.497**	**0.581**	**0.265**	**0.432**	**0.174**	**0.246**	0.000		
		8-AO-CL	**0.555**	**0.516**	**0.597**	**0.280**	**0.445**	**0.209**	**0.213**	**0.251**	0.000	
Hatchery		10-VC	**0.189**	**0.292**	**0.343**	**0.116**	**0.269**	**0.163**	**0.276**	**0.271**	**0.307**	0.000

Significant F_{ST} values are in bold ($P<0.05$).

Table IV*b*. Pairwise F_{ST} values based on 17500 SNP neutral loci dataset between localities.

			1-UR-CR	2-UR-AR	3-UR-UQ	4-NR-RB	9-ML-QC	5-LP-SL	6-AO-BL	7-AO-RL	8-AO-CL	10-VC
Riverine	North	1-UR-CR	0.000									
		2-UR-AR	0.035	0.000								
		3-UR-UQ	0.072	**0.040**	0.000							
	Inter.	4-NR-RB	0.075	**0.100**	**0.134**	0.000						
		9-ML-QC	0.124	0.096	**0.149**	**0.099**	0.000					
Coastal lagoon	South	5-LP-SL	**0.436**	**0.424**	**0.493**	**0.202**	**0.371**	0.000				
		6-AO-BL	**0.518**	**0.477**	**0.561**	**0.255**	**0.417**	**0.196**	0.000			
		7-AO-RL	**0.518**	**0.477**	**0.559**	**0.260**	**0.423**	**0.174**	**0.246**	0.000		
		8-AO-CL	**0.537**	**0.497**	**0.576**	**0.274**	**0.435**	**0.209**	**0.213**	**0.251**	0.000	
Hatchery		10-VC	0.172	**0.274**	**0.320**	**0.115**	0.264	**0.162**	**0.276**	**0.270**	**0.306**	0.000

Significant F_{ST} values are in bold ($P<0.05$).

. **Table IV*c*.** Pairwise F_{ST} values based on 75 SNP outlier loci dataset between localities

			1-UR-CR	2-UR-AR	3-UR-UQ	4-NR-RB	9-ML-QC	5-LP-SL	6-AO-BL	7-AO-RL	8-AO-CL	10-VC
Riverine	North	1-UR-CR	0.000									
		2-UR-AR	**0.116**	0.000								
		3-UR-UQ	**0.356**	-0.005	0.000							
	Inter.	4-NR-RB	0.161	**0.310**	**0.388**	0.000						
		9-ML-QC	0.038	0.149	0.233	0.050	0.000					
Coastal lagoon	South	5-LP-SL	**0.949**	**0.916**	**0.997**	**0.451**	**0.668**	0.000				
		6-AO-BL	**0.944**	**0.911**	**0.997**	**0.434**	**0.655**	0.000	0.000			
		7-AO-RL	**0.944**	**0.911**	**0.997**	**0.435**	**0.655**	0.000	0.000	0.000		
		8-AO-CL	**0.950**	**0.918**	**0.997**	**0.459**	**0.673**	0.000	0.000	0.000	0.000	
Hatchery		10-VC	0.660	**0.754**	**0.882**	0.156	**0.408**	**0.398**	**0.362**	**0.369**	**0.410**	0.000

Significant F_{ST} values are in bold ($P<0.05$).

Discussion

Our study constitutes the first study that analyzes the genetic pattern of *R. quelen* based on thousands of genomic loci genotyped by means of 2bRADseq methodology. In a scenario of hybridization among highly divergent mitochondrial lineages that coexist in Uruguay, Negro and Merin Lagoon basins (*Rq2*, *Rq4* and *Rq6*), the study of genomic differentiation becomes crucial to understand *R. quelen* population genetics history. Here, we considered the possible genomic divergence driven by environmental adaptive selection.

Interbred mitochondrial lineages are widely distributed

Previous studies using cytb and COI mitochondrial markers arrive to important information, but until now there has been a lack of comparative analysis between the mitochondrial lineages identified by the two markers. Our studied area is inhabited by three cytb mitochondrial lineages (*Rq2*, *Rq4* and *Rq6*) that coexist in some basins (Fig. 1) and would have diverged in allopatry 5.94-4.55 million years ago (MYA) (Ríos et al., 2017). According to Ríos et al. (2017), *Rq2* and *Rq4+Rq6* belong to different clades, Clade A and C, respectively. Clade A would have diverged in Amazon and *Rq2* would have spread to LP-PM-AO. Clade C would have diverged in LP-MP-AO, and *Rq4* and *Rq6* would have diverged within LP-PM-AO, consistent with marine transgression in this region (Ríos et al., 2017). In accordance with previous studies (Perdices et al., 2002; Ríos et al., 2017), our COI phylogenetic topology evidenced two major clades that correspond to cis and trans andean clades. Because *R. quelen* is distributed from the extreme northeast of Andes to the center of Argentina (cis andean region), the trans andean specimens that were identified as *R. quelen* (Supplemental File II) should be reviewed. When mitochondrial lineages based on cytb were analyzed using the COI marker, specimens from Miranda river (KU845690- KU845694, Usso et al., 2018; Supplemental File II-III) were assigned to *Rq2* cytb mitochondrial lineage. This is in accordance with Ríos et al. (2017) that have proposed the origin of *Rq2* in the Amazon basin, given that Miranda river is located far north of La Plata basin, close to Amazon basin. Our present results showed that *Rq4* mitochondrial lineage not only would inhabit lower Uruguay river, Merin Lagoon and Negro river basin, but it also would be present in upper Uruguay and Parana river basins. Rq6 mitochondrial lineage was proposed as a putative species and was more abundant in the southern region of LP-PM-AO (Ríos et al., 2017). In the present study, we identified individuals that belong to Rq6 from Pampa plain (Rosso et al., 2012),

which is at the far southwest of LP-PM-AO. However, we also found Rq6 individuals from Parana river (Rosso et al., 2012) as well as hatchery individuals from Santa Catarina (Scaranto et al., 2018). Surprisingly, *R. branneri* (Haseman, 1911) from Iguaçu basin (Ribolli et al., 2017; Scaranto et al., 2018) also belongs to Rq6. *Rhamdia branneri* was considered endemic of Iguaçu river in the far northeast of LP-PM-AO. These results would extend the distribution area of Rq6 and warn about the need to review *R. branneri*. Moreover, *R. branneri* could hybridize with *R. quelen* mitochondrial lineages, not only in hatcheries, as proposed by Scaranto et al. (2018), but also in natural populations, in accordance to Ríos et al. (2019).

Genomic pattern of differentiation

The amount of genetic diversity was similar across all locality samples, but according to Ríos et al. (2019), coastal lagoon samples were less diverse than riverine ones ($H_{EXP,}$ Table III). High and significant F_{IS} values found in Negro river basin (4-NR-RB) and Merin Lagoon basin (9-ML-QC) would not be related to inbreeding, but can be explained by having considered the two clusters inhabiting these basins as one single population (See below). When we split these samples by cluster (4-NR-RB-N, 4-NR-RB-S, 9-ML-QC-N and 9-ML-QC-S; Table I), F_{IS} was not significant in each sub-sample, although caution on F_{IS} should be considered given the small sampling size.
Differentiation is greater among northern samples than among southern ones, while intermediate samples (4-NR-RB and 9-ML-QC) are genetically closer to South samples (Fig. 2*a-c*, Table IV*a-c*). These results are in accordance with Ríos et al. (2019) who suggested a genetic structure pattern according to North-South and East-West distribution. The low pairwise F_{ST} values among 1-UR-CR, 2-UR-AR and 3-UR-QR, along with a genetic pattern that does not follow the North-South distribution, could be due to their common origin from Uruguay river basin (Fig. 2). The low pairwise differentiation between these Uruguay river basin samples (specially 1-UR-CR) and 4-NR-RB is consistent with Ríos et al. (2019), who suggest the existence of gene flow between *R. quelen* populations from Uruguay and Negro river basins. Total and neutral pairwise F_{ST} analysis recovered sensible differentiation among coastal lagoons (Ríos et al., 2019, based on microsatellites: 0.125-0.216; Neutral SNPs dataset: 0.174-0.251, Table IV*b*). However, pairwise F_{ST} of Outlier loci putatively under selection revealed important structuring among samples from North and southern basins (F_{ST} mean: 0.939), and absence of structuring among coastal lagoons (F_{ST}= 0.000). Therefore, the structure observed in Total and Neutral dataset could be indicative of

basin isolation and genetic drift driven differentiation. Structure analyses of the three datasets were similar and revealed the presence of two clusters. Like DAPC, the distribution of these clusters follows a North-South pattern. One cluster (North Cluster) inhabits the basins from the North (Uruguay river basin (UR): Cuareim (1-UR-CR), Arapey (2-UR-AR) and Queguay (3-UR-QR) river basins); the other one (South Cluster) inhabits the South (La Plata (LP): Sauce lagoon (5-LP-SL) basin and Coastal lagoons draining to Atlantic Ocean (AO): Blanca lagoon (6-AO-BL); Rocha lagoon (7-AO-RL) and Castillos lagoon (8-AO-CL)). The aforementioned clusters were detected in Negro river and Merin Lagoon basins, without evidence of significant introgression between them (Fig. 1; Fig. 2*a-c*), therefore, they could be reproductively isolated. Latitudinal differentiation associated with water temperature was evidenced in Chinese Sea Bass (*Lateolabrax maculatus* (Cuvier, 1828)), also using RAD-seq population analysis in continental marginal seas along the Chinese coast (Zhao et al., 2018).

Cluster evolution driven by outlier SNPs under selection

Detected clusters appeared associated with different latitudes; the North Cluster inhabit Uruguay river basin (in the North) and the South Cluster coastal lagoons (in the South). Therefore, the coexistence of both clusters in geographically intermediate basins could be due to a secondary contact. The pairwise F_{ST} analysis based on 75 loci putatively under selection recovered higher values among northern and southern locality samples, in comparison with the values corresponding to the Neutral and Total loci. This could reveal that a strong divergent selection is acting over the 75 loci, and so differentiation of these loci is increased over the rest of the genome. Surprisingly, based on Outlier dataset absence of structure (F_{ST}= 0.000) was unraveled among four coastal lagoon samples (5-LP-SL, 6-AO-BL, 7-AO-RL and 8-AO-CL). This extremely low structure could be caused by balancing or purifying selection acting over these loci. However, all outlier loci were monomorphic in coastal lagoons, which would be suggestive of purifying selection in the adaptation to coastal lagoons environment. Strong selection and genetic drift for small isolated populations could explain the low genetic diversity evidenced in coastal lagoons samples and of the detection of bottleneck events by Ríos et al. (2019) in these samples. The similarity observed in the population structure analyses of the three datasets (Total, Neutral and Outlier), could be explained by two different scenarios: first, divergence in allopatry in the northern basin (Uruguay river) and coastal lagoons (La Plata and Atlantic Ocean); and second, cluster divergence with gene

flow. In the first scenario, two genomic clusters diverged allopatrically, and so divergent selection would be coupled with the potential for genetic drift by geographic isolation. Thus, outlier loci under selection could represent genome regions directly involved in divergence processes (Feder et al., 2013). It would be expected that hybrids between divergent clusters show F_{ST} local divergence in those regions. Alternatively, past hybridization evidenced between mitochondrial lineages could point out a scenario of divergence by selection with gene flow. As a result of basin history, each of these clusters became later isolated to the north and south regions. In this scenario, outlier loci under selection would be considered as an evidence of divergence hitchhiking. According to Feder et al. (2012) speciation with gene flow is divided in four phases. Based on the similitude among the three datasets and the fact that all annotated loci would lie in different chromosomes (Table II), we considered that genomic cluster divergence would be consistent with a phase three. In this phase, divergent selection across several loci allows new mutations to effectively establish across the genome. Thus, variation between regions with high and low divergence decreases. Because divergence occurs across the entire genome, this phase is considered to be governed by genomic hitchhiking. In sum, North and South clusters appeared strongly differentiated and could be in an incipient speciation process, mediated by divergent selection. Berg et al. (2015) proposed a similar scenario in Baltic cod, where ecological speciation driven by genomic islands was suggested, leading even to an effective reproductive barrier.

Annotated loci under selection provide crucial information about *R. quelen* evolution. We can classify these annotated loci in different gene groups under selection: loci associated to cell function, development and reproduction (Table II). Among the loci associated with development processes, we annotated the locus 66502_32 as Homeobox D3 (hoxd3). As other homeobox genes, this gene codifies a transcription factor involved in antero-posterior differentiation and neural development (Glover, 2001; Mungpakdee et al., 2008). We annotated the SNP 449_34 as F-box and WD repeat domain containing 2 (fbxw2), which codifies a protein associated with embryonic development of branchial arches and probably also with limb development (Fowles et al., 2003). Among loci associated with cell function, the SNP 9230_26 was annotated as Cysteine-rich hydrophobic domain-containing protein 2-like (Chic2). The protein encoded by this gene has a secretory function, and its gene expression is associated to regulation of nuclear hormone receptors (Cools et al., 2001). Chic2 gene is expressed in many secretory organs, like testis, ovary, uterus, thyroid, prostate and pineal gland, among others (Cools et al., 2001; Garstang & Ferrier, 2018).

The SNP 48262_4 was annotated as Coronin-1-C (coro1c) gene; the expression of this gene contributes to cellular assembly and organization (Ticianelli et al., 2017). The 67564_7 was anchored to sodium channel protein type 8 subunit alpha-like gene (scn8a). Sodium channel and its scn8a protein are involved in osmoregulation and enable environmental adaptation (Zakon, 2012). In this sense, ion regulation in plasma (homeostasis) plays an important role in adaptation to changing conditions of external salinity (McCormick, 2001). The fact that scn8a gene could be under selection and the fixation of the same allele in coastal lagoons samples, would be evidence of adaptation of lacustrine populations (5-LP-SL, 6-AO-BL, 7-AO-RL and 8-AO-CL) to this environment. As expected, these lagoons are characterized by a higher salinity than the one of riverine environments (Bonilla et al. 2006). Evidences of adaptation to different salinity levels has been documented for several fish species (DeFaveri & Merilä, 2014; Berg et al., 2015; do Prado et al., 2018), and associated with genes mainly involved in osmoregulation. On the other hand, the outlier locus 33517_8 was anchored to Sperm acrosome membrane-associated protein 6 (SPACA6) gene. Differential selection over this gene could promote reproductive isolation between clusters, given that the protein encoded by this gene, together with IZUMO1, could mediate sperm fusion through binding to a yet unknown egg membrane receptor (Lorenzetti et al. 2014). This is in agreement with Bird et al. (2012), which suggests that evolutionary studies in early stages of lineage divergence could reveal details of the mechanisms that generate reproductive isolation. In this sense, Krug (2011), suggested that rapid evolution of sperm and egg recognition proteins could explain the relative amount of sympatric sister gastropod species.

Divergent population compose genomic clusters

Based on 10 microsatellite loci, Ríos et al. (2019) found a two-cluster structure, which could be consistent with scenarios of past hybridization events. However, that scenario differs from the genomic structure elucidated in the present study. While in previous microsatellite results, 6-AO-BL and 8-AO-CL did not appear to belong to the same cluster as 5-LP-SL and 7-AO-RL, the current genomic structure groups these four localities in the same cluster. Ríos et al. (2019) found that the recent structure follows a geographic pattern and is composed by different genetically divergent populations. In this sense, population substructure was unraveled based on Total and Neutral datasets (Fig. 4*a-b*, K= 7). Substructure evidenced major differences in 4-NR-RB-S, 5-LP-SL, 7-AO-RL and 9-ML-QC-N samples. Moreover, Uruguay river basin together with

specimens of Negro river basin that belong to the North cluster (4-NR-RB-N) compose a population significantly divergent from the rest. These five subclades are consistent with the divergent populations proposed by Ríos et al. (2019). The group that consists of 6-AO-BL, 8-AO-CL and 9-ML-QC-S is highly differenced from the rest of the locality samples, but low divergence was observed among the locality samples that form this group. Three divergent populations have been proposed by Ríos et al. (2019) for these localities, however our results do not evidence significant divergence among them. Apart from this discrepancy, the divergent populations recovered in the present study were mostly coincident with Ríos et al. (2019). These divergent populations could be nested into each genomic cluster (North and South cluster), and this becomes evident when population substructure using Total and Neutral dataset are compared against the substructuration based on outlier loci. In other words, divergent populations could have diverged more recently within each genomic cluster, probably by geographic isolation and genetic drift. The two divergent populations detected in Negro river basin as well as in Merin Lagoon basin, evidence notable differences and absence of introgression. The possible reproductive isolation among divergent populations in sympatry could reinforce the idea of an incipient speciation process taking place between clusters, as mentioned above. Ríos et al. (2019) proposed that these divergent populations in sympatry would remain isolated, due to homing behavior or divergent selection. Our results allowed to identify genomic footprints of divergent selection between clusters, and one of the genes (SPACA6) under selection could be directly involved in establishing a reproductive barrier. This might rule out homing behavior as a possible explanation for reproductive isolation, particularly since this behavior has never been described for *Rhamdia*. However, reproductive barrier and homing behavior should be specifically tested by deliberate crosses between genomic clusters and by ecologic studies of reproductive behavior, respectively.

Conclusion

Population genomic analysis allowed to identified two well latitudinally differentiated clusters. The process of differentiation among *R. quelen* populations from North and South (North and South cluster) areas of the system LP-PM-AO could be driven by divergent selection. In this sense, this differentiation could be related with an incipient speciation process. The two major genomic clusters appeared to exhibit restricted gene flow and represent greater adaptive variability. Particularly, the coastal lagoons (Ríos et al., 2019) should be specially considered for conservation and management purposes, given population adaptation to this environment by means of purifying selection. Novel insights into the phylogeographic composition of mitochondrial lineages within LP-PM-AO were found; *Rq2*, *Rq4* and *Rq6* could be more widely distributed within that basin system than was suggested in previous studies. Also, we confirm introgression and hybridization among *R. quelen* mitochondrial lineages and raise alert about *R. branneri* validity, which has been supported by phylogenetic analysis based on mitochondrial markers.

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Supplemental File I. Cytochrome b datasets: GenBank accession number, basin, species and mitochondrial lineage according to Ríos et al., (2017).

Genbank accession number cyt b	Basin	Species	Cyt b lineages
KP798762	Uruguay river	*R. quelen*	Rq2
MH669076	Uruguay river	*R. quelen*	Rq2
KP798651	Uruguay river	*R. quelen*	Rq4
KX379745	Uruguay river	*R. quelen*	Rq4
MH669077	Uruguay river	*R. quelen*	Rq4
KP798763	Uruguay river	*R. quelen*	Rq6
KX379744	Uruguay river	*R. quelen*	Rq6
KX379753	Uruguay river	*R. quelen*	Rq4
KX379754	Uruguay river	*R. quelen*	Rq4
KX379755	Uruguay river	*R. quelen*	Rq4
KX379756	Uruguay river	*R. quelen*	Rq4
KX379757	Uruguay river	*R. quelen*	Rq4
KX379758	Uruguay river	*R. quelen*	Rq4
MH669078	Uruguay river	*R. quelen*	Rq4
MH669079	Uruguay river	*R. quelen*	Rq4
MH669080	Uruguay river	*R. quelen*	Rq4
MH669081	Uruguay river	*R. quelen*	Rq4
MH669082	Uruguay river	*R. quelen*	Rq6
KP798663	Uruguay river	*R. quelen*	Rq4
KX379762	Uruguay river	*R. quelen*	Rq4
KX379763	Uruguay river	*R. quelen*	Rq4
MH669083	Uruguay river	*R. quelen*	Rq4
KX379752	Uruguay river	*R. quelen*	Rq6
KX379761	Uruguay river	*R. quelen*	Rq6
KP798646	Negro river	*R. quelen*	Rq6
KP798647	Negro river	*R. quelen*	Rq6
MH669084	Negro river	*R. quelen*	Rq6
MH669085	Negro river	*R. quelen*	Rq4
MH669122	Negro river	*R. quelen*	Rq4
KX379748	Negro river	*R. quelen*	Rq4
MH669086	Negro river	*R. quelen*	Rq4
MH669087	Negro river	*R. quelen*	Rq6
KP798743	Negro river	*R. quelen*	Rq6
MH669088	Negro river	*R. quelen*	Rq6
KP798742	Negro river	*R. quelen*	Rq6
MH669089	Negro river	*R. quelen*	Rq6
MH669090	Negro river	*R. quelen*	Rq6
MH669091	Negro river	*R. quelen*	Rq6
KP798652	Negro river	*R. quelen*	Rq6

KP798649	Negro river	*R. quelen*	Rq6
KP798650	Negro river	*R. quelen*	Rq6
MH669092	Negro river	*R. quelen*	Rq6
MH669093	Negro river	*R. quelen*	Rq6
MH669094	Negro river	*R. quelen*	Rq6
KX379747	Negro river	*R. quelen*	Rq6
MH669095	Negro river	*R. quelen*	Rq6
MH669096	Negro river	*R. quelen*	Rq6
MH669097	Negro river	*R. quelen*	Rq6
KX379749	Negro river	*R. quelen*	Rq6
MH669098	Negro river	*R. quelen*	Rq6
KP798737	Negro river	*R. quelen*	Rq4
MH669099	Negro river	*R. quelen*	Rq4
KX379742	Negro river	*R. quelen*	Rq6
MH669100	Negro river	*R. quelen*	Rq4
KP798739	Negro river	*R. quelen*	Rq6
KP798738	Negro river	*R. quelen*	Rq6
MH669101	Negro river	*R. quelen*	Rq6
MH669102	Negro river	*R. quelen*	Rq6
MH669103	Negro river	*R. quelen*	Rq6
MH669104	Negro river	*R. quelen*	Rq6
KP798759	La Plata river	*R. quelen*	Rq6
MH669105	La Plata river	*R. quelen*	Rq6
KP798749	La Plata river	*R. quelen*	Rq6
KP798748	La Plata river	*R. quelen*	Rq6
KP798808	La Plata river	*R. quelen*	Rq6
KP798807	La Plata river	*R. quelen*	Rq6
KP798806	La Plata river	*R. quelen*	Rq6
KP798805	La Plata river	*R. quelen*	Rq6
KP798804	La Plata river	*R. quelen*	Rq6
KP798803	La Plata river	*R. quelen*	Rq6
KP798802	La Plata river	*R. quelen*	Rq6
KP798801	La Plata river	*R. quelen*	Rq6
KP798800	La Plata river	*R. quelen*	Rq6
KP798799	La Plata river	*R. quelen*	Rq6
KP798798	La Plata river	*R. quelen*	Rq6
KP798797	La Plata river	*R. quelen*	Rq6
KP798796	La Plata river	*R. quelen*	Rq6
KP798795	La Plata river	*R. quelen*	Rq6
KP798794	La Plata river	*R. quelen*	Rq6
KP798792	La Plata river	*R. quelen*	Rq6
KP798791	La Plata river	*R. quelen*	Rq6
KP798790	La Plata river	*R. quelen*	Rq6
KP798789	La Plata river	*R. quelen*	Rq6
KP798788	La Plata river	*R. quelen*	Rq6

KP798787	La Plata river	*R. quelen*	Rq6
KP798786	La Plata river	*R. quelen*	Rq6
KP798785	La Plata river	*R. quelen*	Rq6
KP798784	La Plata river	*R. quelen*	Rq6
KP798783	La Plata river	*R. quelen*	Rq6
KP798782	La Plata river	*R. quelen*	Rq6
KP798781	La Plata river	*R. quelen*	Rq6
KP798780	La Plata river	*R. quelen*	Rq6
KP798779	La Plata river	*R. quelen*	Rq6
KP798778	La Plata river	*R. quelen*	Rq6
KP798777	La Plata river	*R. quelen*	Rq6
KP798775	La Plata river	*R. quelen*	Rq6
KP798774	La Plata river	*R. quelen*	Rq6
KP798669	La Plata river	*R. quelen*	Rq6
KP798668	La Plata river	*R. quelen*	Rq6
KP798667	La Plata river	*R. quelen*	Rq6
KP798666	La Plata river	*R. quelen*	Rq6
KP798665	La Plata river	*R. quelen*	Rq6
KP798664	La Plata river	*R. quelen*	Rq6
KP798659	La Plata river	*R. quelen*	Rq6
KP798658	La Plata river	*R. quelen*	Rq6
KP798657	La Plata river	*R. quelen*	Rq6
KP798656	La Plata river	*R. quelen*	Rq6
KP798655	La Plata river	*R. quelen*	Rq6
KP798643	La Plata river	*R. quelen*	Rq6
KP798644	La Plata river	*R. quelen*	Rq6
KP798760	Atlantic Ocean SW	*R. quelen*	Rq6
KP798761	Atlantic Ocean SW	*R. quelen*	Rq6
MH669106	Atlantic Ocean SW	*R. quelen*	Rq6
MH669108	Atlantic Ocean SW	*R. quelen*	Rq6
MH669109	Atlantic Ocean SW	*R. quelen*	Rq6
MH669107	Atlantic Ocean SW	*R. quelen*	Rq6
MH669110	Atlantic Ocean SW	*R. quelen*	Rq6
KP798746	Atlantic Ocean SW	*R. quelen*	Rq6
KP798745	Atlantic Ocean SW	*R. quelen*	Rq6
KP798744	Atlantic Ocean SW	*R. quelen*	Rq6
KP798715	Atlantic Ocean SW	*R. quelen*	Rq6
KP798714	Atlantic Ocean SW	*R. quelen*	Rq6
KP798713	Atlantic Ocean SW	*R. quelen*	Rq6
KP798712	Atlantic Ocean SW	*R. quelen*	Rq6
KP798711	Atlantic Ocean SW	*R. quelen*	Rq6
KP798710	Atlantic Ocean SW	*R. quelen*	Rq6
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KP798708	Atlantic Ocean SW	*R. quelen*	Rq6
KP798707	Atlantic Ocean SW	*R. quelen*	Rq6

KP798706	Atlantic Ocean SW	*R. quelen*	Rq6
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KP798704	Atlantic Ocean SW	*R. quelen*	Rq6
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KP798685	Atlantic Ocean SW	*R. quelen*	Rq6
KP798683	Atlantic Ocean SW	*R. quelen*	Rq6
KP798681	Atlantic Ocean SW	*R. quelen*	Rq6
KP798772	Atlantic Ocean SW	*R. quelen*	Rq6
KP798771	Atlantic Ocean SW	*R. quelen*	Rq6
KP798770	Atlantic Ocean SW	*R. quelen*	Rq6
KP798769	Atlantic Ocean SW	*R. quelen*	Rq6
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KP798767	Atlantic Ocean SW	*R. quelen*	Rq6
KP798735	Atlantic Ocean SW	*R. quelen*	Rq6
KP798734	Atlantic Ocean SW	*R. quelen*	Rq6
KP798733	Atlantic Ocean SW	*R. quelen*	Rq6
KP798732	Atlantic Ocean SW	*R. quelen*	Rq6
KP798731	Atlantic Ocean SW	*R. quelen*	Rq6
KP798730	Atlantic Ocean SW	*R. quelen*	Rq6
KP798729	Atlantic Ocean SW	*R. quelen*	Rq6
KP798728	Atlantic Ocean SW	*R. quelen*	Rq6
KP798727	Atlantic Ocean SW	*R. quelen*	Rq6
KP798726	Atlantic Ocean SW	*R. quelen*	Rq6
KP798725	Atlantic Ocean SW	*R. quelen*	Rq6
KP798724	Atlantic Ocean SW	*R. quelen*	Rq6
KP798723	Atlantic Ocean SW	*R. quelen*	Rq6
KP798722	Atlantic Ocean SW	*R. quelen*	Rq6
KP798719	Atlantic Ocean SW	*R. quelen*	Rq6
KP798718	Atlantic Ocean SW	*R. quelen*	Rq6
KP798717	Atlantic Ocean SW	*R. quelen*	Rq6
KP798703	Atlantic Ocean SW	*R. quelen*	Rq6
KP798701	Atlantic Ocean SW	*R. quelen*	Rq6
KP798698	Atlantic Ocean SW	*R. quelen*	Rq6
KP798697	Atlantic Ocean SW	*R. quelen*	Rq6
KP798696	Atlantic Ocean SW	*R. quelen*	Rq6
KP798693	Atlantic Ocean SW	*R. quelen*	Rq6
KP798691	Atlantic Ocean SW	*R. quelen*	Rq6
KP798766	Merin lagoon	*R. quelen*	Rq6
KP798654	Merin lagoon	*R. quelen*	Rq6
KP798653	Merin lagoon	*R. quelen*	Rq6
KX379746	Merin lagoon	*R. quelen*	Rq4
MH669114	Merin lagoon	*R. quelen*	Rq4
MH669117	Merin lagoon	*R. quelen*	Rq4
KX379750	Merin lagoon	*R. quelen*	Rq6
KX379751	Merin lagoon	*R. quelen*	Rq6

KX379759	Merin lagoon	*R. quelen*	Rq6
KX379760	Merin lagoon	*R. quelen*	Rq6
MH669111	Merin lagoon	*R. quelen*	Rq6
MH669112	Merin lagoon	*R. quelen*	Rq6
MH669113	Merin lagoon	*R. quelen*	Rq6
MH669115	Merin lagoon	*R. quelen*	Rq6
MH669116	Merin lagoon	*R. quelen*	Rq6
MH669118	Merin lagoon	*R. quelen*	Rq6
KP798676	Hatchery	*R. quelen*	Rq2
KP798673	Hatchery	*R. quelen*	Rq2
MH669120	Hatchery	*R. quelen*	Rq2
MH669121	Hatchery	*R. quelen*	Rq2
KP798679	Hatchery	*R. quelen*	Rq6
MH669119	Hatchery	*R. quelen*	Rq6
KP798677	Hatchery	*R. quelen*	Rq6
KP798675	Hatchery	*R. quelen*	Rq6
KP798674	Hatchery	*R. quelen*	Rq6
KX379755	Hatchery	*R. quelen*	Rq6
KP798671	Hatchery	*R. quelen*	Rq6
KP798670	Hatchery	*R. quelen*	Rq6
MK511219*	Uruguay river	*R. quelen*	Rq2
MK511196*	La Plata river	*R. quelen*	Rq6
MK511197*	La Plata river	*R. quelen*	Rq6
MK511198*	La Plata river	*R. quelen*	Rq6
MK511199*	Atlantic Ocean SW	*R. quelen*	Rq6
MK511200*	Atlantic Ocean SW	*R. quelen*	Rq6
MK511201*	Atlantic Ocean SW	*R. quelen*	Rq6
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MK511208*	Atlantic Ocean SW	*R. quelen*	Rq6
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MK511211*	Atlantic Ocean SW	*R. quelen*	Rq6
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MK511213*	Atlantic Ocean SW	*R. quelen*	Rq6
MK511194*	Merin lagoon	*R. quelen*	Rq4
MK511195*	Merin lagoon	*R. quelen*	Rq4
AY036742	Paraná river	*R. quelen*	Rq2
EF564743	Paraná river	*R. quelen*	Rq5a
AY036743	Iguaçu river	*R. quelen*	Rq5b

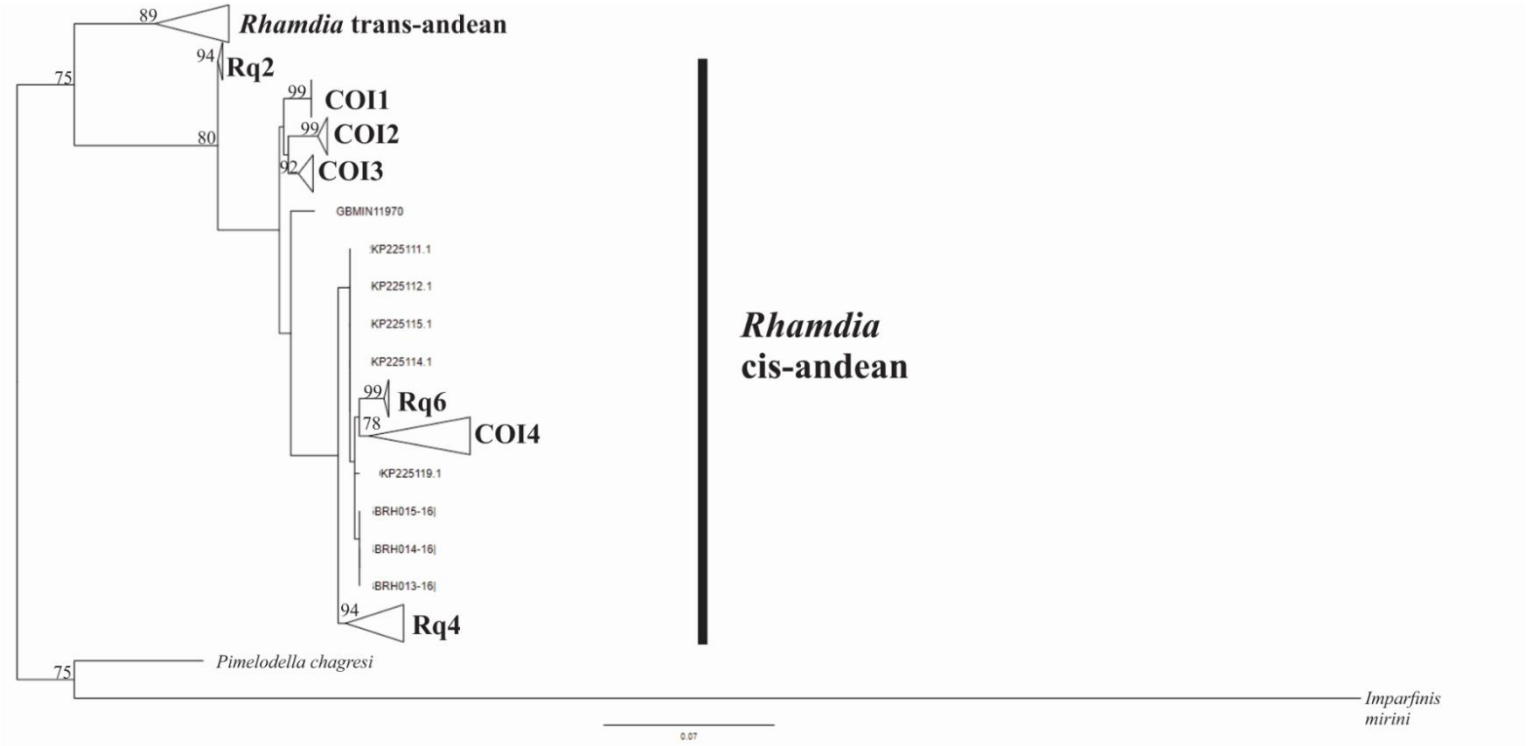
AY036744	Paraná river	*R. quelen*	Rq5b
AY036740	Amazon river	*R. quelen*	Rq3
AY036741	Amazon river	*R. quelen*	Rq3
AY036739	Esssequibio river	*R. quelen*	Rq1
DQ119395	Unknown	*R. quelen*	Rq1
AY036737	Orinoco river	*R. laukidi*	*R. laukidi*
AY036738	Orinoco river	*R. laukidi*	*R. laukidi*
KM489084	Orinoco river	*R. laukidi*	*R. laukidi*
KM489083	Orinoco river	*R. laukidi*	*R. laukidi*
KM489081	Orinoco river	*R. laukidi*	*R. laukidi*
AY036709		*R. laticauda*	*R. laticauda*
AY036708		*R. laticauda*	*R. laticauda*
AY036672		*R. guatemalensis*	*R. guatemalensis*
AY036671		*R. guatemalensis*	*R. guatemalensis*
AY036693		*R. wagneri*	*R. wagneri*
AY036694		*R. wagneri*	*R. wagneri*
AY036725		*R. cabrerae*	*R. cabrerae*
AY036726		*R. cabrerae*	*R. cabrerae*
AY036735		*R. cinerascens*	*R. cinerascens*
AY036736		*R. cinerascens*	*R. cinerascens*
AY036734		*R. rogersi*	*R. rogersi*
AY036733		*R. rogersi*	*R. rogersi*
AY036718		*R. nicaraguensis*	*R. nicaraguensis*
AY036719		*R. nicaraguensis*	*R. nicaraguensis*
KM489075		*R.saijaensis*	*R.saijaensis*
KM489076		*R.saijaensis*	*R.saijaensis*
KM489077		*R.saijaensis*	*R.saijaensis*

Supplemental file II. Cytochrome c oxidase subunit I dataset: sample code of each repository, taxa identification, haplotype, basin, country and mitochondrial lineages or clade. (*) Specimens that belong to Rq2, Rq4 or Rq6 mitochondrial lineage based on phylogenetics analysis of cytochrome b.

Bold Code or GenBank Accession number	Repository	Taxon	Basin, country	Mitochondrial lineages or Major clade
BRH002	BOLD	*Rhamdia branneri*	Iguaçu river, Brazi	*Rq6*
BRH009	BOLD	*Rhamdia branneri*	Iguaçu river, Brazi	*Rq6*
BRH013	BOLD	*Rhamdia voulezi*	Iguaçu river, Brazi	
BRH014	BOLD	*Rhamdia voulezi*	Iguaçu river, Brazi	
BRH015	BOLD	*Rhamdia voulezi*	Iguaçu river, Brazi	
BSB006	BOLD	*Rhamdia branneri*	Grande river, Brazil	*Rq4*
BSB025	BOLD	*Rhamdia quelen*	Grande river, Brazil	*Rq4*
BSB062	BOLD	*Rhamdia quelen*	Grande river, Brazil	*Rq4*
BSB108	BOLD	*Rhamdia quelen*	Grande river, Brazil	*Rq4*
BSB209	BOLD	*Rhamdia quelen*	Grande river, Brazil	*Rq4*
BSB377	BOLD	*Rhamdia quelen*	Grande river, Brazil	*Rq4*
BSFFA450	BOLD	*Rhamdia sp.*	Bayano river, Panama	*Rhamdia trans andean*
BSFFA451	BOLD	*Rhamdia sp.*	Canas river, Costa Rica	*Rhamdia trans andean*
BSFFA452	BOLD	*Rhamdia sp.*	Coto river, Costa Rica	*Rhamdia trans andean*
BSFFA453	BOLD	*Rhamdia sp.*	Sixaola river, Costa Rica	*Rhamdia trans andean*
BSFFA454	BOLD	*Rhamdia sp.*	Yape river, Panama	*Rhamdia trans andean*
BSFFA455	BOLD	*Rhamdia sp.*	Cocle del Norte river, Panama	*Rhamdia trans andean*
BSFFA456	BOLD	*Rhamdia sp.*	Mandinga river, Panama	*Rhamdia trans andean*
BSFFA457	BOLD	*Rhamdia sp.*	Ipeti river, Panama	*Rhamdia trans andean*
BSFFA458	BOLD	*Rhamdia sp.*	Esti river, Panama	*Rhamdia trans andean*
BSFFA461	BOLD	*Rhamdia sp.*	Cocle del Sur river, Panama	*Rhamdia trans andean*
BSFFA463	BOLD	*Rhamdia sp.*	San Juan river, Costa Rica	*Rhamdia trans andean*
BSFFA728	BOLD	*Rhamdia sp.*	Chagres river, Panama	*Rhamdia trans andean*
BSFFA729	BOLD	*Rhamdia sp.*	Playa Alta river, Panama	*Rhamdia trans andean*
BSFFA812	BOLD	*Rhamdia sp.*	Tranquera river, Nicaragua	*Rhamdia trans andean*
BSFFA813	BOLD	*Rhamdia sp.*	Santa Maria river, Panama	*Rhamdia trans andean*
BSFFA814	BOLD	*Rhamdia sp.*	Hato Grande river, Nicaragua	*Rhamdia trans andean*
BSFFA873	BOLD	*Rhamdia sp.*	Santa Maria river, Panama	*Rhamdia trans andean*
BSFFA883	BOLD	*Rhamdia sp.*	Membrillo river, Panama	*Rhamdia trans andean*
FARG317	BOLD	*Rhamdia quelen*	Mar Chiquita coastal lagoon, Argentina	*Rq6*
FARGB235	BOLD	*Rhamdia quelen*	El Divisorio stream, Argentina	*Rq6*
FARGB236	BOLD	*Rhamdia quelen*	El Divisorio stream, Argentina	*Rq6*
FARGB250	BOLD	*Rhamdia quelen*	El Divisorio stream, Argentina	*Rq6*
FARGB297	BOLD	*Rhamdia quelen*	Rojas river, Argentina	*Rq6*
FARGB365	BOLD	*Rhamdia quelen*	Parana river, Argentina	*Rq6*
FPSR094	BOLD	*Rhamdia quelen*	Paraiba do Sul Basin, Brazil	*COI4*
FPSR095	BOLD	*Rhamdia quelen*	Paraiba do Sul Basin, Brazil	*COI4*
FPSR096	BOLD	*Rhamdia quelen*	Paraiba do Sul Basin, Brazil	*COI4*
FPSR097	BOLD	*Rhamdia quelen*	Paraiba do Sul Basin, Brazil	*COI4*
FPSR098	BOLD	*Rhamdia quelen*	Paraiba do Sul Basin, Brazil	*COI4*

FUPR777	BOLD	*Rhamdia quelen*	Upper Parana Basin, Brazil	*Rq4*
FUPR778	BOLD	*Rhamdia quelen*	Upper Parana Basin, Brazil	*Rq4*
FUPR779	BOLD	*Rhamdia quelen*	Upper Parana Basin, Brazil	*Rq4*
GBGC7572	BOLD	*Rhamdia quelen*	No data available	*COI4*
GBGCA7590	BOLD	*Rhamdia quelen*	Sao Paulo, Brazil	*Rq4*
GBMN119705	BOLD	*Rhamdia quelen*	Bolivia	
GBMN132214	BOLD	*Rhamdia quelen*	No data available	*COI1*
GBMN132215	BOLD	*Rhamdia quelen*	No data available	*COI1*
ITAPE403	BOLD	*Rhamdia quelen*	Itapecuru river, Brazil	*COI3*
ITAPE404	BOLD	*Rhamdia quelen*	Itapecuru river, Brazil	*COI3*
KP225111	GenBank	*Rhamdia quelen*	Peri Lagoon, Brazil	
KP225112	GenBank	*Rhamdia quelen*	Peri Lagoon, Brazil	
KP225113	GenBank	*Rhamdia quelen*	Peri Lagoon, Brazil	*Rq4*
KP225114	GenBank	*Rhamdia quelen*	Peri Lagoon, Brazil	
KP225115	GenBank	*Rhamdia quelen*	Peri Lagoon, Brazil	
KP225116	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225117	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225118	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225119	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	
KP225120	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225121	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq4*
KP225122	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225123	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225124	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225125	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq4*
KP225126	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq4*
KP225127	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225128	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225129	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225130	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225131	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225132	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq4*
KP225133	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq6*
KP225134	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq4*
KP225135	GenBank	*Rhamdia quelen*	Commercial origin, fish farm	*Rq4*
KP225136	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225137	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225138	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225139	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225140	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225141	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225142	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225143	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225144	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225145	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*

KP225146	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225147	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225148	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225149	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225150	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225151	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225152	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225153	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225154	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225155	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225156	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225157	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KP225158	GenBank	*Rhamdia quelen*	Upper Uruguay river, Brazil	*Rq4*
KU845687	GenBank	*Rhamdia quelen*	Quexada river, Brazil	*Rq4*
KU845688	GenBank	*Rhamdia quelen*	Quexada river, Brazil	*Rq4*
KU845689	GenBank	*Rhamdia quelen*	Quexada river, Brazil	*Rq4*
KU845690	GenBank	*Rhamdia quelen*	Miranda river	*Rq2*
KU845691	GenBank	*Rhamdia quelen*	Miranda river	*Rq2*
KU845692	GenBank	*Rhamdia quelen*	Miranda river	*Rq2*
KU845693	GenBank	*Rhamdia quelen*	Miranda river	*Rq2*
KU845694	GenBank	*Rhamdia quelen*	Miranda river	*Rq2*
LARGI368	BOLD	*Rhamdia quelen*	Parana river, Argentina	*Rq6*
MNCE190	BOLD	*Rhamdia quelen*	San Francisco river, Brazil	*COI1*
MNCE191	BOLD	*Rhamdia quelen*	San Francisco river, Brazil	*COI1*
MNCE192	BOLD	*Rhamdia quelen*	San Francisco river, Brazil	*COI1*
MUCU033	BOLD	*Rhamdia quelen*	Atlantic Ocean. Brazil	*COI4*
MUCU083	BOLD	*Rhamdia quelen*	Atlantic Ocean. Brazil	*COI4*
MK511191*	GenBank	*Rhamdia quelen*	Rocha lagoon, Uruguay	*Rq6*
MK511192*	GenBank	*Rhamdia quelen*	Hatchery, Uruguay	*Rq2*
MK511193*	GenBank	*Rhamdia quelen*	Cueareim river, Uruguay	*Rq4*
TZGAA005	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
TZGAA016	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
TZGAA027	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
TZGA039	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
TGZAA051	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
TZGAA063	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
TZGA075	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
TZGAA087	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
TZGAA088	BOLD	*Rhamdia quelen*	Essequibo river, Guyana	*COI2*
UDEA184	BOLD	*Rhamdia quelen*	Campoalegre river, Colombia	*Rhamdia trans andean*
UDEA185	BOLD	*Rhamdia quelen*	Campoalegre river, Colombia	*Rhamdia trans andean*
UDEA186	BOLD	*Rhamdia quelen*	Campoalegre river, Colombia	*Rhamdia trans andean*
UDEA187	BOLD	*Rhamdia quelen*	Cienega river, Colombia	*Rhamdia trans andean*
UDEA188	BOLD	*Rhamdia quelen*	Cienega river, Colombia	*Rhamdia trans andean*



Supplemental File III. Phylogenetic analysis of the genus *Rhamdia* in the Neotropical region. Tree topology generated based on 131 sequences of cytochrome c oxidase subunit I gene on PhyML 3.1 (Guindon & Gascuel 2003). Numbers above nodes refer to the bootstrap support values from Maximum Likelihood. The bottom bar reflects 0.07 of genetic differences. *Rhamdia quelen* mitochondrial lineages are indicated and numbered (Rq2, Rq4 and Rq6 in accordance with Ríos et al., (2017); and COI1, COI2 and COI3 novel mitochondrial lineages).

Discusión General

Rhamdia quelen es un taxón de importancia en la ictiofauna Neotropical, de amplia distribución, constituyendo un importante recurso para la acuicultura y la pesca artesanal. En base a esto ha sido propuesta como una especie prioritaria para la conservación en Uruguay (Loureiro et al., 2013). En esta tesis se abordó la compleja sistemática de *R. quelen* mediante análisis filogeográficos y de genética de poblaciones, empleando diferentes marcadores (genes mitocondriales, microsatélites y SNPs (en inglés, "Single Nucleotide Polymorphism*"*)). Este análisis incluye un amplio muestreo en las cuencas conformadas por el Río de la Plata, el sistema de lagunas Patos-Merín, así como arroyos y lagunas que desembocan en la costa suroeste del Océano Atlántico (LP-PM-AO), con 246 especímenes de *R. quelen* analizados procedentes de 24 localidades de muestreo en el área de estudio. Además, para el análisis filogeográfico se integraron datos del género *Rhamdia* de la región Neotropical publicados en estudios previos (Perdices et al., 2002; Vergara et al., 2008; Hernández et al., 2015; Ribolli et al., 2017; Scaranto et al., 2018; Usso et al., 2018). Los distintos marcadores moleculares empleados, en su mayoría han sido desarrollados en el marco de esta tesis, y tienen potencial aplicación en estudios de diversidad genética y trazabilidad genealógica asociados al manejo reproductivo de *R. quelen* y su posible mejoramiento genético en la acuicultura.

Capítulo I. Delimitación de linajes mitocondriales en el complejo de especies *R. quelen*

En este primer capítulo se abordó el análisis filogeográfico de *Rhamdia* a distintas escalas geográficas: macro, meso y microescala. A nivel macrogeográfico se analizó la región Neotropical, enfocando principalmente hacia la región cis-andina, donde se distribuye el complejo de especies *R. quelen*. En el análisis filogenético y filogeográfico a distintas escalas se utilizó el marcador mitocondrial citocromo b (cytb), un marcador ampliamente utilizado en este tipo de estudios. Un total de 184 individuos fueron secuenciados y analizados junto a secuencias del gen cytb disponibles en bases de datos por estudios previos (Perdices et al., 2002; Vergara et al., 2008; Hernández et al., 2015). En el análisis a macroescala geográfica de este complejo de especies, se aplicó un análisis filogenético mediante los métodos de reconstrucción filogenética; Máxima Parsimonia, Máxima Verosimilitud y Análisis Bayesiano. Las reconstrucciones filogenéticas se analizaron junto a los algoritmos GMYC (del inglés, "Generalized Mixed Yule Coalescent" (Pons

et al., 2006)) y ABGD (del inglés, "Automatic Barcode Gap Discovery" (Puillandre et al., 2012)), con el fin de definir posibles especies dentro del género *Rhamdia*. GMYC evidenció un mayor número de especies que el algoritmo ABGD. Ya que GMYC suele identificar una mayor cantidad de especies que el resto de los algoritmos comúnmente utilizados (Kekkonen et al., 2015), en el presente estudio se adoptó un criterio conservador siguiendo los resultados arrojados por el algoritmo ABGD para delimitar las posibles especies del género *Rhamdia*. Esto llevó a proponer que el género *Rhamdia* está compuesto por 16 hipotéticas especies, que se agrupan en ocho linajes trans-andinos y ocho cis-andinos. *Rhamdia quelen* en particular está integrado por siete linajes mitocondriales altamente diferenciados (*Rq1*, *Rq2*, *Rq3*, *Rq4*, *Rq5a*, *Rq5b* y *Rq6*) los que de acuerdo a su grado de divergencia podrían soportar la hipótesis de que este taxón sería un complejo de especies.

Las reconstrucciones filogenéticas obtenidas soportaron la monofilia del género *Rhamdia*, compuesto por dos grandes clados que se corresponden con las especies trans-andinas y las de la región cis-andina. La divergencia de estos grandes clados habría sido por vicarianza asociada al levantamiento de la cadena montañosa de los Andes (Lundberg et al., 1998). El clado cis-andino se subdivide en cuatro sub-clados (A-D). El sub-clado D diverge del nodo más basal y está representado únicamente por la especie *R. laukidi* que habita la cuenca del Río Orinoco. Esta especie habría divergido 8,28 (7,06-9,44 (densidad posterior más alta al 95%) Ma (Millones de años atrás)) en la cuenca del Río Orinoco. Los tres restantes sub-clados están integrados por linajes mitocondriales de *R. quelen* y su divergencia es consistente con sucesivos eventos en alopatría en grandes cuencas de América del Sur. De esta manera el sub-clado A (*Rq1*) habría divergido en la cuenca del Río Essequibio, el sub-clado B (*Rq2* y *Rq3*) en la cuenca del Río Amazonas; mientras que el sub-clado C (*Rq4*, *Rq5a*, *Rq5b* y *Rq6*) habría divergido en el sistema de cuencas LP-PM-AO. Además, la presencia del linaje *Rq2* en el sistema de cuencas LP-PM-AO estaría explicado por una conexión secundaria entre las cuencas del Río Amazonas y LP-PM-AO durante el Plioceno (4,07; 3,07-5,02 Ma). La divergencia entre los linajes mitocondriales *Rq2* y *Rq3*, que integran el sub-clado B sería consecuencia del contacto entre las cuencas del Amazonas y LP-PM-AO, donde *Rq2* divergió en LP-PM-AO y *Rq3* en Amazonas. En tanto, la divergencia de los linajes mitocondriales del sub-clado C estaría relacionada con las transgresiones marinas que habrían limitado la conexión entre las cuencas (Lundberg et al., 1998).

El análisis poblacional a escala mesogeográfica se enfocó en los linajes que habitan el sistema de cuencas LP-PM-AO. Como resultado de este análisis, se propuso un escenario de expansión poblacional para el linaje mitocondrial *Rq6*, aunque no se pudo descartar la incidencia de la selección como explicación al polimorfismo observado. Finalmente, el primer abordaje de genética de poblacionales a microescala geográfica se basó en marcadores de tipo microsatélites y mitocondriales (cyt b). En este nivel microgeográfico se analizaron tres lagunas costeras del suroeste del Océano Atlántico; como resultado se identificaron tres poblaciones genéticamente diferenciadas que se correspondieron con las lagunas analizadas: Laguna del Sauce, Laguna de Rocha y Laguna Castillos. Estas tres poblaciones fueron propuestas como potenciales unidades de manejo (en inglés, "Management Units" (MUs)). Previo a este estudio, el único antecedente de análisis de la diversidad genética por medio de marcadores microsatélites en *R. quelen*, consistía en la evaluación genética de reproductores capturados en el Río Uruguay alto, en un emprendimiento de acuicultura en Brasil (Ribolli & Zaniboni-Filho, 2009). Por lo tanto, el análisis presentado en este capítulo representó el primer análisis de estructuración genética poblacional de *R. quelen* en poblaciones naturales en base a marcadores microsatélites, utilizando cinco loci disponibles en la literatura (Ribolli & Zaniboni-Filho, 2009), cuatro de ellos con amplificación cruzada de secuencias heterólogas aisladas en otras especies (PC17 y PC97; Moeser & Bermingham, 2005; Pcor y Pcor2; Revaldaves et al., 2005). Los resultados de este estudio aportaron información novedosa de tipo filogeográfico y de estructura genética a diferentes escalas geográficas que permitió identificar diferentes fenómenos geográficos implicados en la evolución del complejo de especies *R. quelen.* El creciente desarrollo de nuevas tecnologías de secuenciación abre perspectivas para obtener nuevos marcadores a escala genómica que permitan profundizar en los análisis de estructura genético poblacional, debido a la complejidad de la problemática abordada en esta especie.

Capítulo II. Desarrollo de marcadores de tipo microsatélite mediante tecnologías de secuenciación masiva

Con objeto de caracterizar un conjunto específico de marcadores microsatélites en *R. quelen*, se planteó realizar un rastreo genómico de este tipo de secuencias repetidas mediante tecnología de pirosecuenciación de Roche de nueva generación. A partir de 1/16 de carrera en un secuenciador Roche 454-GS-FLX, se obtuvieron 54.694 secuencias de un promedio de 324 pb de longitud. Según Fenerich et al. (2004), el genoma de *R. quelen* tendría un tamaño de aproximadamente 1.000 Mb (Valor C: 1,04 pg), por lo tanto, se habrían secuenciado cerca del 0,02 % del genoma de la especie. A partir del análisis de esta fracción aleatoria del genoma, se estimó que *R. quelen* tiene una densidad de microsatélites de un locus cada 5.446 pb. En cuanto a los motivos de repetición, los microsatélites dinucleótidos fueron los más frecuentes, seguido por los tetra-, tri, penta- y hexanucleótidos. Estos resultados son en general similares a los observados en otros Siluriformes como *Ictalurus punctatus* (Rafinesque, 1818), aunque esta especie presenta mayor número de microsatélites con motivos tri- que tetranucleótidos (Serapion et al., 2004).

De un total de 13.552 secuencias de motivos repetidos tipo microsatélite encontrados, 30 loci fueron seleccionados para el diseño de oligonuclótidos y finalmente 10 marcadores (Rhq2, Rhq7, Rhq8, Rhq13, Rhq15, Rhq16, Rhq20, Rhq26, Rhq28 y Rhq29) fueron elegidos por su viabilidad técnica, polimorfismo, facilidad para la amplificación y genotipado; para conformar el primer panel específico de loci microsatélites de *R. quelen*. Los 10 loci fueron caracterizados mediante genotipado en una muestra poblacional de 24 especímenes procedentes de la Laguna del Sauce, mostrando altos niveles de polimorfismo y buen ajuste general a las proporciones genotípicas esperadas en equilibrio de Hardy-Weinberg. No se detectaron señales significativas de errores de genotipado comunes a este tipo de marcadores por tartamudeo (“stuttering”) o amplificación diferencial de alelos de gran tamaño (“drop-out”). Por los indicios de presencia de alelos nulos (amplificación fallida por mutación en secuencias cebadoras flanqueantes) en el genotipado de Rhq8 se recomendó el uso de este marcador, pero con cautela. El panel de loci microsatélites generado en esta Tesis tiene utilidad para la aplicación tanto en análisis de genética de poblaciones de la especie *R. quelen*, así como para realizar asesoramiento en acuicultura, mediante la asignación de parentesco y trazabilidad genealógica en programas de mejoramiento genético.

Capítulo III. Hibridación histórica entre linajes mitocondriales y patrones de diversidad genética de *R. quelen* en cuencas de Uruguay

El análisis poblacional en el sistema de cuencas LP-PM-AO de los linajes mitocondriales (*Rq2*, *Rq4* y *Rq6*) basado en los 10 microsatélites desarrollados específicamente en *R. quelen* (Ríos et al., 2013), permitió evidenciar huellas de hibridación e introgresión, al menos entre *Rq4* y *Rq6*. Los análisis de hibridación también revelaron la presencia de individuos con evidencia de introgresión en todas las cuencas analizadas, sugiriendo eventos históricos de hibridación entre estos linajes, en paralelo con la evolución de las cuencas hidrográficas y las últimas transgresiones marinas (Ochoa et al., 2015). La hibridación entre los linajes mitocondriales altamente divergentes podría ser una de las explicaciones de la compleja sistemática de *R. quelen* en donde análisis basados en caracteres morfológicos, citogenéticos y moleculares han sido contradictorios (e.g. Silfvergrip, 1996; Perdices et al., 2002; Ríos et al., 2017; Angrizani & Malabarba, 2018). En este sentido, se propuso que la delimitación de especies en el complejo de especies *R. quelen* requiere, no sólo del soporte de análisis de genes mitocondriales, sino que también son necesarios análisis de genética de poblaciones basados en marcadores nucleares.

Los análisis de estructura poblacional evidenciaron dos patrones diferentes de estructuración: el primero es explicado por una relación pasada de las poblaciones, mientras que el segundo se explica en gran parte por la estructura actual de las cuencas habitadas por *R. quelen* en LP-PM-AO. La estructura poblacional en el pasado está representada por dos clústeres, en donde uno es integrado por las muestras de la Laguna del Sauce y la Laguna de Rocha, mientras que el otro agrupa el resto de las localidades analizadas. En este sentido, se propuso que esta relación pasada entre las dos lagunas, podría tener su explicación en un contacto entre estas dos poblaciones en la última regresión marina entre 14.000 y 6.000 años atrás (Laborde et al., 1996). Por otro lado, la estructura genética actual de *R. quelen*, sigue un patrón Norte-Sur y Este-Oeste y estaría conformado por siete clústeres poblacionales, que fueron propuestos como posibles unidades de manejo (MU) genéticamente diferenciadas. Cuatro de ellas se corresponden con las cuatro lagunas costeras analizadas (i.e. Laguna del Sauce, Laguna Blanca, Laguna de Rocha y Laguna Castillos); otra habita mayormente, y se habría originado, en la cuenca del Río Uruguay, aunque también se ha registrado su presencia en las cuencas del Río Negro y Laguna Merín; también se detectó una MU endémica de la cuenca de la Laguna Merín; y otra MU que está presente en las cuencas del

Río Negro, Río de la Plata y Laguna Merín. En este capítulo, por tanto, se amplió el análisis poblacional en la región de estudio, confirmando los resultados obtenidos en el primer capítulo (Ríos et al., 2017) respecto a la diferenciación genética entre las tres lagunas analizadas como unidades de manejo y conservación.

Además, en este capítulo se encontraron evidencias de diferencias en la historia demográfica entre las poblaciones que habitan lagunas y ambientes fluviales. Las poblaciones de las lagunas costeras presentaron menor diversidad genética y mayor nivel promedio de parentesco molecular entre pares de individuos, que las poblaciones fluviales. Además, en las poblaciones de las lagunas se identificaron señales de reducción de tamaño efectivo poblacional asociadas a cuellos de botella en su historia evolutiva reciente, lo que fue propuesto como posible explicación a la alta diferenciación de estas lagunas con respecto al resto de las poblaciones analizadas de *R. quelen* en cuencas fluviales En este sentido, ambos ambientes aparecen relacionados con los patrones observados de estructura genética de las poblaciones y podrían estar asociados a variación adaptativa de *R. quelen*.

Capítulo IV. Evidencias de selección e incipiente especiación en *R. quelen*

El avance en tecnologías de genotipado por secuenciación (GBS) utilizando plataformas de nueva generación de secuenciación ha hecho posible el rastreo de variabilidad genética para miles de marcadores SNP distribuidos por todo el genoma en especies no modelo (Robledo et al., 2017). El análisis genómico de las poblaciones de *R. quelen* a través de la técnica de 2bRAD-seq (siguiendo el protocolo de Wang et al. (2012), con modificaciones descriptas por Maroso et al. (2018)) buscando huellas de selección fue realizado mediante la comparación de las muestras del Norte de Uruguay (cuenca del Río Uruguay) y el Sur del país (Laguna del Sauce, Laguna Blanca, Laguna de Rocha y Laguna Castillos). Como resultado del análisis, de un total de 17.575 loci de tipo SNPs, 75 resultaron "outliers" significativos, asociados a huellas de selección; y los restantes 17.500 fueron considerados presuntamente neutrales en el escenario muestral evaluado. La comparación de los valores de diferenciación genética (F_{ST}) y la estructura poblacional basados en el panel de loci "outlier" bajo selección y loci presuntamente neutrales, permitieron proponer que los 75 primeros estarían bajo selección divergente. Se identificaron dos clústeres genómicos altamente diferenciados asociados con dicha selección divergente. La asociación geográfica disjunta de estos

dos clústeres genómicos sugiere posible divergencia en alopatría, uno asociado a la cuenca del Norte (Río Uruguay) y otro en el Sur, en las lagunas costeras (Laguna del Sauce, Laguna Blanca, Laguna de Rocha y Laguna de Castillos). El patrón de diferenciación genética sigue un patrón geográfico Norte-Sur de distribución de las localidades analizadas. La similitud entre las estructuras obtenidas mediante el grupo de SNP totales, utilizando los SNPs presuntamente neutrales y considerando sólo los "outliers", podría estar influenciado por diferentes razones técnicas y biológicas (Bernatchez et al., 2010), entre ellas estar evidenciando un efecto de arrastre ("hitchhiking"). La selección divergente actuando sobre ciertos loci promueve su diferenciación, pero a su vez por efecto del ligamiento genético promueve la diferenciación de los loci cercanos. Alternativamente, podría estar representando el acoplamiento entre la selección divergente y el efecto de la deriva genética en alopatría. La anotación de las secuencias que contienen los marcadores "outliers" y posterior comparación con el genoma de *I. punctatus*, permitió sugerir que las regiones de mayor diferenciación podrían estar distribuidas en varios cromosomas.

El análisis de los SNPs presuntamente neutrales evidenció estructuración entre las lagunas costeras, apoyando los análisis realizados en el capítulo III. Por otro lado, cuando se analizó la estructura genética por medio de los loci bajo selección, no se detectó diferenciación genética entre las lagunas, con fijación de las mismas variantes alélicas de los SNP "outliers" en todas las poblaciones de las lagunas costeras. Estas evidencias sugieren que la selección purificadora habría actuado en el proceso de adaptación a las lagunas costeras. Además, esto contribuiría a explicar la menor diversidad genética propuestos para estas localidades en el capítulo III.

A pesar del tamaño pequeño de las etiquetas generadas mediante RADseq (36 pb), el análisis de anotación funcional de los SNP "outliers" por homología de secuencia respecto a la base de datos del GenBank permitió identificar algunos posibles genes candidatos a estar sujetos a selección divergente, relacionados con el desarrollo, con la estructura y organización celular, y reproducción. Entre los genes identificados se destaca el que codifica para proteína 8 de la subunidad alfa del canal de sodio (scn8a), que podría estar involucrado en la adaptación a las lagunas costeras, ya que estos ambientes presentan mayor salinidad y este gen participa en la osmorregulación (Zakon, 2012). Además, este trabajo evidenció que otro de los genes bajo selección divergente codifica para la proteína 6 asociada a la membrana acrosomal del espermatozoide (SPACA6). Resulta interesante que esta proteína fue propuesta como posible mediadora en la fusión del esperma con

otra proteína aún desconocida de la membrana del ovocito (Lorenzetti et al., 2014). Este resultado podría estar evidenciando la presencia de algunos genes de importancia relacionados con los mecanismos de aislamiento reproductivo entre los clústeres, que explicarían por qué no se comportan como una población panmíctica en las cuencas del Río Negro y la Laguna Merín.

Las diferentes poblaciones identificadas en el sistema de cuencas LP-PM-AO en el capítulo III habrían divergido dentro de los clústeres genómicos. En el clúster del Norte se observaron dos sub-clústeres diferenciados, uno integrando las cuencas de los ríos Uruguay y Negro, y otro particularmente divergente en la cuenca de la Laguna Merín. En el clúster del Sur se detectó sub-estructuración asociada a la Laguna del Sauce, la Laguna de Rocha y la cuenca del Río Negro, que conformarían tres poblaciones diferenciadas. Finalmente, se detectó un sub-clúster adicional integrando una fracción de individuos de la Laguna Merín con dos lagunas del clúster del Sur (Laguna Blanca y Laguna de Castillos) sugerentes de pertenencia a una misma población ya que no presentan grandes niveles de diferenciación entre las tres localidades.

El análisis integrado de secuencias de genes mitocondriales (citocromo b y subunidad I de la citocromo oxidasa) permitió comparar y ampliar la distribución de los linajes mitocondriales en el contexto filogeográfico descrito en estudios previos (Perdices et al., 2002; Hernández et al., 2015; Ribolli et al., 2017; Ríos et al., 2017; Scaranto et al., 2018; Usso et al., 2018). En este sentido, el reanálisis con el marcador COI de los linajes cytb descritos por Ríos et al (2017) mostró evidencias de distribución adicional de Rq2 en la región noroeste del sistema de cuencas LP-PM-AO, y de Rq4 en toda la cuenca del Río Uruguay. Además, Rq6 detectado en la región sur del sistema de cuencas LP-PM-AO, se identificó también en la cuenca del río Iguazú. Sorprendentemente, haplotipos descritos como *R. branneri* en base a COI (Scaranto et al., 2018) comparten el linaje Rq6 de *R. quelen*, con lo cual se sugiere una revisión sistemática del género *Rhamdia* en la región cis andina.

A modo de perspectiva de este capítulo, sería interesante contrastar el patrón de diferenciación genética con características de las localidades analizadas (e,g, latitud, longitud, temperatura y salinidad) de tal manera de realizar análisis de correlación y asociación con variables ambientales como modo de evidenciar qué factores estarían influenciando el patrón de diferenciación genética observada.

Conclusiones Generales

-Los análisis filogeográficos a escalas macro, meso y microgeograficas con diferentes tipos de marcadores moleculares identificaron siete linajes mitocondriales que componen el complejo de especies *Rhamdia quelen*. Cinco de estos linajes (*Rq2*, *R4*, *Rq5a*, *Rq5b* y *Rq6*) habitan el sistema de cuencas LP-PM-AO (conformado por el Río de la Plata, el sistema de lagunas Patos-Merín, así como arroyos y lagunas que desembocan en la costa suroeste del Océano Atlántico) y tres habitan las cuencas de Uruguay (*Rq2*, *Rq4* y *Rq6*).

-Mediante secuenciación genómica de nueva generación se generó un panel de 10 microsatélites específicos de la especie *R. quelen*, de utilidad para abordar análisis de diversidad genética y de parentesco molecular en estudios evolutivos y aplicaciones en acuicultura en esta especie.

-Los linajes mitocondriales *Rq4* y *Rq6* de *R. quelen* identificados en las cuencas de Uruguay habrían hibridado en el pasado, previo a la conformación geológica actual de las cuencas.

-Las poblaciones de *R. quelen* de Uruguay analizadas en lagunas costeras presentan características de diversidad genética e histórico demográficas diferentes a las poblaciones de ambientes fluviales.

-El análisis de estructuración genética mediante genotipado por secuenciación de miles de loci SNP permitió identificar dos clústeres genómicos altamente diferenciados en las cuencas de Uruguay, asociados al patrón de distribución geográfica siguiendo un eje Norte-Sur, desde la cuenca del Río Uruguay a las lagunas costeras de la cuenca del Río de la Plata y Océano Atlántico. Estos clústeres estarían en un proceso incipiente de especiación y bajo selección divergente.

-Se han identificado algunos genes candidatos ligados a huellas de selección divergente asociados al desarrollo, a funciones celulares y reproducción. Entre ellos, el gen codificante para la proteína 6 asociada a la membrana acrosomal del espermatozoide (SPACA6) podría estar implicado directamente en el aislamiento reproductivo entre los dos clústeres genómicos divergentes. También se han detectado indicios de selección purificadora que habría mediado la adaptación de las poblaciones del complejo de especies *R. quelen* a las lagunas costeras.

-El análisis integrado de marcadores moleculares reveló una subestructuración subyacente a los dos clústeres genómicos principales, detectando distintas poblaciones diferenciadas que deberían

ser tomadas en cuenta como unidades de manejo para su conservación por sus posibles adaptaciones locales.

Finalmente, el estudio llevado a cabo en esta tesis permite rechazar la hipótesis planteada originalmente ya que, si bien el complejo de especies *R. quelen* está compuestos por varios linajes mitocondriales, estos habrían hibridado en el pasado. La estructura poblacional estaría principalmente determinada por dos clústeres genómicos en incipiente especiación que, sin embargo, presentan un patrón de distribución semejante al de los linajes mitocondriales.

Perspectivas

En el marco de esta Tesis se han generado diferentes recursos genómicos, que estarán a disposición para futuros estudios en la especie *R. quelen*, así como en peces Neotropicales. Adicionalmente, aprovechando la disponibilidad de los transcriptomas generados mediante tecnología RNAseq en un proyecto paralelo a esta Tesis, se realizará un análisis comparativo de los dos clústeres genómicos diferenciados detectados en cuencas del Norte y Sur de Uruguay. Este análisis, que se encuentra actualmente en desarrollo, persigue evidenciar posibles genes y transcritos que estén afectados por selección divergente. De este modo, se pretende identificar *in silico* secuencias génicas que presenten polimorfismo diferencial significativo entre los dos clústeres genómicos (fijación de alelos alternativos o frecuencias alélicas diferenciales). Este análisis nos permitirá dilucidar de manera más completa el posible proceso de especiación, así como el proceso de adaptación en las lagunas costeras. Como un resultado transversal de estos estudios de aplicación en genómica comparada de peces, se generará el primer transcriptoma de la especie que podría ser explotado para el rastreo y diseño de marcadores SNP asociados con genes de interés productivo (e.g., reproducción, sistema inmune, crecimiento, osmorregulación), para su posible aplicación en estudios de asociación familiar y poblacional, y en programas de mejoramiento genético asistido por marcadores. A su vez, el transcriptoma de la especie permitirá una mejor anotación e identificación de los loci 2bRADseq genotipados en esta Tesis.

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