EVALUACIÓN GENÉTICA DE ORGANISMOS ACUÁTICOS Y SU POTENCIAL USO COMO BIOMARCADORES EN LA CUENCA ALTA DEL RÍO CHINCHINÁ, CALDAS, COLOMBIA

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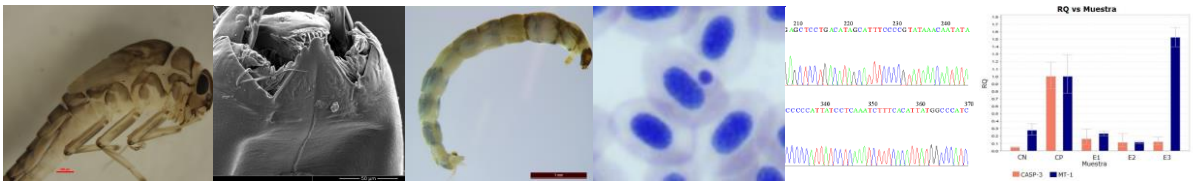

Maestría en Ciencias Biológicas

Facultad de Ciencias Exactas y Naturales

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Trabajo de grado presentado como requisito parcial para optar al título de Magíster en Ciencias Biológicas

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Manizales, 06 de septiembre de 2017

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CONTENIDO

Página

RESUMEN

En la actualidad el gran impacto generado en los ecosistemas fluviales por la contaminación, producto de diversas actividades antropogénicas como la industrialización, minería, agricultura, ganadería; causan alteraciones en éstos ecosistemas y pueden afectar los organismos que están expuestos a desechos tóxicos y desencadenar alteraciones morfológicas y moleculares. Entre los organismos acuáticos, se encuentran especies de macroinvertebrados y vertebrados, que se han adaptado a los ecosistemas fluviales y que presentan aspectos como su exigencia en la calidad de hábitat, sensibilidad ante la degradación ambiental y facilidad de estudio, que los han convertido en modelos para el desarrollo de métodos en el biomonitoreo hídrico. Estos organismos pueden proporcionar información sobre la calidad del agua y otras características del medio fluvial y pueden mostrar plasticidad en sus rasgos vitales, como respuestas a cambios en las condiciones ambientales.

En éste contexto, la evaluación y monitoreo de los sistemas hídricos necesitan de la aplicación de herramientas integradoras, como las que se plantean en el presente trabajo, donde se evaluó genéticamente algunas especies de macroinvertebrados y vertebrados en afluentes impactados por minería en la cuenca alta del Río Chinchiná, Caldas. En la evaluación morfológica de *Andesiops peruvianus* (Ephemeroptera: Baetidae), *Género 1* sp. *2* y *Cricotopus (Oliveiriella) rieradevallae* (Díptera: Chironomidae), se tuvieron en cuenta caracteres diagnósticos de las especies registradas en la literatura y se evaluaron posibles cambios morfológicos (incidencia de deformaciones y/o variaciones morfométricas) en *Género 1* sp. *2*, empleando microscopía de luz (ML) y microscopía electrónica de barrido (MEB). Se analizaron secuencias de ADN de los genes mitocondriales 16S ADN*r* y Citocromo oxidasa I (COI). Así mismo se realizaron pruebas genotóxicas y de expresión génica en *Danio rerio* (Cypriniformes: Cyprinidae), evaluando la presencia de micronúcleos (MN), anormalidades nucleares (AN) y PCR en tiempo real (*qPCR*) para medir la expresión de los genes metalotioneína (MT-1) y caspasa 3 (CASP-3).

Los resultados determinaron que *A. peruvianus* es un posible complejo de especies, representada por al menos cuatro grupos con variaciones morfológicas en branquias y uñas tarsales, así como por las distancias intraespecíficas de los genes 16S y COI. El *Género 1* sp. 2 se confirmó morfológica y molecularmente, asociando larvas con pupas y registrando algunos organismos con deformidad parcial o total en dientes. No se presentó una diferencia significativa entre la frecuencia de las deformidades y la contaminación minera y solo se registró para el análisis morfométrico una medición “área dorsal de la cabeza” con diferencias significativas. Adicionalmente, se colaboró para la confirmación morfológica y molecular de

una nueva especie: *C.* (*O.) rieradevallae*, Prat & Paggi 2016. Los análisis genéticos preliminares en *D. rerio*, mostraron que micronúcleos, núcleos *blebbed*, índices de proliferación y expresión génica de los genes MT-1 y CASP-3, son afectados por aguas procedentes de las estaciones con impacto evidente por minería de la cuenca alta del Río Chinchiná. En general los resultados obtenidos exploran diferentes herramientas que pueden contribuir al conocimiento de éstas especies y su posicionamiento como potenciales biomarcadoras de la calidad de agua.

Palabras clave

ADN, Anormalidades nucleares, Biomonitoreo hídrico, contaminación, expresión génica, evaluación morfológica, micronúcleos.

INTRODUCCIÓN GENERAL

La región Neotropical cuenta con una gran riqueza en recursos hídricos y consecuentemente con una exuberante biodiversidad acuática, que en conjunto proporcionan diversos servicios ecosistémicos (disponibilidad de agua, descomposición de desechos, producción de alimentos, regulación del clima, entre otros) que se traducen en beneficios para la calidad de vida en los asentamientos humanos (Oscoz et al., 2006; Acosta et al., 2009; Sabater & Elosegui, 2009). Sin embargo, en las últimas décadas la creciente urbanización e industrialización han generado una fuerte presión como consecuencia de las actividades antropogénicas asociadas a las cuencas hidrográficas, principalmente la transformación del paisaje, sobreexplotación del recurso y cambios en el uso de la tierra (agricultura, ganadería y minería) provocando cambios en los ecosistemas acuáticos y terrestres (Segnini, 2003; Gamboa et al., 2008; Ramírez et al., 2008).

Éstas transformaciones crecen exponencialmente y tienen un papel importante en la economía, pero también en la producción de desechos tóxicos depositados principalmente en los ecosistemas de agua dulce, que juntamente con los estuarios, son los sistemas más baratos y fáciles para descartar efluentes (Odum, 1988). Frente a esto es necesario realizar una evaluación y monitoreo de los sistemas hídricos, considerando la aplicación de herramientas que integren la medición de parámetros fisicoquímicos, microbiológicos y el uso de macroinvertebrados acuáticos, donde se pueden evaluar deformidades morfológicas, asimetría fluctuante, *fitness* de los adultos, paleolimnología, rasgos biológicos y estudios etológicos (Giacometti & Bersosa, 2006; Matsumoto et al., 2006; Alba-Tercedor, 2007; Prat et al., 2009). Recientemente, algunas investigaciones destacan la importancia de la inclusión de cambios morfológicos teniendo en cuenta las deformidades y las fluctuaciones en los tamaños corporales como un criterio de valoración biológica de calidad de agua (Vuori & Kukkonen, 2002; Skinner & Bennett, 2007; Prommi, 2011). Éstas fluctuaciones morfológicas son consideradas por muchos autores como respuestas subletales de los organismos a agentes estresantes (Vermeulen, 1995), ya que los primeros cambios en el ambiente ocurren en los niveles inferiores de la organización biológica, principalmente de carácter bioquímico y fisiológico (Servia, 2002; Linde et al., 2007).

Teniendo en cuenta su potencial uso como biomarcador en las alteraciones ecosistémicas, insectos de amplia distribución geográfica como Chironomidae y Ephemeroptera han sido muy empleados (Hamalainen, 1999; Linde et al., 2007; Prat et al., 2009; Biasi & Restell, 2010).

En los ephemerópteros se ha determinado presencia de deformidades en las branquias cuando están expuestos a bajos niveles de oxígeno, alta demando biológica de oxígeno (DBO) (Pescador & Rasmussen, 1995) y algunas respuestas genéticas por la exposición crónica al mercurio (Snyder & Hendricks, 1997).

Entre tanto, los quironómidos manifiestan una notable respuesta morfológica a los agentes contaminantes asociados a sedimentos y residuos industriales, conformando el grupo con mayor incidencia de deformidades, en estructuras de la capsula cefálica larvaria, como piezas bucales y antenas (Vermeulen 1995; Hamalainen 1999; Servia et al., 1999a; 1999b; Servia 2002; Nazarova et al., 2004; Biasi & Restello, 2010). Su caracterización molecular y variaciones genéticas han sido evaluadas en organismos capturados en campo y en bioensayos mediante el uso de microsatélites (*SSR-simple sequence repeat*), ADN-código de barras (gen *Citocromo Oxidasa I*), 18S y 28S del ADN*r* (Salman et al., 2009; Cranston et al., 2012). También se ha estimado el efecto de los metales pesados mediante la aparición de aberraciones cromosómicas en larvas de Chironomidae, presentando alteraciones funcionales y estructurales en los organismos, que pueden ser fácilmente identificados y utilizados para la detección de agentes tóxicos en el medio ambiente (Michailova et al., 2012).

El uso de peces como indicadores de toxicidad en ecosistemas de agua dulce tienen una larga historia (Szarek et al., 2000). Según Fontanetti et al. (2012), el reflejo del compromiso ambiental de un ecosistema acuático puede ser evidenciado utilizando peces, debido a su alto nivel trófico y su gran importancia en la dieta alimenticia del ser humano, características que contribuyen para que los peces sean especies blanco de investigaciones de evaluación de impactos en el ambiente acuático y/o evaluación del riesgo, en las que sus órganos analizados son utilizados como biomarcadores. Al tratarse de organismos vertebrados, son normalmente considerados como buenos representantes de especímenes de mayor complejidad y por lo tanto, los efectos tóxicos observados en ellos son más fáciles de comprender e interpretar (Martínez & Espinosa, 2002).

Los biomarcadores pueden ser bioquímicos, fisiológicos, histológicos (daños en tejidos) o genéticos (daños en el material hereditario), detectados y evaluados empleando diferentes técnicas (Evangelista, 2012). En éste sentido, las variaciones ó alteraciones morfológicas encontradas en los organismos, se hace necesario buscar cambios transitorios o permanentes en ciertas vías metabólicas, propiedades del material genético y daños celulares. El test de micronúcleos (MN) es ampliamente utilizado, y asociado a anomalías nucleares (AN), evaluación de daños en el ADN y otras alteraciones que parecen estar relacionadas con fallos en la división celular, muerte celular, genotoxicidad y/o mutagenicidad, empleadas en el diagnóstico de la toxicología genética en ambientes acuáticos, en pruebas con células eucariotas (Faust et al., 2004; Collins 2004; Hoshina et al., 2008; Evangelista, 2012).

Actualmente las herramientas moleculares, como los niveles de expresión de genes, potencialmente contribuyen en éste tipo de investigaciones. Sin embargo, la articulación se debe hacer entre las respuestas moleculares presentadas en los individuos (Poynton et al., 2011). La expresión génica también puede ser cuantificada y puede ser evaluada como respuesta de los organismos ante determinadas condiciones ambientales, algunos genes como *Melatonin receptor* (MT-1), *cytochrome P450, subfamily XXVIA, polypeptide 1* (cyp26a1), *caspase 3* (CASP-3) han sido registrados en diferentes taxa, cuya expresión está inducida principalmente por exposición a metales pesados y la regulación de esa expresión no ocurre de la misma manera en todos los organismos (vertebrados e invertebreados), por lo que pueden estar expuestos a diferentes condiciones y de ello dependen sus respuestas fisiológicas, celulares, etc (Swain et al., 2004; Quirós et al., 2007; Deng et al., 2009; Jin et al., 2010; Zhang et al., 2015).

Frente a la reconocida importancia de las investigaciones integrales en la evaluación de la contaminación del recurso hídrico, el propósito de la presente investigación fue el de evaluar y describir los posibles cambios en *A. peruvianus* (Ephemeroptera: Baetidae), *Género 1* sp. 2, *Cricotopus (Oliveiriella) rieradevallae* (Díptera: Chironomidae: Orthocladiinae) y *D. rerio* (Cypriniformes: Cyprinidae) en quebradas con intervención minera en la cuenca alta del Río Chinchiná (Caldas, Colombia), que puedan ser tenidas en cuenta como potenciales herramientas de diagnóstico en la calidad del agua.

OBJETIVOS

Objetivo general

Evaluar genéticamente especies acuáticas en afluentes de la Cuenca alta del Río Chinchiná, Departamento de Caldas (Colombia).

Objetivos específicos

- Evaluar la variabilidad genética de la especie *Andesiops peruvianus* (Ephemeroptera: Baetidae).

- Evaluar morfológica, morfométrica y molecularmente las especies *Género 1* sp. *2* y *Cricotopus (Oliveiriella) rieradevallae* (Díptera: Chironomidae).

- Cuantificar los efectos genotóxicos y la expresión génica en *Danio rerio* (Cypriniformes: Cyprinidae) expuesto a afluentes contaminados por minería.

RESULTADOS

Los resultados integrales del trabajo de investigación, se presentan a manera de capítulos. Cada capítulo consta de al menos un artículo científico, aceptado, sometido o en preparación para ser publicado en revista internacional especializada en el área estudio. En éste sentido y a manera de complemento, solo se realiza un pequeño apartado de consideraciones al final del documento.

CAPÍTULO 1

Estatus: Publicado, 2017. DOI 10.1007/s10750-017-3321-1

PAULA A. OSSA-LÓPEZ, MARIA I. CAMARGO-MATHIAS AND FREDY A. RIVERA-PÁEZ. *Andesiops peruvianus* (Ephemeroptera: Baetidae): a species complex based on molecular markers and morphology.

Revista: *Hydrobiologia,* ISSN: 0018-8158 (Impresa) 1573-5117 (*Online*).

Factor de Impacto: 2,056

CAPÍTULO 2

Estatus: Sometido

PAULA A. OSSA-LÓPEZ, ERIKA M. OSPINA-PÉREZ, GABRIEL J. CASTAÑO-VILLA, NARCÍS PRAT, GHENNIE T. RODRIGUEZ-REY AND FREDY A. RIVERA-PÁEZ**.** Taxonomy of *Genus 1* sp. *2* (Diptera: Chironomidae: Orthocladiinae) and the potential use of its larvae as bioindicators. **Revista:** *Insects***,** ISSN: 2075-4450.

Factor de Impacto: 1,94

ANEXO (Abstract)

Estatus: Publicado, 2017. DOI 10.1007/s13744-017-0548-5

N. PRAT, A. PAGGI, C. RIBERA, R. ACOSTA, B. RÍOS-TOUMA, C. VILLAMARÍN, F. RIVERA, **P. OSSA,** M. RIERADEVALL. The *Oliveiriella* (Diptera Chironomidae) of the High Altitude Andean streams, with the description of a new species, *Cricotopus* (*Oliveiriella) rieradevallae*, Prat & Paggi 2016.

Revista: *Neotropical Entomology***,** ISSN: 1519-566X (Impresa) 1678-8052 (*Online*)

Factor de Impacto: 0,756

CAPÍTULO 3

Estatus: Aceptado, 2017.

PAULA A. OSSA-LÓPEZ; GABRIEL J. CASTAÑO-VILLA; FREDY A. RIVERA-PÁEZ. Genotoxic effects and gene expression in Danio rerio (Hamilton 1822) (Cypriniformes: Cyprinidae) exposed to mining-impacted tributaries in Manizales, Colombia.

Revista: *Environmental Monitoring and Assessment,* ISSN: 1573-2959 (*Online*).

Factor de Impacto: 1,687

CAPÍTULO 1

Andesiops peruvianus (Ephemeroptera: Baetidae): a species complex based on molecular markers and morphology

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Andesiops peruvianus *(Ephemeroptera: Baetidae): a species complex based on molecular markers and morphology*

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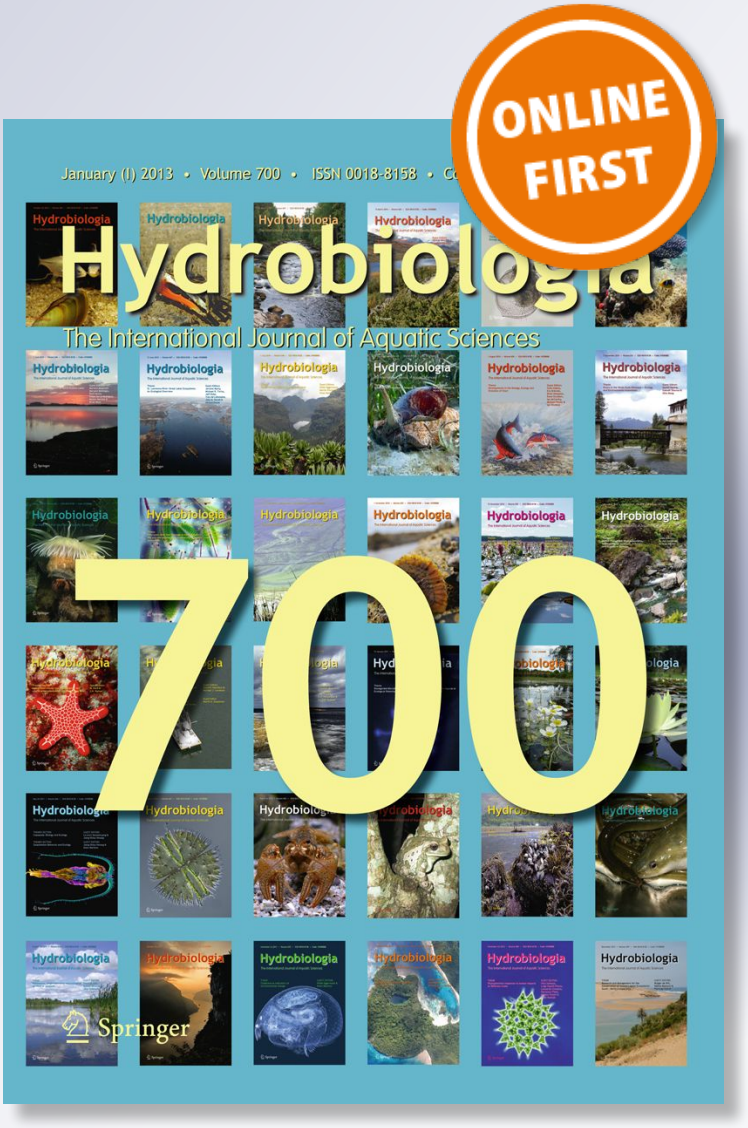

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PRIMARY RESEARCH PAPER

Andesiops peruvianus (Ephemeroptera: Baetidae): a species complex based on molecular markers and morphology

Paula A. Ossa-López · Maria I. Camargo-Mathias · Fredy A. Rivera-Páez

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Abstract *Andesiops peruvianus* is widely distributed in the Andean region and has been preliminarily used as a bioindicator of good water quality. Given the morphological variations that are reported for the species, this study aimed to address whether such morphological variability in nymphs captured in tributaries of the Upper Chinchiná River Basin, Caldas-Colombia, could be connected to genetic differences, suggesting the existence of hidden, hitherto unknown taxonomic diversity. The morphological evaluation allowed confirming the presence of 73 females and 83 males belonging to what can be considered *A. peruvianus* sensu lato. However, Automatic Barcode Gap Discovery and Poisson Tree Processes modeling identified four different taxonomic units. The genetic distances found for specimens of *A. peruvianus* were higher than expected for conspecific organisms, and DNA analyses allowed to separate *A. peruvianus* into taxonomic units, which were further supported by morphological characters (shape and size of the abdominal gills and number of denticles of the tarsal claws). The results of this study show that *A. peruvianus* represents a species complex, with four putative species inferred, contributing to the growing knowledge of the existence of pseudocryptic species. These new findings could influence the conservation status of these rivers.

Keywords Bioindicator · DNA · Morphological characters · Putative species · Pseudocryptic species

Introduction

The Neotropical region has a great water resource richness and vast aquatic biodiversity. However, part of this biodiversity has not been thoroughly studied. Dijkstra et al. (2014) mention that nearly 100,000 species belonging to 12 orders spend one or more life stages in freshwater, yet little is known about how this remarkable diversity arose. Allopatric speciation and ecological adaptation are thought to be primary mechanisms involved, as freshwater habitats are highly susceptible to environmental change and exhibit marked ecological gradients (Gill et al.,

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2016). Ephemeroptera is an insect order with an aquatic nymphal stage, encompassing approximately 3,000 species that belong to 42 families and over 400 genera (Domínguez et al., 2006; Barber-James et al., 2008; Gutiérrez & Dias, 2015). Most of the species of this order are considered water quality indicators, because they do not tolerate variations in water quality and have been widely used and accepted as a biomonitoring tool (Roldán, 1999; Bonada et al., 2006; Menetrey et al., 2008; Zúñiga, 2009; Rutschmann et al., 2017).

The genus *Andesiops* Lugo-Ortiz & McCafferty, 1999 family Baetidae, reported exclusively in South America, was initially composed of a single species: *A. peruvianus* Ulmer, 1920. Currently, the genus consists of four species, *A. angolinus* Navás, 1933, *A. ardua* Lugo-Ortiz & McCafferty, 1999, *A. torrens* Lugo-Ortiz & McCafferty, 1999 and *A. peruvianus* Lugo-Ortiz & McCafferty, 1999, which are distributed in Argentina, Peru, and Colombia (Nieto, 2004; Domínguez et al., 2006). Adults and nymphs described as *A. peruvianus* are distributed at high altitudes between Colombia and Argentina, along the Andean highlands (Nieto, 2004). Considering the broad geographic distribution of *A. peruvianus* in the Andean region and its apparent morphological variability (Finn et al., 2016; Gill et al., 2016), a molecular evaluation is warranted to investigate whether the species may actually constitute a group of closely related species, i.e., a species complex. For example, molecular data has revealed multiple cryptic species within the ephemeropteran genus *Baetis* (e.g., *Baetis rhodani* and *Baetis alpinus*) (Williams et al., 2006; Finn et al., 2014; Múrria et al., 2014).

Molecular tools have been widely used to discriminate aquatic insect species, mainly when morphological characters are not informative (Moore, 1995; Pereira-da-Conceicoa et al., 2012; Santos et al., 2016). Several studies (Williams et al., 2006; Virgilio et al., 2010; Gill et al., 2014; Hoyos et al., 2014; Rutschmann et al., 2014) have highlighted the existence of cryptic species in Ephemeroptera. A 658 bp fragment of the mtDNA cytochrome C oxidase subunit I (COI) gene has been standardized as the animal DNA barcode (Hebert et al., 2003).

In this study, we tested the hypothesis that *A. peruvianus* includes more than one species, or conversely, it is a single species with an elevated phenotypic plasticity, based on the examination of morphological features and sequence analysis of two mtDNA markers (16S rRNA and COI genes) in specimens collected from different affluents in the rural zone of the municipalities of Manizales and Villamaría, Caldas-Colombia, considering the morphological variability of *A. peruvianus.*

Materials and methods

Study area

The study region included six stations located in the Chinchiná River Basin (Fig. 1). Three sampling stations were located in La Elvira stream, municipality of Manizales—Caldas (05°03′10.9″N 75°24′33.6″W; 05°03′4.4″N 75°24′33.1″W; 5°1′53″N 75°24′43.8″W) and three stations in the municipality of Villamaría—Caldas, Romerales stream (04°59′22″N 75°25′58″W), California stream (04°59′5″N 75°26′35″W), and Toldafría stream (4°59′08″N 75°26′43″W). These stations are located between 2275 and 2766 m.a.s.l. and have similar physical habitat characteristics, such as an undulating topography, the presence of riparian vegetation, and open canopy cover (0–25% of the streams are shaded). A total of six sampling events were carried out between February 2014 and February 2015; with months of high-rainfall (February, July and November 2014 and February 2015) and low-rainfall (April and September 2014). Additionally, to try to correlate environmental variables with the occurrence of hidden diversity in the studied group, the following physical and hydrobiological variables were measured in situ: water temperature, pH, conductivity, dissolved oxygen, oxygen saturation, average depth, width, and water flow velocity. Also, the following chemical variables were evaluated in the laboratory: chemical oxygen demand (COD), biological oxygen demand (BOD5), total coliforms, fecal coliforms, total suspended solids (TSS), total solids (TS), cyanide (CN), boron (B), lead (Pb), mercury (Hg), ammoniacal nitrogen (NH_3-N), phosphate (PO_4), sulfate (SO_4), iron (Fe), chloride (Cl^-), lipids and oils, nitrates (NO_3), nitrites (NO_2), and aluminum (Al) (Appendix 1—Supplementary Material).

Two sampling stations can be considered references (areas without an evident mining impact), one located in La Elvira stream (05°03′10.9″N, 75°24′33.6″W) E1 and the other in Romerales stream

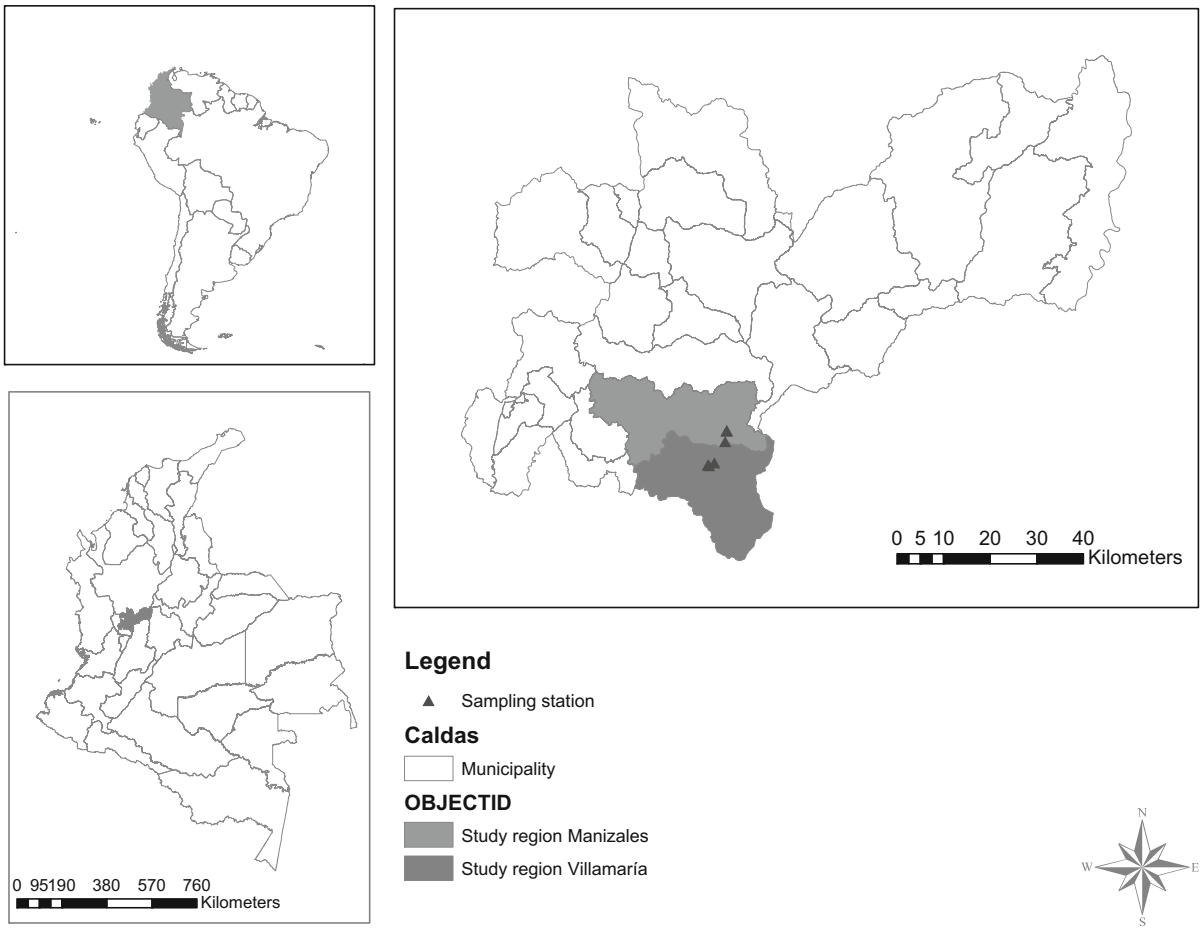


Fig. 1 Sampling stations in the Chinchiná River Basin, municipalities of Manizales and Villamaría in the department of Caldas, Colombia. (Adapted and modified from ESRI, 2014)

(04°59′22″N, 75°25′58″W) E4. The other four sampling stations (E2, E3, E5, E6) are impacted by waste disposal generated by gold mining, according to Jiménez-Pérez et al. (2014) (Table 1). Due to the lack of normal distribution of the data, we compared the median of the physical, hydrobiological, and chemical variables between the reference and mining stations using the Wilcoxon two-sample test (*W*) (Zar, 1999), implemented in R version 2.15.3 (R Development Core Team, 2013).

Specimen sampling

Nymphs were collected from water using a Surber net (30.5 cm × 30.5 cm; 250 µm mesh size) and manual strainers, as well as from sediment, rocks, and fallen leaves samples. Specimens were labeled (with date, location, and coordinates), preserved in absolute ethanol, and deposited in the Entomological Collection of the Biology Program of the Universidad de Caldas (CEBUC for its initials in Spanish) in Manizales, Caldas-Colombia. This collection is registered in the Alexander von Humboldt Institute as collection number 188.

Morphological examination

Nymph morphology, sex, and aquatic stage determination was conducted using the keys and descriptions available for head, antennae, tarsal claws, labium palp, lingua, and eyes for *A. peruvianus* (Domínguez et al., 2006; Wilson & Kennedy, 2012; Gutiérrez & Dias, 2015). Specimens were examined under a Leica M205C stereomicroscope equipped with a MC170HD

Table 1 Nymphs of *Andesiops peruvianus* sensu lato (s. l.) in each sampling station

Sampling station	*A. peruvianus* s. l.								Total
	Group 1		Group 2		Group 3		Group 4		
	Male	Female	Male	Female	Male	Female	Male	Female	
E1	6	5	5 (E67)[a]	6 (E7)[a]	5	7	8	4	46
E2	2	1	–	4	3	2	1 (E63)[a]	–	13
E3	1	–	1	–	–	1	–	1 (E12)[a]	4
E4	8 (E86)[a]	6	7 (E13)[a]	8	9 (E28)[a]	6	6	4	54
E5	9	6 (E90)[a]	4	3	2 (E31)[a]	4	2	1	31
E6	2 (E37)[a]	–	2	2	–	1	–	1	8
Total	28	18	19	23	19	21	17	11	156

E1, E2 and *E3* La Elvira streams, *E4* Romerales stream, *E5* California stream, *E6* Toldafría stream

[a] Code of sequenced specimens deposited in GenBank

digital camera. Taxonomically important structures for the species were examined (such as labrum, dorsal surface, left and right mandible, posterior margin of terga, maxillae and maxillae palpi, dented plates distributed on tarsi, tibiae, and femora), in addition to mouthparts, tarsal claws, and abdominal gills, which were analyzed in search of genetic diversity within *A. peruvianus*. Euparal mountings were done, according to the procedures described by Waltz & McCafferty (1987), and were examined under a Leica DM500 microscope.

In addition, we observed 20 individuals with the help of scanning electron microscopy (SEM). For this, nymphs were subjected to five-min washes in increasing concentrations of acetone (50, 75, 90, 95, and 100%); the last concentration was repeated twice. The material was brought to critical level, mounted on stubs, and metalized with gold. Subsequently, the material was analyzed and photo-documented on a Hitachi TM3000 scanning electron microscope at the *Laboratorio de Microscopia Eletrônica do Departamento de Biologia do Instituto de Biociências da UNESP de Rio Claro* (Sao Paulo, Brazil).

Molecular evaluation

After the morphological identification, a total of 10 specimens from the six collection sites (six males and four females), previously identified based on shape and size of the abdominal gills and number of denticles of the tarsal claws, were individually processed for molecular analyses at the Molecular Biology Laboratory of the Department of Biological Sciences of Universidad de Caldas (Manizales, Caldas-Colombia). Tissue samples were obtained from the thorax and abdomen (the remaining body structures were preserved and mounted). Genomic DNA was extracted with the DNeasy Blood and Tissue Kit (QIAGEN®), following the standard protocol indicated by the manufacturer. DNA quality and quantity were measured using a NanoVue™ Plus spectrophotometer.

PCR amplification was performed using universal primers flanking the two target mtDNA genes widely employed in aquatic insects, including Ephemeroptera (Alexander et al., 2009; Pereira-da-Conceicoa et al., 2012; Rutschmann et al., 2014). The 16S rRNA gene was amplified using the primer pair 16Sa (F) 5′-GCCTGTTTATCAAAAACAT-3′ and 16Sb (R) 5′-CTCCGGTTTGAACTCAGATCA-3′ (Ogden & Whiting, 2005). Primers LCO1490 (F) 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198 (R) 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ were used to amplify a fragment of the COI gene (Folmer et al., 1994). The final amplification reaction volume was 40 µl, which contained 21.4 µl ultrapure water, 8 µl 5× buffer, 2.4 µl $MgCl_2$ (25 mM), 3.2 µl dNTP mix (10 mM), 0.8 µl of each primer (25 µM), 2 U of GoTaq® Flexi DNA Polymerase (Promega), and 3 µl DNA (approximately 100–130 ng of DNA). The amplifications were performed on a Techne TC-PLUS thermocycler, according to the following

conditions: initial denaturation at 94°C for 2 min, followed by 7 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 45 s, followed by 28 cycles at 94°C for 30 s, 48°C for 30 s, and 72°C for 45 s, completing the reaction with a final extension cycle at 72°C for 7 min, for the 16S rRNA gene. Initial denaturation at 95°C for 5 min, followed by 5 cycles at 94°C for 5 min, 46°C for 1 min30 s, and 72°C for 1 min 30 s, followed by 35 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min, completing the reaction with a final extension cycle at 72°C for 5 min, for the COI gene. All PCR products were visualized by horizontal electrophoresis on 1% agarose gels with 1× TBE pH 8.0 running buffer at 110 v/50 mA. Gels were stained with ethidium bromide and visualized on a GelDoc-It®2 310 Imager (UVP). PCR products were purified using the QIAquick PCR purification kit (QIAGEN®), according the manufacturer's instructions, and sequenced at Macrogen Advancing Through Genomics—South Korea.

Sequences obtained were evaluated and edited with the programs Geneious Trial v8.14 (Drummond et al., 2009) and Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). To further compare sequence divergence, we downloaded sequences of closely related taxa available in GenBank and BOLD databases. Specifically, we obtained COI gene sequences corresponding to *Andesiops peruvianus* (accessions KU710334–KU710343) (Finn et al., 2016) and *Andesiops torrens* (accessions GU175993–GU176002) (Sabando et al., 2011); while, as outgroup, we used *Baetis* sp. (Ephemeroptera: Leptohyphidae) (accession KR134666) (Múrria et al., 2015). There were no 16S rRNA gene sequences for *Andesiops* available in the public databases. The COI gene sequences were aligned using ClustalW (Thompson et al., 1997), included in the program MEGA version 7 (Tamura et al., 2013), and the 16S sequences were aligned using MAFFT (Multiple Alignment using Fast Fourier Transform), with Q-ins-i settings (Katoh et al., 2002) (http://mafft.cbrc.jp/alignment/server/).

Different taxonomic units were identified using two different approaches. First, we used Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012), using an intraspecific prior divergence ranging from 0.001 to 0.1 and the K2P evolution model. Second, we used an evolutionary approach based on relative branch lengths. The Poisson Tree Processes (PTP) model identifies putative species boundaries on a given phylogenetic input tree (Zhan et al., 2013). The input tree was obtained by an updated version of the original maximum likelihood PTP (maximum likelihood PTP search result is part of the bPTP results), which adds Bayesian support (BS) values to species delimited on the input tree. A higher BS value on a node indicates that all descendants from this node are more likely to belong to a single species. Intraspecific nucleotide divergences between individuals and taxonomic units were estimated using the Kimura 2-Parameter distance model (K2P; Kimura, 1980), with the program MEGA. Species confirmation was carried out through a similarity analysis based on Maximum Likelihood (ML), with the K2P model and 1,000 bootstrap replications, using the program MEGA.

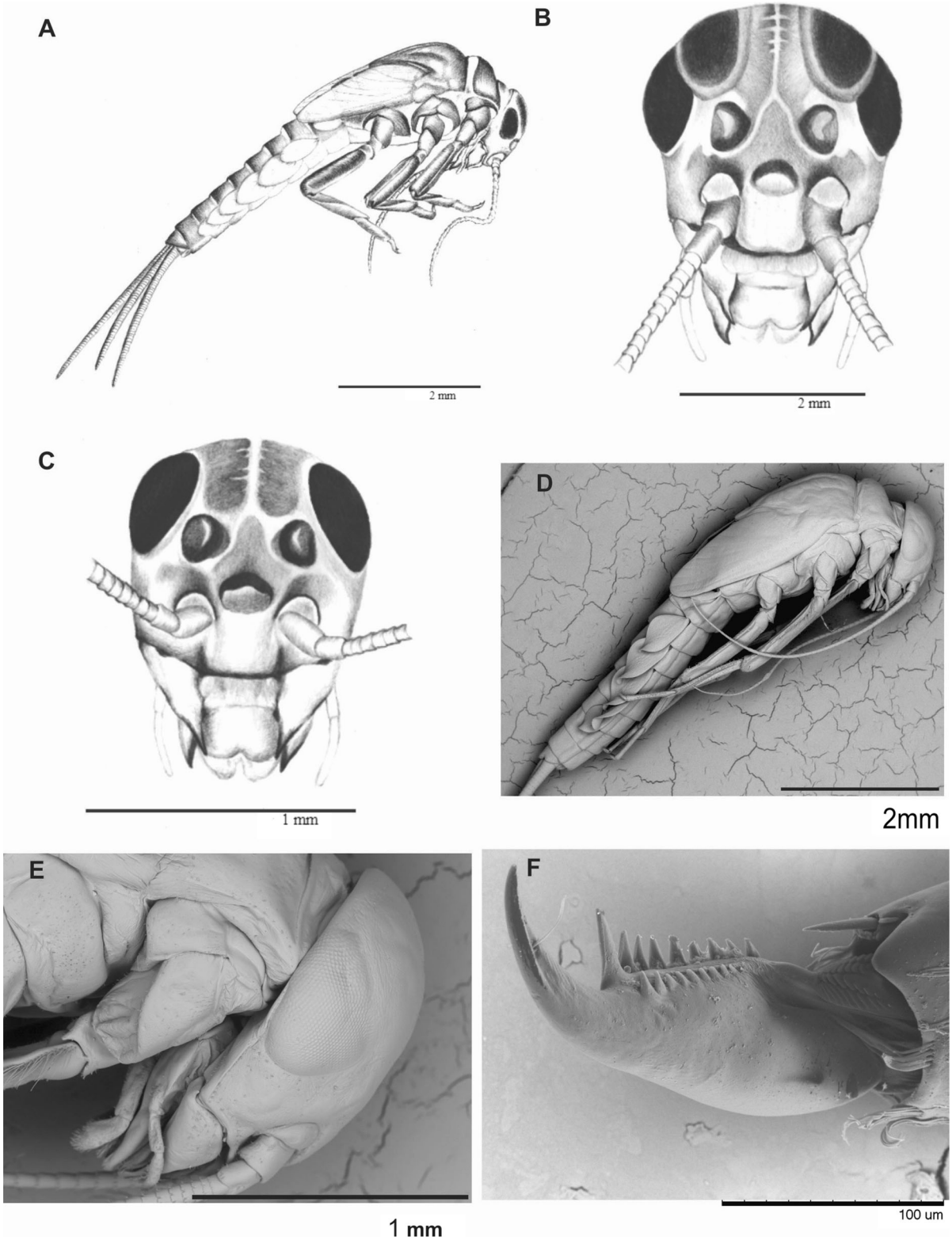
Results

Morphological data

A total of 156 nymphs in larval stage V were collected (Table 1). Specimens from both sexes possessed a head longer than broad, antennae three times longer than the head capsule (Fig. 2A–E); tarsal claws with an apical pair of fine setae and two rows of denticles, the first row well developed, and the second row reduced in size or in number of denticles (Figs. 2F, 3A); segment II of the labium palp with a small distomedial projection (Fig. 3C); lingua apically with a sharp-pointed projection (Fig. 3H). Finally, 73 females (Fig. 2B) and 83 males (Fig. 2C) were sexed, based on the presence of turbinate eyes in males.

The following structures did not show variation across sampling stations: labrum longer than wide with anteromedial cleft and a small central lobe, dorsal surface with two kinds of apically bipectinate marginal setae: basally bifid near the midline of the labrum and apically bifid near the lateral setae (Figs. 3B, 4B); left mandible, prostheca robust, apically denticulate (Fig. 3F); right mandible with incisors cleft apically, prostheca bifid (Fig. 3G); posterior margin of terga serrate (Fig. 4A).

In contrast to the characters previously reported for the species, the following two new features were recorded (with no differences observed among the *A. peruvianus* individuals examined): (1) top edge of the maxillae forked and setae randomly covering the short



◀**Fig. 2** Nymph, determined as *A. peruvianus*: **A** Male (illustration); **B** Male head (illustration); **C** Female head (illustration); **D** Male; **E** Male head; **F** Tarsal claw with apical setae and two rows of denticles (Scanning Electron Microscopy—SEM)

maxillae palpi (Figs. 3D–E, 4C, D); (2) dented plates distributed on tarsi, tibiae, and femora, alternated with short setae. In addition, variations in the number of denticles and abdominal gills revealed four groups:

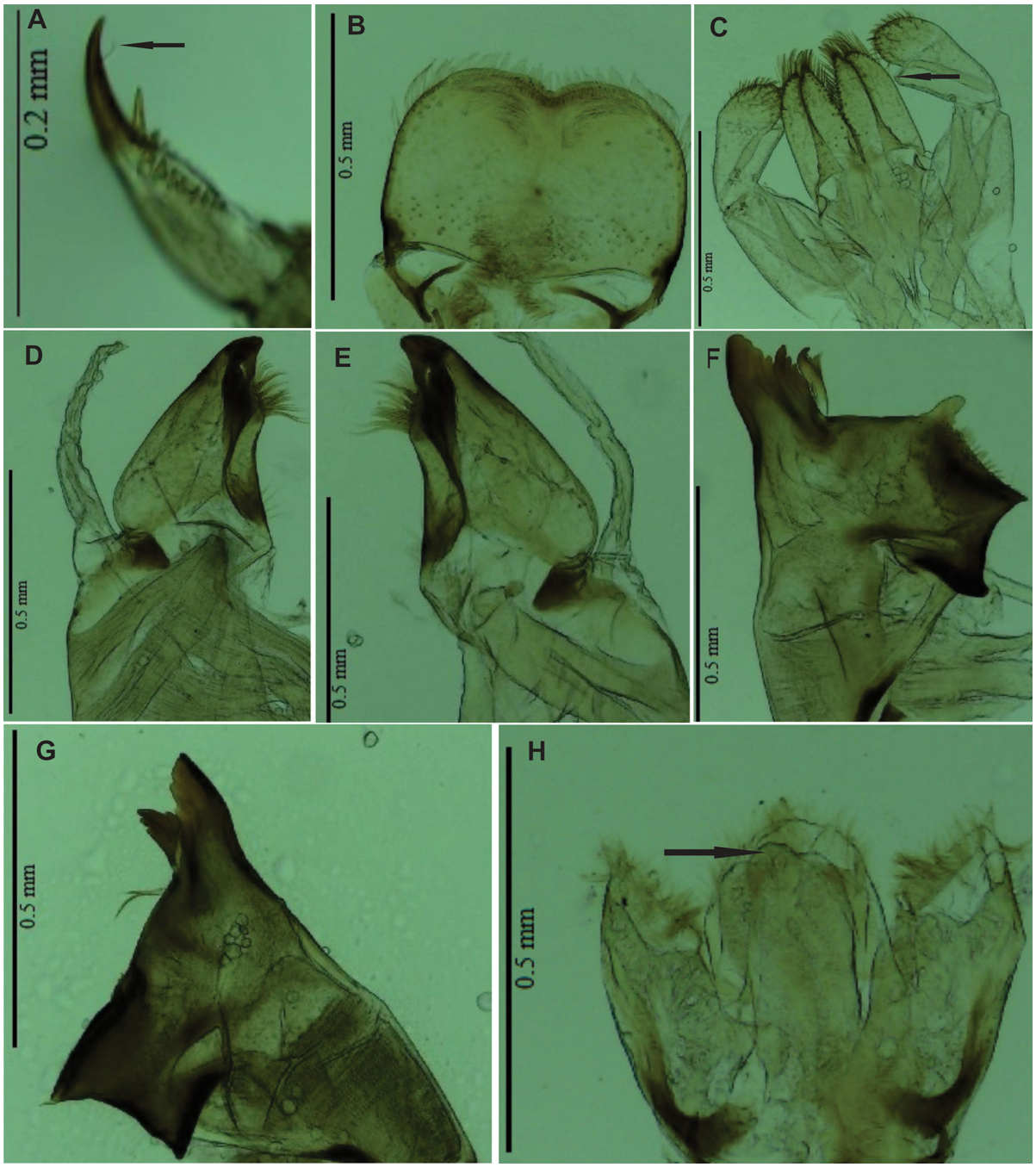


Fig. 3 Nymph, characterized as *A. peruvianus*: **A** Tarsal claw with two rows of denticles, the *arrow* indicates apical setae; **B** Labrum in ventral view (v.v.); **C** Labium v.v.; **D** Left maxilla v.v.; **E** Right maxilla v.v.; **F** Left mandible v.v.; **G** Right mandible v.v.; **H** Hypopharynx in dorsal view (d.v.), the *arrow* indicates the projection of the lingua (Light microscopy)

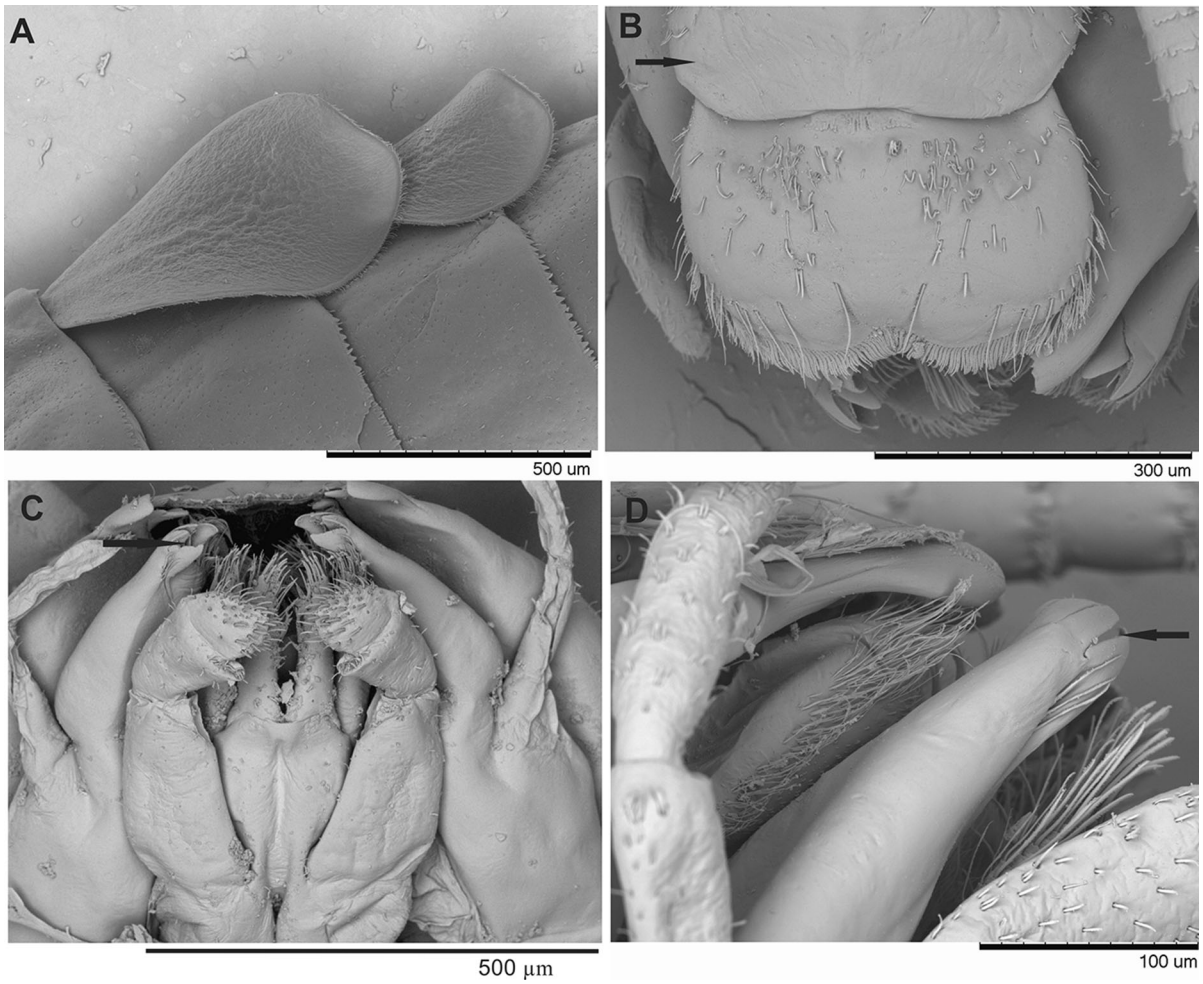


Fig. 4 Nymphs, characterized as *A. peruvianus*: **A** Serrated margin in the posterior margin of the tergum and microsetae in the margin of the abdominal gills; **B** Labrum in dorsal view (d.v), the arrow indicates the clypeus; **C**, **D** Labium, the *arrow points* to the forked termination of the maxilla, labium palpi with short setae (Scanning Electron Microscopy—SEM)

Group 1: Denticles 8/7–9 and with broadly tracheated gills; Group 2: Denticles 9/7–9 and with broadly tracheated gills (less marked than for Group 1); Group 3: Denticles 9/8–10 and very soft tracheated gills; and Group 4: Denticles 10–11/8–9 and without tracheae (Fig. 6; Table 1). There was no association between the sampling stations and the four groups of *A. peruvianus* (Table 1).

For the physical, hydrobiological and chemical analyses, significant differences were observed only for total suspended solids (TSS) ($W = 60$, $P = 0.027$), total solids (TS) ($W = 61$, $P = 0.018$), sulfate (SO_4) ($W = 63$, $P = 0.013$), and iron (Fe) ($W = 66$, $P = 0.003$), according to the non-parametric Wilcoxon test. In addition, there were differences in organism abundance of *A. peruvianus* in relation to the reference and mining stations.

Molecular data

The sequences obtained in this study were deposited in GenBank and the Barcode of Life Data Systems (BOLD) under accession numbers KT625446–KT625464. Automatic Barcode Gap Discovery (ABGD) identified four different taxonomic units, based on the 16S rRNA sequences and using an intraspecific prior divergence between 0.001 and 0.0599 (Appendix 2A—Supplementary Material). Six taxonomic units were identified from the COI gene sequences, across an intraspecific prior

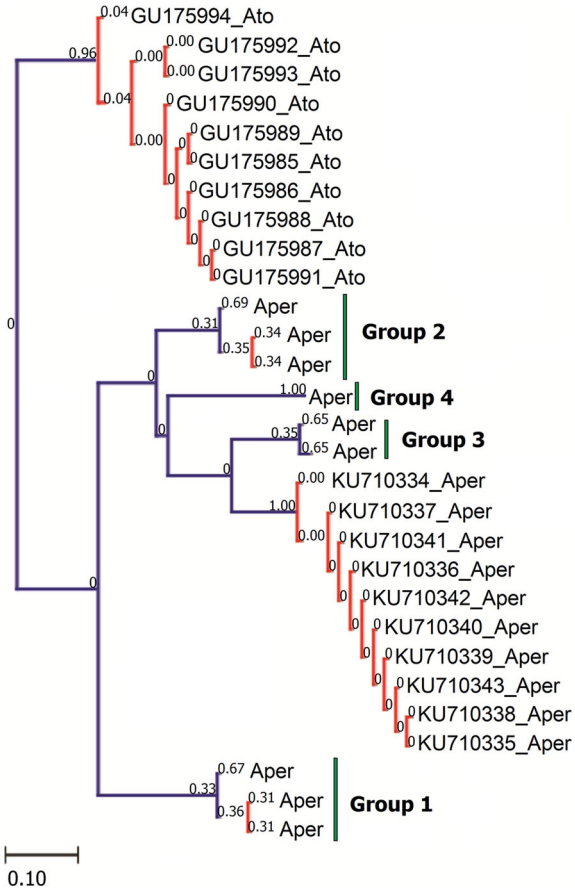


Fig. 5 Poisson Tree Processes (PTP) model to infer putative species boundaries on a given phylogenetic input tree mtDNA COI gene. Branches are the Maximum likelihood partition support values from the Poisson Tree Processes (PTP) model given for each species identified by the model

divergence range between 0.001 and 0.1 (Appendix 2B—Supplementary Material). These taxonomic units corresponded to sequences of *A. torrens* and *A. peruvianus,* and four taxonomic units corresponding to the specimens from the Chinchiná River Basin (Caldas-Colombia), sequenced in the present study. The same taxonomic units were also recovered by the PTP model (Fig. 5).

Based on the intraspecific genetic distance of the molecular taxonomic units, *A. peruvianus* showed average distances between 0.0 and 0.0–0.9% for the 16S and the COI fragments, respectively (Tables 2, 3), while genetic divergence values between the taxonomic units of *A. peruvianus* varied between 7.3–12.4 and 17.4–24.5% for 16S and COI, respectively (Table 2, 3).

In addition, the Maximum Likelihood consensus trees obtained for the two genes clearly show a series of taxonomic units with low internal genetic divergences for the sequences of *A. peruvianus*. Four well-supported molecular groups are formed for *A. peruvianus* from the department of Caldas, based on the mtDNA 16S rDNA gene (Fig. 6), and five well-supported molecular groups are formed for *A. peruvianus* from the mtDNA COI gene, including the sequences deposited in GenBank (Fig. 5).

Discussion

We analyzed 156 nymphs in larval stage V, collected from six stations located in the Chinchiná River Basin, that showed characteristics registered by Domínguez et al. (2006), Wilson & Kennedy (2012), Gutiérrez & Dias (2015) for *A. peruvianus*: head longer than broad, antennae three times longer than the head capsule (Fig. 2A–E); tarsal claws with an apical pair of fine setae and two rows of denticles, the first row well developed, and the second row reduced in size or in number of denticles (Figs. 2F, 3A); segment II of the labium palp with a small distomedial projection (Fig. 3C); lingua apically with a sharp-pointed projection (Fig. 3H), and the presence of turbinate eyes in males.

Nevertheless, we found that these morphological characteristics encompass organisms belonging to the *A. peruvianus* complex, based on the presence of four morphological groups, showing clear distinctions in shape and size of the abdominal gills and number of denticles of the tarsal claws (Table 1; Fig. 6). Variation in the number of denticles on the tarsal claws can be due to their function in substrate adhesion (Ditsche-Kuru et al., 2012), and the variation observed in tracheae branching relates to their importance in respiration, osmoregulation, and locomotion (Pruthi, 1927; Morgan & Grierson, 1932; Wingfield, 1939; Wichard et al., 1972; Notestine, 1994; Zhou, 2010).

The analyses show that these sampling stations have some type of anthropic impact, as evidenced by the physical, hydrobiological, and chemical analyses, where significant differences were observed only for total suspended solids (TSS) ($W = 60$, $P = 0.027$), total solids (TS) ($W = 61$, $P = 0.018$), sulfate (SO_4) ($W = 63$, $P = 0.013$), and iron (Fe) ($W = 66$, $P = 0.003$), according to the non-parametric

Table 2 Average intraspecific (on the diagonal) and interspecific (below the diagonal) distances based on Kimura two parameters for the mtDNA 16S gene

Species	*A. peruvianus* (1)	*A. peruvianus* (2)	*A. peruvianus* (3)	*A. peruvianus* (4)
Andesiops peruvianus (1)	0.000[a]			
Andesiops peruvianus (2)	0.096[a]	0.000[a]		
Andesiops peruvianus (3)	0.073[a]	0.124[a]	0.000[a]	
Andesiops peruvianus (4)	0.082[a]	0.090[a]	0.094[a]	0.000[a]

Group 1: Denticles 8/7–9 and with broadly tracheated gills; Group 2: Denticles 9/7–9 and with broadly tracheated gills (less marked than for Group 1); Group 3: Denticles 9/8–10 and very soft tracheated gills and Group 4: Denticles 10–11/8–9 and without tracheae

[a] There is no variability and minimum, average, and maximum distances

Table 3 Average (minimum and maximum) intraspecific (on the diagonal) and interspecific (below the diagonal) distances based on Kimura two parameters for the mtDNA COI gene

Species	*A. p* (1)	*A. p* (2)	*A. p* (3)	*A. p* (4)	*A. peruvianus* (Finn)	*A. torrens*	*Baetis* sp.
A. peruvianus (1)	0.000 (–)						
A. peruvianus (2)	0.205 (–)	0.000 (–)					
A. peruvianus (3)	0.202 (0.199–0.205)	0.199 (–)	0.009 (–)				
A. peruvianus (4)	0.245 (–)	0.214 (–)	0.200 (0.198–0.202)	–			
A. peruvianus (Finn)	0.229 (0.226–0.232)	0.178 (0.175–0.180)	0.174 (0.165–0.183)	0.213 (0.209–0.216)	0.004 (0.00–0.009)		
A. torrens	0.233 (0.221–0.253)	0.238 (0.226–0.262)	0.260 (0.247–0.280)	0.265 (0.254–0.288)	0.267 (0.250–0.294)	0.016 (0.004–0.033)	
Baetis sp.	0.251 (–)	0.242 (–)	0.216 (–)	0.258 (–)	0.250 (0.249–0.252)	0.272 (0.262–0.293)	–

Group 1: Denticles 8/7–9 and with broadly tracheated gills; Group 2: Denticles 9/7–9 and with broadly tracheated gills (less marked than for Group 1); Group 3: Denticles 9/8–10 and very soft tracheated gills and Group 4: Denticles 10–11/8–9 and without tracheae

Wilcoxon test. A great amount of total and suspended solids were found, with a high content of sulfates and metals such as Fe, which are characteristic of mining disposals and can have negative effects on exposed organisms (Aduvire, 2006).

The molecular results further indicate a deep genetic structuring within *A. peruvianus*, showing that this species indeed constitutes a species complex, supporting the morphological results found (Table 1; Fig. 6). Four different taxonomic units were identified, using two different approaches: Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012) for both 16S and COI, and PTP Zhan et al. (2013) for COI (Fig. 5). Accordingly, four putative species were inferred for *A. peruvianus* in the Chinchiná River Basin (Caldas-Colombia) (Appendix 2B—Supplementary Material), showing genetic distances between 0 and 24.5% for the COI gene, which represent higher than expected genetic distances for conspecific individuals, in comparison to the intraspecific genetic distances obtained for *A. torrens* (Table 3). Based on genetic divergences greater than 19% reported for *B. rhodani* haplogroups (Williams et al., 2006), Buckley et al. (2001), Gattolliat et al. (2015) suggested that such high divergences correspond to cryptic species. Avise (2000), Ball et al. (2009), Zhou et al. (2009) reported a 2% divergence criterion, exceeded only rarely by members of the same species and which is historically congruent with the morphological identification of aquatic insects.

Pereira-da-Conceicoa et al. (2012) suggest that *Baetis harrisoni* (Ephemeroptera), as is currently recognized, is not a single species with a wide geographic range and pH tolerance, but rather may encompass up to five species under the phylogenetic species concept, each with limited pH-tolerances;

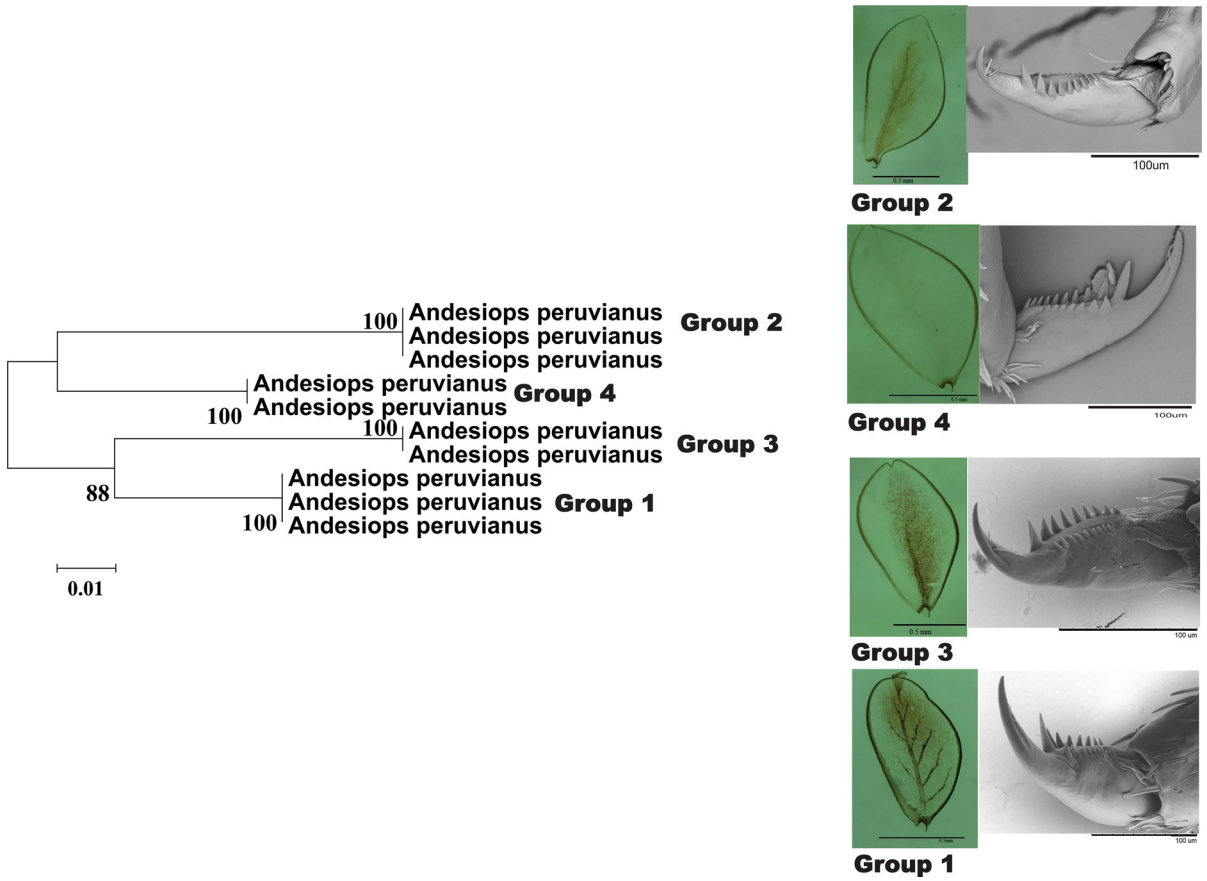


Fig. 6 ML consensus tree with *A. peruvianus* samples, based on mtDNA 16S gene sequence distances. Bootstrap values are only indicated for nodes with support greater than 70%; Abdominal gills and variation in number of denticles of the tarsal claws of *A. peruvianus* nymphs revealed four groups: Group 1: Denticles 8/7–9 and with broadly tracheated gills; Group 2: Denticles 9/7–9 and with broadly tracheated gills (less marked than for Group 1); Group 3: Denticles 9/8–10 and very soft tracheated gills and Group 4: Denticles 10–11/8–9 and without tracheae. ML bootstrap values are shown at the nodes

thus, the *B. harrisoni* species group is in need of taxonomic review. Further, Macher et al. (2016) studied the mayfly *Deleatidium*, in search of cryptic species using the COI gene, finding that *Deleatidium* consisted of 12 molecularly distinct clades that likely represent.

These previous findings support that the high genetic divergences obtained in *A. peruvianus* could correspond to confamiliar organisms belonging to a pseudocryptic species complex, as seems to occur with *B. rhodani* (Williams et al., 2006). Nevertheless, there was no association between the sampling stations and the four groups of *A. peruvianus* in the Chinchiná River Basin (Caldas-Colombia). COI gene studies in *B. rhodani* show that the species has at least 13 morphologically cryptic haplogroups, with local coexistence of cryptic species and their ability to adapt to different temperatures and food resources, justifying some of the differences observed in the relationship between water temperature, growth rates, and phenology documented from field studies (Lucentini et al., 2011; Rutschmann et al. 2014).

Tropical fauna is relatively unknown, therefore, comparing species richness, cryptic diversity, and altitudinal ranges of mayflies (Ephemeroptera) is crucial for understanding global biodiversity patterns (Bickford et al., 2007; Monaghan et al., 2009; Gill et al., 2014, 2016; Finn et al., 2016). Gill et al. (2016) provided evidence suggesting that the high species richness of tropical mayflies may be due not only to allopatric speciation, but also to parapatric speciation along single altitudinal gradients, because limited

thermal tolerance restricts the dispersal ability of these species.

This research highlights the value of performing a detailed taxonomic classification of organisms with potential use as bioindicators of good water quality, using complementary tools such as morphology and molecular biology. The molecular data show the presence of four different taxonomic units supported by morphological characters, where the members of each group are not associated to a given study station. This agrees with reports by Finn et al. (2016); Gill et al. (2016), and with the presence of multiple cryptic o pseudocrytic species within the ephemeropteran genus *Baetis* (e.g., *Baetis rhodani* and *Baetis alpinus*) (Williams et al., 2006; Finn et al., 2014; Múrria et al., 2014). Nevertheless, it is necessary to continue exploring new tools and diagnostic characters, such as associations between nymphs and adults, reproductive mechanisms, mouthparts and tarsal claws in nymphs, and adult morphology such as wing venation, structures of the male genitalia with penis, forceps socket, forceps, and subgenital plate (Nieto, 2004; Domínguez et al., 2006). In addition, other molecular and biogeographic studies, as well as broader sampling efforts in the Chinchiná River basin could prove the existence of more groups within *A. peruvianus* s. l. (Pereira-da-Conceicoa et al., 2012; Rutschmann et al., 2014; Finn et al., 2016).

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CAPÍTULO 2

Taxonomy of Genus 1 sp. 2 (Diptera: Chironomidae: Orthocladiinae) and the potential use of its larvae as bioindicators

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Abstract

Chironomidae belongs to the most abundant macroinvertebrates in samples for water quality assessment and displays a wide tolerance range to contaminants, which makes it an excellent bioindicator. The species *Genus 1* sp. *2*, included among the larval keys of the Cricotopus-Oliveiriella complex, is difficult to determine based on its larval instar using the current morphological keys, which makes it necessary to use pupae for a species-level identification. In this study, 103 organisms in the IV larval instar were collected from tributaries of the high Chinchiná river basin, along with eight organisms at the pupal level (reared in the laboratory). The organisms were morphologically identified, the presence of mentum deformities was assessed, and 13 structure measurements were taken, with the aim of finding possible variations among specimens from different sampling stations. An analysis of the genes 16S and COI was performed in order to confirm and associate larvae-pupae. A total of 18 specimens showed partial or total teeth deformity and only dorsal head area showed significant differences. The results obtained allow for a molecular determination and association of larvae-pupae, and lead to proposing new morphological measurements in larvae that can aid in determining variations resulting from contaminant agents and contributing to establishing this species as a water quality bioindicator.

Keywords: deformities; molecular; morphology; morphometry.

Introduction

The subfamily Orthocladiinae (Diptera: Chironomidae) is one of the richest in genera and species. In Andean rivers above 2000 meters of altitude, the subfamily Orthocladiinae is very abundant, with multiple genera present in the high Andean region, and of which some have yet to be described [1]. Furthermore, species of the same genus share many larval characteristics, making it nearly impossible to distinguish them, even at a genus level [1]. Nevertheless, pupal forms are specific for a genus, and even for a species, and there are several records in which larvae and pupae have been associated in rivers of the high Andean region between Colombia and Peru [1].

Among the most abundant genera of Orthocladiinae in the high Andean region, there are larvae described as *Genus 1* by Roback and Coffman [2], a genus found exclusively in the Andean region. Its larvae belong to the *Cricotopus-Oliveiriella* complex

[1,3]; complicating its identification even at the genus level, and at the species-level, the differences between species have not been studied yet. Its pupae, however, are very characteristic and very different from the genus *Cricotopus*; therefore, a species-level description can be achieved. Larvae belonging to this taxon are abundantly found in the Chinchiná River (Colombia), and based on studies related to the association between macroinvertebrates and contamination, the possibility of using these larvae as contamination indicators has led to a complete morphological and genetic study in order to establish its possible use as bioindicators.

The use of aquatic macroinvertebrates currently constitutes a tool for the biological and integral characterization of water quality [4,5]. All aquatic organisms can be considered as bioindicators, however, the evolutionary adaptations to different environmental conditions and the tolerance limits to a given disturbance are responsible for the characteristics that classify them as sensitive organisms, whether they do not endure changes in their environment or they are tolerant to stress conditions [4,6]. Chironomidae (Insecta: Diptera), with nearly 20000 species distributed throughout all the continents, from the Antarctic region to the Tropics, inhabit lakes, streams and rivers during their larval and pupal developmental stages [1,7,8].

The family Chironomidae is considered to be tolerant to water contamination with organic matter, heavy metals, pesticides, aromatic polycyclic hydrocarbons, and organic solvents, displaying subletal responses such as morphological variations represented by morphometric changes and deformities as a result of exposure to these conditions [4,9,10-16]. Regarding these tolerance characteristics, Warwick [10], Alba-Tercedor [17], Servia et al. [18,19], Giacometti and Bersosa [6], Arambourou et al. [16] report Chironomidae as organisms with a potential use in water quality bioindication. Nevertheless, one of the current limitations for the use of Chironomidae is an insufficient knowledge of their taxonomy, which in many cases is not straightforward, due to phenotypic plasticity or shared characters between several species of a genus, or even between genera [1,6,20].

The present study aimed to determine morphologically the larvae of *Genus 1* sp. *2* Roback and Coffman [2], through the diagnostic characters, as well as to record the frequency of deformities in the sampling stations (reference and mining) by assessing possible morphometric variations in organisms of the IV larval instar, associated to the

sampling zones. Further, larvae and pupae of *Genus 1* sp. *2* were molecularly determined and associated based on the study of mitochondrial genes. Overall, the results support a clearer contributing to the establishment of this species as a water quality bioindicator.

Materials and Methods

Study area

The study area included six sampling stations located in the Chinchiná River basin in the department of Caldas (Colombia), which were set up as part of the project: "Assessment of the impact of mining, agriculture and livestock farming through ecological and genetic responses of aquatic macroinvertebrates". Two sampling stations were selected as reference (areas without an evident mining impact), one located in La Elvira stream (05°03'10.9" North, 75°24'33.6" West), municipality of Manizales, and the other in Romerales stream (04°59'22" North, 75°25'58" West), municipality of Villamaría. The other four sampling stations are impacted by waste disposal generated by gold mining [21,22]. Two of the stations were located in El Elvira stream, Manizales (05°03'4.4" North, 75°24'33.1" West; 5°1'53" North, 75°24'43.8" West), another was located in California stream, Villamaría (04°59'5" North, 75°26'35" West), and the last sampling station was in Toldafría stream, Villamaría (4°59'08" North, 75°26'43" West) (Fig. 1). The six sampling stations stood between 2275 and 2766 meters of altitude, and had similar physical habitat characteristics, such as a wavy topography, the presence of riparian vegetation, and an open canopy cover (0-25% of the stream was shaded).

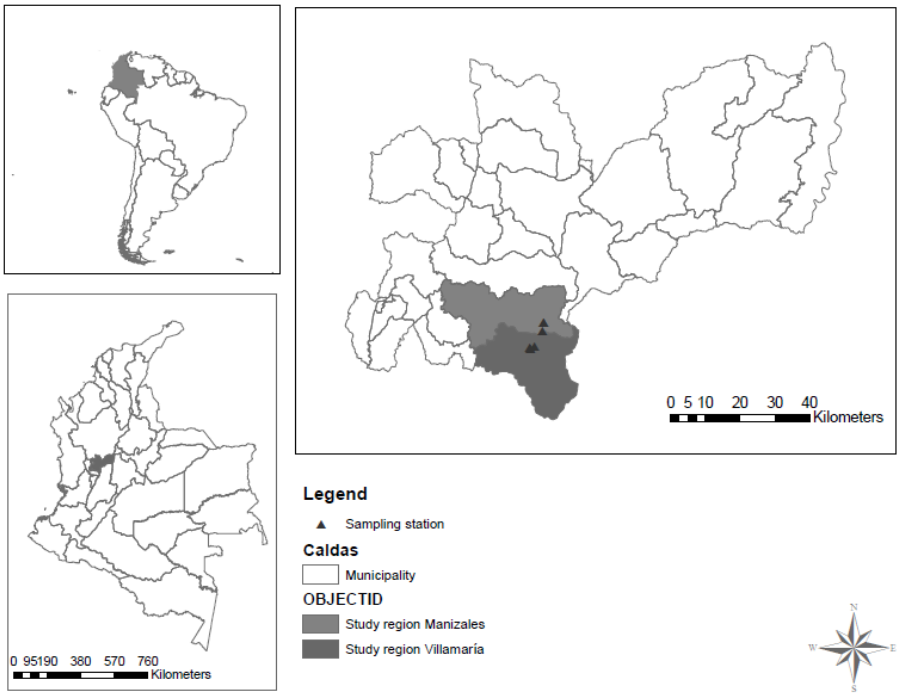

Figure 1. Sampling stations in the Chinchiná River basin, municipalities of Manizales and Villamaría in the department of Caldas – Colombia. Adapted and modified from ESRI [23].

Specimen collection

A total of six sampling events were conducted from February 2014 to February 2015. Larvae collection was carried out with a Surber net, with 30.5 x 30.5 x 8 cm dimensions and a mesh size of 250 µm, and manual drainers (the samples were taken from sediments, rock washes and leaf litter). The specimens were preserved in absolute ethanol with their corresponding information (date, location, and coordinates). In the laboratory, several specimens were conditioned in aquariums with water from the sampling stations, under constant oxygenation, and were fed with TetraMin® until pupae were obtained for species confirmation.

Additionally, the following physical and hydrobiological variables were measured in situ in three of the six sampling events: water temperature, pH, conductivity, dissolved oxygen, oxygen saturation, average depth, width, and water flow velocity. Also, the following chemical variables were evaluated in the laboratory: chemical oxygen demand (COD), biological oxygen demand (BOD5), total coliforms, fecal coliforms, total

suspended solids (TSS), total solids (TS), cyanide (CN), boron (B), lead (Pb), mercury (Hg), ammoniacal nitrogen (NH3-N), phosphate (PO4), sulfate (SO4), iron (Fe), chloride (Cl-), lipids and oils, nitrates (NO3), nitrites (NO2), and aluminum (Al). These variables were analyzed by ACUATEST S.A. (Table S1). Comparisons of the physical, hydrobiological, and chemical variables between the reference and mining stations were performed using the Wilcoxon test (*W*).

Morphological evaluation

The ethanol-preserved organisms were examined and identified based on the keys of Prat et al. [1,3], using a Leica M205C stereomicroscope equipped with a MC170HD digital camera. Next, the head of each specimen, both the larvae and pupae reared in the laboratory, were dissected and placed in hot 10% KOH. They were then washed, dehydrated, and mounted on microscope slides with Euparal® for their subsequent observation, following the light microscopy (LM) techniques described by Epler [24], and the head capsule and pupal keys of Prat et al. [1,25].

In addition, several structures were re-assessed using an electron scanning microscope (ESM). For this, the heads were mounted on stubs and metalized in gold, then; the material was analyzed and photo-documented on a FEI QUANTA 250, ESEM electron scanning microscope at the Laboratory of the Institute of Stratigraphy Research –IIES of the Universidad de Caldas in Manizales, Caldas (Colombia). Both processes, the head capsule mounts with ML, as well as the ESM observations, were analyzed in order to evaluate the possible existence of mouth deformities according to the descriptions of Warwick [9] and Groenendijk et al. [26]. The association between deformity occurrence and the reference or mining stations was examined through Fisher's Exact Test.

Larval morphometric analysis

In order to find possible variations between specimens of the reference and mining stations, 13 structure measurements were recorded (mm or mm^2 for areas), reported by Cranston and Krosch [27], Prat et al. [28] for larvae of the genus *Barbadocladius* (Diptera: Chironomidae) and other genera explored in this study, which were: lateral head length (LH), lateral head width (LHW), lateral head length from the base (LHB), thorax length (TL), width of III thorax segment (WTS), width of IV abdominal segment (WAS), total

body length (TB), body area (BA), body perimeter (BP), dorsal head length (DHL), dorsal head width (DHW), dorsal head area (DHAr), and dorsal head perimeter (DHP). The body measurements were compared between reference and mining sites, through the non-parametric Wilcoxon test (*W*). Statistical analyses were performed using R version 3.1.1 [29].

Molecular evaluation

The molecular evaluation was carried out in the Laboratory of Molecular Biology of the Department of Biological Sciences of the Universidad de Caldas in Manizales (Caldas, Colombia). DNA was extracted from the thorax and abdomen of 11 larvae, as well as two mature pupae, using the DNeasy Blood and Tissue Kit (Qiagen®), according to the manufacturer's instructions. The quality and quantity of the DNA was assessed by a NanoVueTM Plus spectrophotometer. PCR amplification was performed with primers flanking two mtDNA genes, which have been widely used to determine the systematics of aquatic insect groups [20,30]. The 16S rRNA gene was amplified using the primer pair 16Sa (F) 5'-GCCTGTTTATCAAAAACAT-3´ and 16Sb (R) 5'-CTCCGGTTTGAACTCAGATCA-3´ [30]. Primers LCO1490 (F) 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 (R) 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' were used to amplify a fragment of the COI gene [31]. The PCR products were purified with the QIAquick PCR purification kit (Qiagen®), according to the manufacturer's instruction, and were shipped for sequencing at Macrogen Inc. Korea. The sequenced fragments were evaluated and edited using Geneious Trial v8.14 [32] and Sequencher 4.1 [33]. In addition, the sequences were search by MegaBlast against the public databases and deposited in Genbank and Barcode of Life Data Systems (BOLD) (Genbank accessions KY568875-KY568909).

There are no available sequences for species of the genus *Genus 1* in the public databases; therefore, the analysis of the mtDNA COI gene included sequences from eight species of *Cricotopus*, a genus with very similar larvae to *Genus 1* [1]. The reason for including eight species from different subgenera of *Cricotopus* was to be able to more clearly establish the position of larvae of *Genus 1* sp. *2* within the *Cricotopus-Oliveiriella*

complex, since, similarly to what happened with the genus *Oliveiriella*, it is suspected that the larvae of *Genus 1* of Roback are actually a subgenus within *Cricotopus*.

Moreover, the species *Limnophyes* sp. was included as an outgroup. For the mtDNA 16S rDNA gene analyses, sequences from a species of *Cricotopus* and the species *Cardiocladius* sp. were used as outgroups. The sequences for each gene were aligned using ClustalW [34], included in the program MEGA version 7 [35], and the alignments were visually reviewed and edited when necessary.

Intraspecific nucleotide divergences were estimated with the program MEGA, using the Kimura 2-Parameter distance model (K2P) [36]. Automatic Barcode Gap Discovery (ABGD) was used to infer the number of putative species [37], using an intraspecific divergence prior ranging from 0.001 to 0.1 and the K2P evolutionary model. Species confirmation was carried out through a similarity analysis based on Neighbor-Joining (NJ), with the K2P model and 1000 bootstrap replications, using the program MEGA.

Results and Discussion

Morphological evaluation

The morphological evaluation was based on the collection of 103 organisms of the morphotype *Genus 1* sp. *2* (Table 1), corresponding to the IV larval instar (Fig. 2 A-B), as well as eight pupae reared in the laboratory. The larvae of *Genus 1* are morphologically characterized by a white colored body in young larvae, and darker areas in the thorax in more mature larvae (Fig. 2A). However, it has been noted that color variations cause difficulties for species determination [38]. Fourth instar larvae of *Genus 1* sp. *2* show equal abdominal setae of length corresponding to half of the width of the abdominal segment, and anal tubules shorter than the posterior pseudopods (Fig. 2B). Head with no pattern, very dark solid color with lighter areas close to the lateral border in the frontal and medial sections; very dark occipital border (Fig. 2 C-D) and short antennae (Fig. 2 D-E). Mentum with second lateral tooth smaller than the first; first lateral tooth as wide as second and narrower in the lower part (Fig. 2 E-F). Mandible with upper tooth shorter and narrower than the first (Fig. 2A). All morphological characteristics agree with those reported by Prat et al. [1,3].

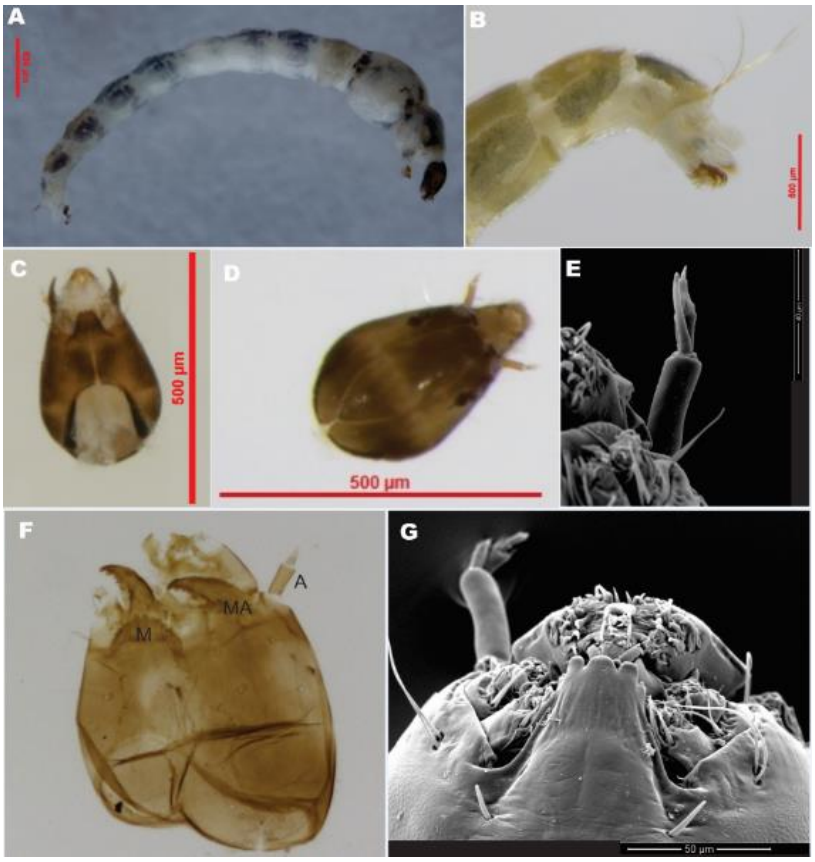

Figure 2. *Genus 1* sp. *2*. IV larval instar. (**A**) Larva in IV instar (LM)*; (**B**) Abdominal termination (LM)*; (**C**) Head cavity (ventral view) (LM)*; (**D**) Head cavity (dorsal view) (LM)*; (**E**) Antenna lateral view (ESM)*; (**F**) Head cavity. Mentum (M), Mandible (MA), Antenna (A), (LM)*; (**G**) Mentum (M) (ESM)**
* LM (Light microscopy); ** ESM (Electron scanning microscopy).

Some of the characteristics previously mentioned appear in many larval forms, including the genus *Cricotopus*, of which several subgenera and morphotypes, along with the genus *Genus 1*, are included in keys of the *Cricotopus-Oliveiriella* complex [1]. Therefore, it is almost impossible to differentiate them at the species level based on a larval instar [8]. However, this morphology is associated with very different pupal forms, which allowed us to reach a species level determination, following the key of Prat et al. [25] and the indications of the original description of pupae for *Genus 1* sp. *2* in Roback and Coffman [2]. Prat et al. [25] report that it is common to find *Genus 1* in pupae forms in high Andean rivers, where these are very characteristic and very different from *Cricotopus*. In the eight pupae evaluated, terga ornamentation showed two rows of anteriorly-oriented

spines in the II-V abdominal segments (SA) (Fig. 3A); anal lobe reduced, with a similar size to the genital sacs (Fig. 3B). Respiratory organ (OR) rounded at the end and often with diverse folds on the surface (Fig. 3 C-D) and without middle spines in the II tergite, although present in the III-IV terga (Fig. 3A).

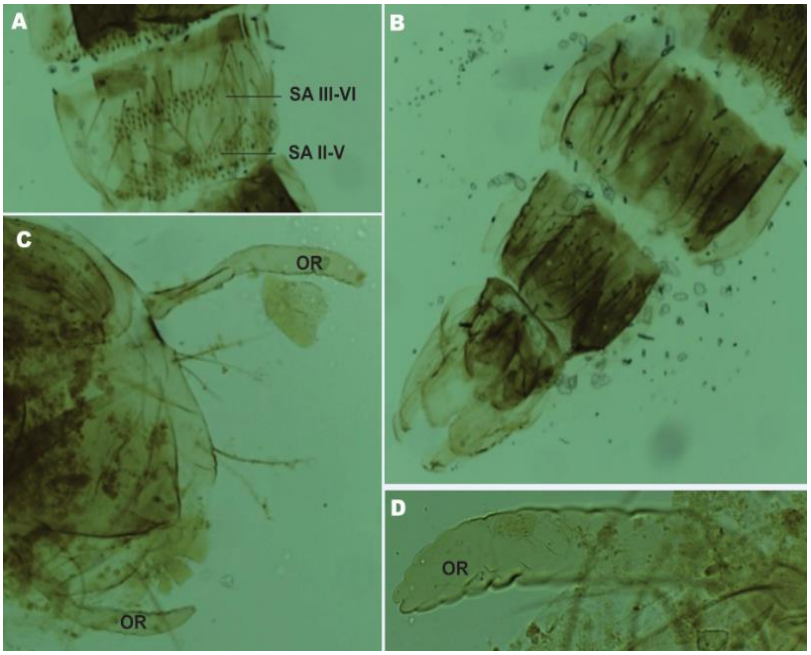

Figure 3. *Genus 1* sp. *2*; pupae. (**A**) Mount of IV abdominal segment (SA). Campo de espinas central (SA III-VI), ganchos posteriores (SA II-V); (**B**) Last abdominal segments and anal lobes; (**C-D**) Respiratory organs (OR) (Light microscopy – LM).

Mentum deformities were observed in 18 of the 103 specimens evaluated (Table 1; Fig. 4A-D). Partial wear of the teeth was evident (Fig. 4A), as well as total wear or tooth loss (Fig. 4B-D). The presence of deformities in Chironomidae larval instars is considered to result from exposure of these organisms to diverse contaminant agents (13,39,40]. Due to their tolerance, Chironomidae are considered excellent water quality bioindicators, since they have regulation mechanisms for metals such as Cu, Ni, Zn, Cd, Pb, Hg, and Mn, and for which they employ a homeostatic control for the uptake of essential and toxic metals through metallothioneins [15,16,41,42]; consequently, allowing them to survive in contaminated conditions [43].

No significant differences were found for deformity occurrence in relation to the reference and mining stations (Fisher's Exact Test, p = 0.669). Of the total organisms collected in the reference stations, 23.1% showed deformities (Table 1), indicating that these sampling stations have some type of anthropic impact, as evidenced by the physical, hydrobiological and chemical analyses, significant differences were observed only for total suspended solids (TSS) (W=60, p = 0.027), total solids (TS) (W=61, p = 0.018), sulfate (SO4) (W=63, p = 0.013) and iron (Fe) (W=66, p = 0.003) according to the non-parametric Wilcoxon test, between the reference and mining stations. A great amount of total and suspended solids were found, with a high content of sulfates and metals such as Fe, which are characteristic of mining disposals and can have negative effects on exposed organisms [44]. Nevertheless, according to Arambourou et al. [40], there is missing information regarding the study of the origin of these abnormalities. Servia et al. [13,14] and Arambourou et al. [40] mention that, to date, there are no studies that allow for discarding the possibility that this type of malformations appear spontaneously due to natural developmental defects. Further, it cannot be ignored that changes in the mentum can be due to the substrate or contamination [39,45].

In addition, there were differences in organism abundance of *Genus 1* sp. *2* in relation to the reference and mining stations (Table 1). The organisms from *Genus 1* sp. *2* showed a greater abundance in stations with mining (n=90) compared to the reference stations (n=13). Considering that genera of the order Diptera are typical of disturbed areas [5], *Genus 1* sp. *2* can be considered as having potential for water quality bioindication, due to its tolerance to environmental stress, similar to other species of the family Chironomidae [6,10,13,14,16,18,19].

Table 1. Larvae (IV instar) of *Genus 1* sp. *2* with presence or absence of deformities in each sampling station. E1: La Elvira stream (reference area), E2: La Elvira stream (mining), E3: La Elvira stream (mining), E4: Romerales stream (reference area), E5: California stream (mining), E6: Toldafría stream (mining).

Genus 1* sp. *2			
Sampling station	**With deformities**	**Without deformities**	**Total**
E1	1	4	5

E4	2	6	8
E2	6	31	37
E3	4	27	31
E5	4	12	16
E6	1	5	6
Total	18	85	**103**

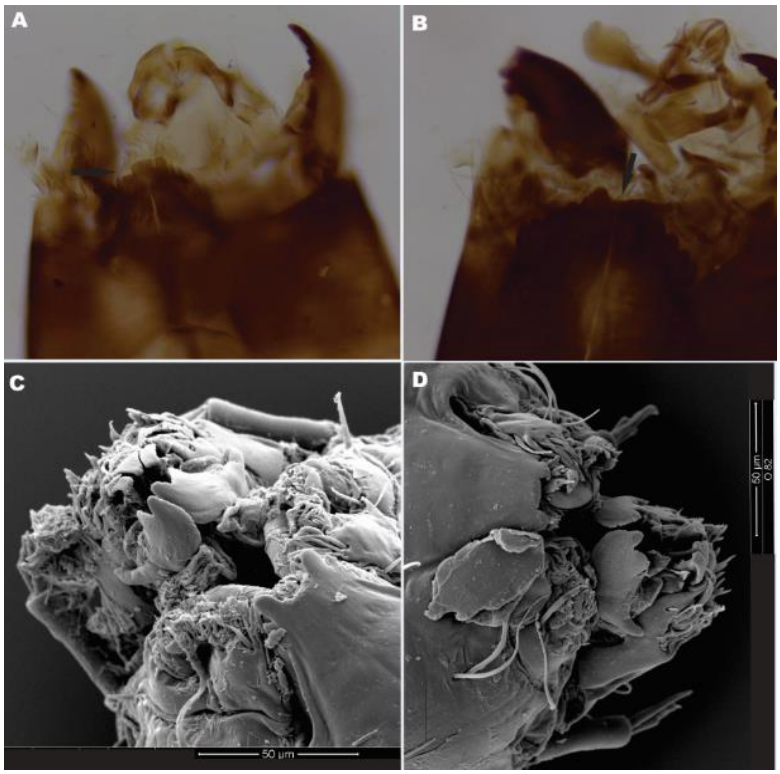

Figure 4. *Genus 1* sp. *2*. Head cavity, arrows indicate teeth deformities in the mentum. (**A**) Teeth wear; (**B-D**) Total loss of several dental pieces (Light microscopy – LM and electron scanning microscopy – ESM).

Larval morphometric analyses

Of the 13 structure measurements assessed, significant differences were observed only for dorsal head area (DHAr) between the specimens found in the reference and mining stations, according to the non-parametric Wilcoxon test (W= 382.5, p = 0,04). Larval measurements have been used to differentiate larval instars and sexual dimorphism [46,47], pupal and exuviae stages [27,28], exposure to contaminant agents by evaluating size

variations in head parts [9,13,14,18,19,26,48,49], and morphometric variations in adults in different regional gradients [50]. In this study, only dorsal head area (DHAr) is informative; therefore, more research is necessary in order to determine if the differences found in this study are due to genetic variability, stress type (essential and/or toxic substances), the structures studied or the morphometric data used, or to a combination of all of these variables [13,14,39,40].

Molecular evaluation

The molecular alignment analyses of the mtDNA COI and 16S rDNA genes, respectively, confirmed the results obtained by the morphological determination. Based on the initial partition and an intraspecific divergence prior between 0.001 and 0.1 for the COI gene and between 0.0028 and 0.0599 for the 16S gene, the ABGD species delimitation method identified that the larval and pupal sequences obtained for *Genus 1* sp. *2* belong to a single species out of the nine species identified with the COI gene and the two species identified with the 16S gene.

Additionally, the consensus trees obtained from the two genes, based on the Neighbor-Joining method, clearly show that the larval and pupal sequences of *Genus 1* sp. *2* constitute a well-supported monophyletic clade (Fig. 5-6), with a mean intraspecific divergence of 0.95%, based on the COI gene, and 0.11% on the 16S gene. These intraspecific divergence values observed for *Genus 1* sp. *2* are similar to the mean values found for the *Cricotopus* species analyzed; with 0% for *C. trifascia* and 2.31% for *C. bicinctus* with the COI gene, and 0.20% para *Cricotopus (Oliveiriella)* with the 16S gene.

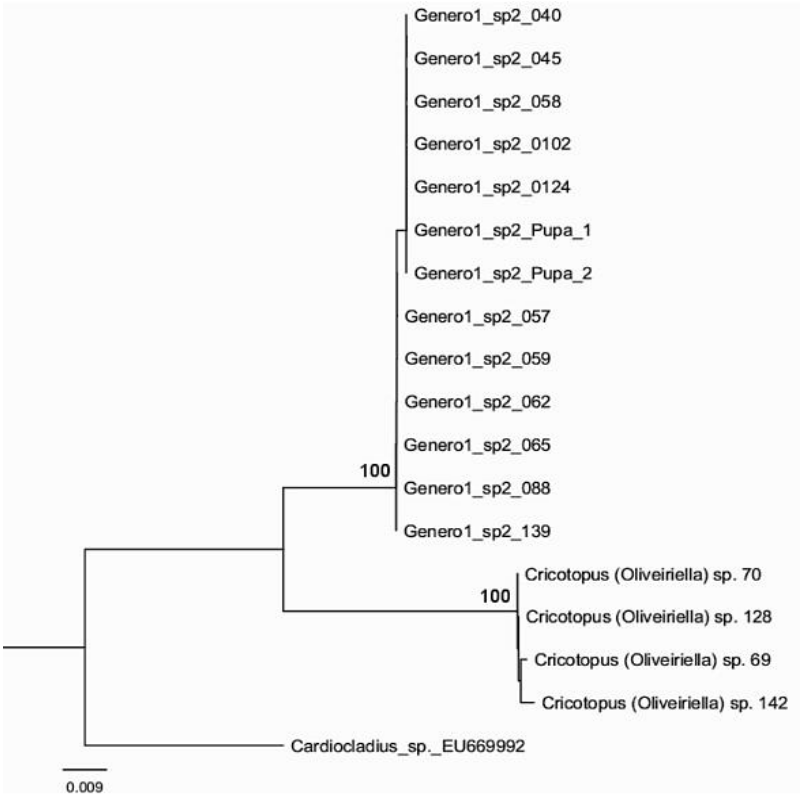

Figure 5. Consensus NJ tree with samples of *Genus 1* sp. *2*, based on distances of the mtDNA 16S gene. Bootstrap values are indicated only for nodes with support greater than 70%.

The intraspecific divergence values found between the species analyzed varied between 4.73% and 19.2% with gene COI; while the observed divergence between *Genus 1* sp. *2* and *Cricotopus (Oliveiriella)* with gene 16S is 7.49%. Previous studies have reported interspecific distances for Diptera similar to those reported here; Shouche and Patole [51] observed interspecific distances with gene 16S between 1% and 9% in three species of Diptera. Ekrem et al. [52] reported interspecific divergences of 16.2% for gene COI in the family Chironomidae. However, this reference value for species identification is not enough, given that these studies mainly comprise specimens from the Holarctic region, and there are still few studies that analyze specimens from the Neotropics, including members of the subfamily Orthocladiinae.

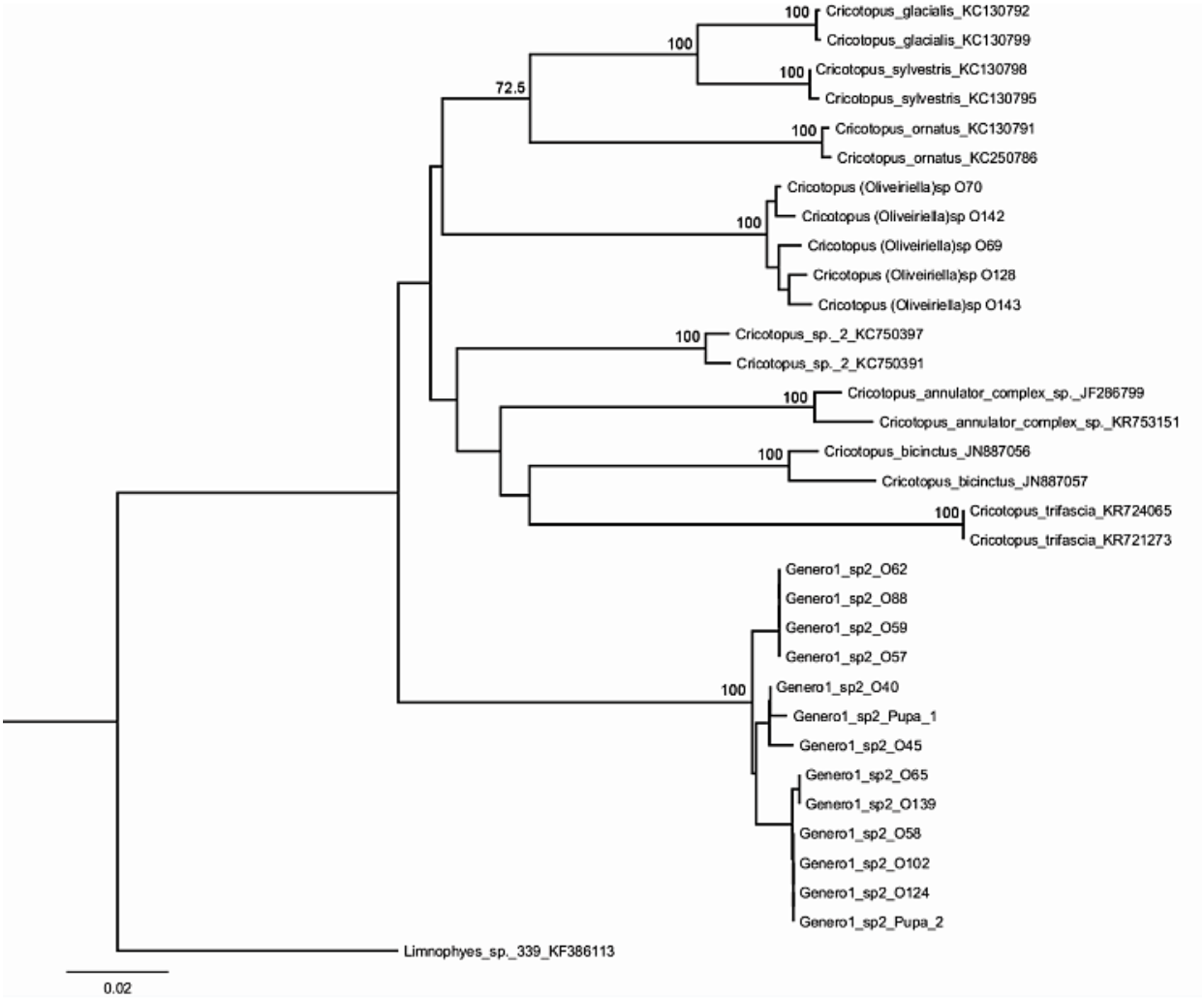

Figure 6. Consensus NJ tree with samples of *Genus 1* sp. *2*, based on distances of the mtDNA COI gene. Bootstrap values are indicated only for nodes with support greater than 70%.

The comparison of the molecular data of *Oliveiriella* and *Genus 1* with other subgenera of *Cricotopus* confirms the findings of Andersen et al. [53], which are also confirmed by Prat et al. [54] in that *Oliveiriella* is a subgenera of *Cricotopus* and *Genus 1* of Roback and Coffman is also a subgenera.

Therefore, it is necessary to name this subgenus, since it is clearly differentiated from the other subgenera. This is currently being carried out by Dr. Prat's group at the Universidad de Barcelona and the name *Cricotopus (Rieradevallia)* is suggested for the

generic name of *Genus 1* of Roback and Coffman [2], which will be described in a more detailed manner in subsequent studies by Dr. Prat's group.

Conclusions

The results obtained allow the molecular determination of Genus 1 sp. 2 (Roback and Coffman), support the morphological data, and associate larvae and pupae, contributing to a better understanding of the taxonomical limits in Chironomidae, specifically the subfamily Orthocladiinae, where there are many difficulties in the taxonomic determination of its species [8,20,43,55,56]. Moreover, the results support the establishment of this species as a water quality bioindicator.

Supplementary Materials: The following are available online at www.mdpi.com/link, Table S1: Physical, hydrobiological, and chemical characteristics of the streams assessed. The values correspond to the mean values of the parameters measured in each sampling station.

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Author Contributions: PAOL collection of specimens, morphological and molecular studies, design and writing of manuscript; EMOP storage of specimens and writing of manuscript; GJCV statistical analysis, writing and review of manuscript; NP morphological studies (taxonomic determination) writing and final correction of manuscript; GTRR phylogenetic analysis and writing of manuscript; and FARP edition the photographs, design and writing of manuscript, and final correction.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials: Table S1. Physical, hydrobiological, and chemical characteristics of the streams assessed. The values correspond to the mean values of the parameters measured in each sampling station. E1: La Elvira stream (reference area), E2: La Elvira stream (mining), E3: La Elvira stream (mining), E4: Romerales stream (reference area), E5: California stream (mining), E6: Toldafría stream (mining).

Variables	Parameter	Measurement units	Sampling station					
			E1	E2	E3	E4	E5	E6
Physical	Water temperature	°C	12.7	13.8	13.8	12.5	13.73	14.4
	pH		7.64	7.8	7.89	7.2	7.35	7.25
	Conductivity	µS	206	291	195	252.7	87.1	376.9
	Dissolved oxygen	mg/L	7.55	6.03	4.75	4.9	5.11	4.73
	Oxygen saturation	%	99.2	65.3	65.27	65.4	67.5	67.3
Hydrobiological	Average depth	cm	10.7	10	13.8	16.5	19.11	10.9
	Width	m	2.23	2.43	3.05	6.4	5.01	5.75
	Water flow velocity	m/s	0.43	0.64	0.5	0.73	0.69	0.55
Chemical	Chemical oxygen demand	mg/L	23.3	20.3	106	32.67	91.7	59.7
	Biological oxygen demand	mg/L	3.21	3.21	10	3.21	6.17	3.21
	Total coliforms	UFC/100mL	2983	9257	341733	2286	3977	1E+05
	Fecal coliforms	UFC/100mL	540	873	2740	504	1546	636
	Total suspended solids	mg/L	34.17	306	1384	6.67	242.3	18.1
	Total solids	mg/L	110.7	395	1497,3	85.3	569	161.3
	Cyanide	mg/L	0.09	0.093	0.093	0.09	0.09	0.093

	Boron	mg/L	0.83	0.83	0.83	0.83	0.83	0.83
	Lead	mg/L	0.05	0.085	0.05	0.02	0.02	0.02
	Mercury	mg/L	0.33	0.33	0.34	0.33	0.33	0.33
	Ammoniacal nitrogen	mg/L	0.10	0.21	0.22	0.11	0.35	0.11
	Phosphate	mg/L	0.7	1.2	3.5	0.3	2.3	0.2
	Sulfate	mg/L	21	56	103.3	7.67	45.7	19.3
	Iron	mg/L	0.4	1.34	3.68	0.163	1.87	0.53
	Chloride	mg/L	2.5	2.9	9.2	3.0	7.3	2.5
	Lipids and oils	mg/L	0.5	0.9	0.63	0.5	0.5	0.5
	Nitrates	mg/L	1	1.1	1.4	1	1	1
	Nitrites	mg/L	0.07	0.3	0.72	0.07	0.34	0.07
	Aluminum	mg/L	0.11	0.6	8.02	0.1	2.3	0.15

ANEXO

The *Cricotopus (Oliveiriella)* (Diptera Chironomidae) of the High Altitude Andean Streams, with Description of a New Species, *C. (O.) rieradevallae* Prat & Paggi 2016

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SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

The *Cricotopus* (*Oliveiriella*) (Diptera: Chironomidae) of the High Altitude Andean Streams, with Description of a New Species, *C.* (*O.*) *rieradevallae*

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Keywords
Biogeography, taxonomy, Cox1 mitochondrial sequences, Midges, Orthocladiinae, South America

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Abstract
The genus *Oliveiriella* (Chironomidae, Orthocladiinae) was erected by Wiedenbrug & Fittkau (1997). The adults have characteristic black spots on their wings and other characteristics similar to the genus *Cricotopus*. Pupal skins are very characteristic with strong short spines in the anal lobe instead of setae, while larvae are distinguishable by the long anal papillae and the intense blue color of their body. However, Andersen *et al* (2013) consider *Oliveiriella* as a subgenera of *Cricotopus*. In this paper, using the sequences of the *cox1* gene, we conclude that *Oliveiriella* should be considered a subgenus within *Cricotopus*, confirming its status in Andersen *et al* (2013). Furthermore, we describe *Cricotopus* (*Oliveiriella*) *rieradevallae* Prat & Paggi sp. n. from the Saltana river (Ecuador). The adult males, females, and preimaginal stages of the two species of subgenus *Oliveiriella* known from South America *Cricotopus* (*O.*) *almeidai* n. comb. from Peru, Brazil, and Argentina and *Cricotopus* (*O.*) *sanjavieri* n. comb. from Argentina are compared with those of *Cricotopus* (*O.*) *rieradevallae* sp. n. from Ecuador. The differences allow the distinction of the three species. The *cox1* gene reveals that at least three different undescribed species of the same subgenus are present in the high-altitude tropical Andes. The morphology of the available pupae and pupal exuviae reveals the presence of several morphotypes that are candidates to be described as new species. A key used to distinguish these pupal morphotypes is provided, including the three described species. Additionally, the distribution of the subgenus is discussed.

Introduction

The genus *Oliveiriella* was erected by Wiedenbrug & Fittkau (1997) from material studied from Rio Grande do Sul (southern Brazil) by Oliveira (1946), described originally as *Spaniotoma* (*Stictocladius*) *almeidai*. Years later, Tejerina & Paggi (2009) described a new species from Argentina, *Oliveiriella sanjavieri*, in all stages. In the same year, the range of *Oliveiriella* was extended to North America by Krestian *et al* (2009), which found larvae and pupae of this

species in Arizona and New Mexico. Today, due to morphological similarities with *Cricotopus*, *Oliveiriella* is considered a synonym of *Cricotopus* but is maintained as a separate subgenus according to Andersen *et al* (2013). Therefore, in this paper, we will use *Oliveiriella* as a subgenus of *Cricotopus*. Andersen *et al* (2013) provided a key to distinguish between the different *Cricotopus* subgenera larvae. Prat *et al* (2014) provided a key for the pupae of the high-altitude Andean Chironomidae genus where *Oliveiriella* can be easily distinguished from other genera and subgenera of Orthocladiinae.

Larvae of *Oliveiriella* have been found in lotic habitats of montane and submontane streams from southern to southeastern Brazil, and on the eastern slopes of the Andes in Ecuador and Peru by Wiedenbrug & Fittkau (1997), but the authors do not provide the altitude at which the larvae were found. *Oliveiriella sanjavieri* was found by Tejerina & Paggi (2009) and Tejerina & Malizia (2012) from several streams in the region of Tucumán (Argentina) in altitudes ranging from 825 to 1275 m a.s.l. and in two streams located at 411 and 505 m a.s.l. in the region of Jujuy (Argentina). Finally, pupae of *Oliveiriella* were collected by Krestian *et al* (2009) in Arizona and New Mexico, from two rivers at elevations between 1180 and 1449 m a.s.l. In all cases, streams where the larvae have been found until now are of clear and oligotrophic waters. During our studies in the high-altitude tropical streams in Colombia, Ecuador, and Peru, we found larvae, pupae, and pupal exuviae of *Cricotopus* (*Oliveiriella*) in Peru, Ecuador, and Colombia. In this paper, we used all this material to (1) present a phylogenetic tree of the *Oliveiriella* species using COI sequences that support that *Oliveiriella* is a subgenus of *Cricotopus*; (2) describe a new species of this subgenus found in Saltana creek (Pichincha, Ecuador); (3) report the distribution of the subgenus in the high-altitude Andean rivers; (4) study the morphological variation of larvae and pupae of this subgenus; and (5) provide a preliminary key of the different morphotypes of pupal exuviae.

Material and Methods

Study area

Samples were taken in the tropical highland Andean streams (over 2500 m a.s.l) from northern Colombia to southern Peru. A total of 185 sites were studied in four different research projects from 2007 to 2011 (Table 1). From all the sites sampled, the subgenus *Oliveiriella* was found only in eight sites. The sites with *Oliveiriella*, the date, country, basin, stream, position, and altitude are listed in Table 1. In this table, we also indicate the research project in which the site was studied. Details of the projects and sites may be found at http://www.ub.edu/riosandes/. Each site was visited once, except site GUAY 04 from the FUCARA project when samples were taken from April 2009 to November 2010. Physicochemical, habitat (IHF index), and riparian vegetation (QBR index) characteristics of the sampled sites are listed in Table 2. Material collected from one site in Colombia (VR-6; Table 1) by two of the authors (FR, PO) has been included in the molecular studies. The two species currently known, *Cricotopus* (*O.*) *almeidai* and *Cricotopus* (*O.*) *sanjavieri*, have also been used for comparison with the description of *Cricotopus* (*O.*) *rieradevallae* sp. n. Larvae and pupae of *C.* (*O.*) *almeidai* were collected from Los Sosa river, Tucumán, Argentina. We were unable to amplify the DNA of the larvae of *C.* (*O.*) *sanjavieri* sampled at San Javier stream, Tucumán, Argentina. For this reason, it was not possible to include this species in the phylogenetic tree.

Sampling, sorting, and preservation of material

Sampling of larvae and pupae or pupal exuviae was done using a Surber sampler in the CERA and BIQURA projects (Villamarín *et al* 2013, Prat *et al* 2013) and a Hess sampler at Saltana creek in the FUCARA project (Ríos-Touma *et al* 2011). Surber samples were taken in different substrates following the protocol described by Villamarín *et al* (2013). To enhance the collection of pupae and pupal exuviae, drift samples were also taken. At least two drift nets were left in the river to filter the water in each site during the time used for sampling of benthos with the Surber sampler (2–3 h). In all cases, the net mesh was 250 μm. In the case of the site GUAY04 (Saltana creek, FUCARA project), Hess samples were taken randomly, every month from April 2009 to August 2010 using the methods reported in Ríos-Touma *et al* (2012).

Except in the BIQURA project, samples were fixed with formalin in the field, taken to the laboratory, where, after elimination of formalin from the sample, Chironomidae were sorted. At least 100 individuals per sample were collected. In all cases, chironomid larvae were identified at genera or morphotype level under the stereoscope following the key of Prat *et al* (2012). Up to 10 individuals of each genera or morphotype were prepared for microscope examination. The larvae were digested in hot 10% KOH, washed, dehydrated, and mounted in Euparal® on a slide following the usual techniques for optical microscopy described by Epler (2001).

In the BIQURA project, the main objective was to obtain the barcode of the maximum taxa possible. Live larvae were collected in the field (at least 100 individuals) picking the organisms from a combined sample of 12 Surbers including all the inhabitants found in the stream. The midges were then fixed in the field using 96% alcohol that was changed at least once before the DNA analyses were carried out. The body of the larvae was used for DNA extraction, while the head capsule was prepared following the methods previously described. The pupae (or pupal exuviae) were prepared after the DNA extraction (described further on in the text) in Euparal® using the same protocol of larvae. All the larvae were classified using the key of Prat *et al* (2012) and other papers referred to by the authors. For pupae, the key of Prat *et al* (2014) was used.

Table 1 High-altitude Andean sites and basins where *Cricotopus* (*Oliveiriella*) have been found. In the last column, we indicate the number of individuals examined as larvae (L) or pupae (P). In brackets, number of individuals used the molecular analysis. As the individuals of CERA project were fixed in formaldehyde, they cannot be included in the molecular analysis. More information about the research projects and each site can be found in Table 2 and at http://www.ub.edu/riosandes/.

Site code	Project	Date	Country	Drainage	Basin	Stream name	Longitude	Latitude	Altitude (m.a.s.l.)	Material studied
SA10	CERA	18/10/2007	Peru	Pacific	Santa	Llanganuco bajo laguna	77°25.552′	09°10.886′	3846	7P; 8L
SA12	CERA	18/10/2007	Peru	Pacific	Santa	Río Llullan	77°46.109′	09°01.937′	2746	1P
MO16	CERA	23/10/2007	Peru	Amazon-Atlantic	Mosna	Vaqueria	77°33′23.32″	9°2′37.92″	4010	1L
AGU14	CERA	20/2/2008	Ecuador	Pacific	Napo	S/N	77°41.323′	00°37.169′	3387	1P
NA02	BIQURA	23/10/2011	Ecuador	Amazon-Atlantic	Napo	Papayacta	78°01.675′	00°24.722′	2457	1L (1)
CHIN02	BIQURA	14/9/2011	Colombia	Caribbean-Atlantic	Magdalena	Romerales	75°25.745′	04°59.172′	2485	3
MO08	BIQURA	4/7/2011	Peru	Amazon-Atlantic	Mosna	Purhuay	77°11.513′	09°19.316′	3192	7L (7)
GUAY04	BIQURA FUCARA	22/10/2011 2009/2010	Ecuador	Pacific	Esmeraldas	Saltana	78°13′8.8″	00°19′1.80″	3869	5P (1); 29L (7)
VR-6	EIMAG-REG-MA	09/09/2014	Colombia	Caribbean-Atlantic	Magdalena	Romerales	75°25′58″	04°59′22″	2305	6L (6)

Table 2 Physicochemical characteristics of the basins where *Cricotopus* (*Oliveiriella*) larvae or/and pupae have been found in the high-altitude Andean rivers. For BIQURA project, only field data is available. BIQURA streams are reference streams without pollution.

Site code	*T* (°)	pH	Conductivity (µS/cm)	O_2 (%)	O_2 (mg/l)	Ammonia (mg/l)	Phosphate (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	QBR	IHF
SA10	12.00	7.84	32.80	73.10	7.93	0.03	0.00	0.02	0.20	95	85
SA12	14.30	7.47	102.00	80.10	8.18	0.11	0.04	0.02	0.10	45	57
MO16	8.2	8.08	17.3	73.9	8.17	0.06	0.00	0.0	0.2	100	50
AG14	8.00	7.87	10.70	77.50	9.16	0.00	0.02	0.00	0.00	100	53
NA02	10.6	7.48	46.1	71.1	7.91					100	69
CHIN02	13.6		61.5							95	59
MO08	11.6	6.64	26.2	75.7	8.2					95	75
GUAY04	5.6	7.46	42.9	63.3	8.02					100	76
	6.3[a]	7.12[a]	68.9[a]	76.2[a]	9.18[a]	0.27[a]	0.07[a]	0.00[a]	0.29[a]	100	100
VR-06	12.5	7.22	67	65.4	4.9	0.11		0.07			

QBR riparian quality index, *IHF* fluvial habitat index.

[a] Mean values from 15 months.

Adults were collected only at the Saltana creek (FUCARA Project), with insect traps following the methods described in Ríos-Touma *et al.* (2012). Three malaise traps were deployed in the river banks for 24 h on a monthly basis. Table 3 lists the total number of adults of *C.* (*Oliveiriella*) *rieradevallae* sp. n. found on different dates. The studied material was preserved in 80% ethanol cleared in 10% KOH and slide-mounted in Euparal® for microscopic observation, following the methods outlined in Paggi (2009). General terminology and abbreviations follow Saether (1980). Total and wing lengths are given in millimeters, and other measurements in micrometers; the measurements are given as range, followed by mean, and the number of specimens in parenthesis. Material examined was deposited at the La Plata Museum of Natural Science (MLP), La Plata, Argentina, and at the Institute of Limnology "Dr. R. A. Ringuelet" (ILPLA), La Plata, Argentina, and in the Museo Ecuatoriano de Ciencias Naturales del Instituto Nacional de Biodiversidad (MECN), Quito, Ecuador.

DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

Currently, the molecular identification of species using DNA-based tools represents a successful method to assess macroinvertebrate biodiversity (especially Chironomidae) in high-altitude streams (Montagna *et al* 2016). In our study, total genomic DNA was extracted from samples preserved in 96% ethanol using the QIamp® DNA Mini Kit (QIAGEN) or SPEEDTOOLS® Tissue DNA Extraction Kit (BIOTOOLS) following the manufacturer's protocol. A partial fragment of the cytochrome oxidase I (*cox1*) gene was selectively amplified in polymerase chain reactions (PCR) that were conducted following Krosch et al (2011), Prat *et al* (2013) and sequenced in the Macrogene sequence center in the Netherlands. Raw sequences were edited and assembled with Geneious 4.6.5 (Drummond *et al.* 2009). All new sequences obtained were deposited in GenBank for localities and GenBank accession numbers of all used samples).

Table 3 Adults of *Cricotopus* (*Oliveiriella*) *rieradevallae* Prat & Paggi sp. n. caught in the Malaise traps of the river Saltana in 10 successive months.

Data	*Oliveiriella*	Ind/trap
02/10/2009	3f	30.0
21/10/2009	1f	20.0
20/11/2009	0	20.7
17/12/2009	1m	30.3
14/01/2010	1m 7f	37.7
10/02/2010	1f	35.5
11/03/2010	9m 3f	63.7
08/04/2010	1m 13f	97.3
09/05/2010	4f	68.7
08/06/2010	0	1.0

f female, *m* male.

Alignment of the *cox1* gene fragments was trivial due to the absence of length polymorphisms. Sequences were translated into amino acids, and no indels or stop codons were found. We obtained the uncorrected pairwise (p)-distance genetic divergences between terminals using MEGA v.7.0.14 (Kumar *et al* 2016) removing alignment gaps in pairwise comparisons. A maximum likelihood (ML) analysis was performed using RAxML 7.4.2 (Stamatakis 2006) through the graphical front-end RAxMLGUI 1.3 (Silvestro & Michalak 2012).

GTR+G+I nucleotide substitution model was applied, and nonparametric bootstrap support analysis of 1000 pseudoreplicates was conducted. *Limnophyes* species was used to root the tree.

To test if *Oliveiriella* is a valid genus or it is a subgenus of *Cricotopus* according to Andersen *et al* (2013), we included in the analysis several sequences of *Cricotopus* available at GenBank. In addition, we included in the analysis *Oliveiriella almeidai* from larval and pupal material collected in Argentina (see "Material and Methods" section). In Table 1, we summarized the localities of all the analyzed sites. Because some *Cricotopus* species appear paraphyletic with respect to some *Limnophyes* used as outgroup, a typology of outgroups was enforced.

Results

The species of Oliveiriella *in the high-altitude Andean streams and rivers and the identity of* Oliveiriella *as genus: molecular data*

Specimens, locality, and sequences with corresponding GenBank accession numbers analyzed in the present study are listed in Table 10 in supplementary material. The matrix used in the phylogenetic analysis includes 35 terminals and 454 aligned characters. Figure 1 shows the ML tree obtained. The genus *Cricotopus* appears paraphyletic with respect to the *Oliveiriella* species included, although with moderate bootstrap support. Within the *C.* (*Oliveiriella*) clade, two clusters are present. The first one is formed by *C.* (*O.*) *almeidai* plus the BIQURA samples from Peru (MO08 samples), together with specimens from Colombia (VR-6). The uncorrected p-distances (Table 4) from *O. almeidai* and MO08 plus VR-6 (12.5%) suggest that there are two different species, while MO08 and the sequences from Colombia (VR-6) must be considered as different populations of the same species (uncorrected p-distance = 2.3%). The second cluster shows two putative species, one from river Saltana (GUAY04) described below in this paper as *C.* (*O.*) *rieradevallae* sp. n. and another one from Napo River (NA02) (uncorrected p-distance = 14.4%) (Table 4).

In summary, Fig 1 suggests that *Oliveiriella* cannot be considered as a separate genus in the Orthocladiinae, but just as a subgenus of *Cricotopus* as indicated by Andersen *et al*

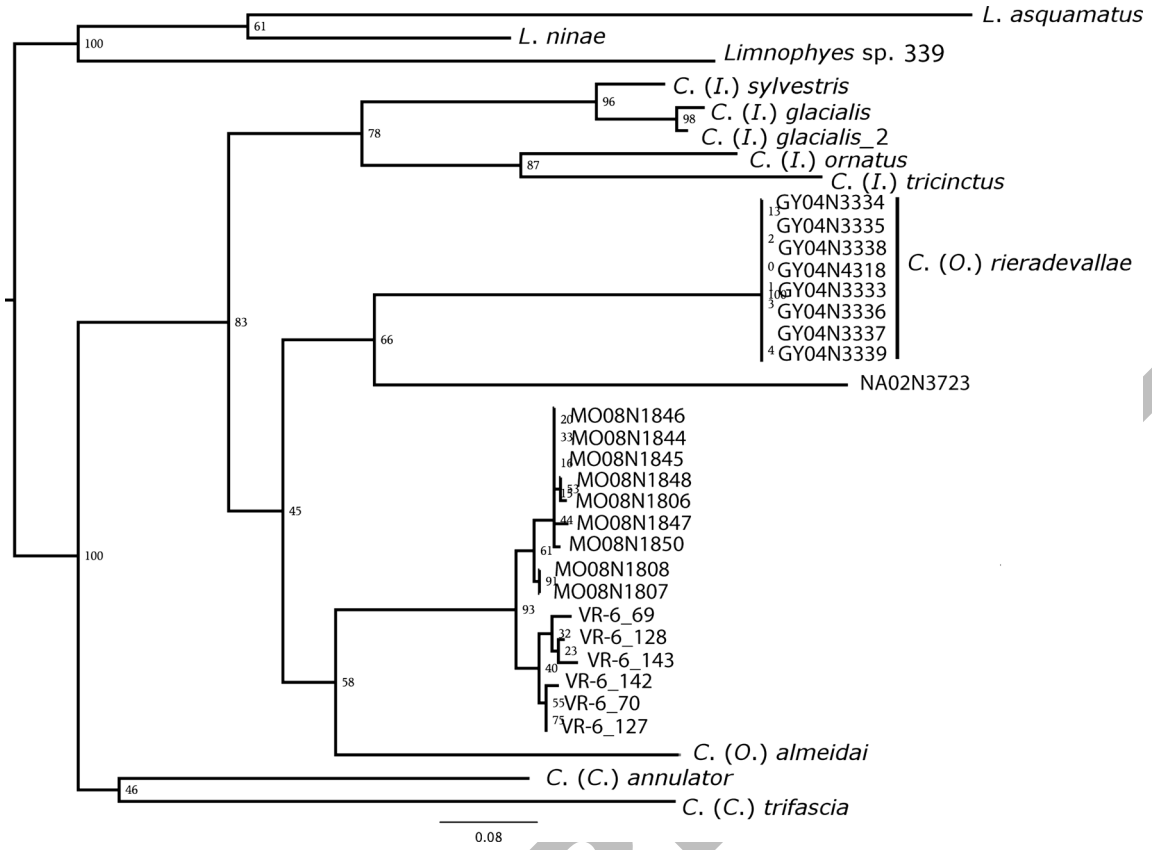

Fig 1 ML tree of the *Oliveiriella* samples using partial *cox1* gene sequences. Numbers in 604 nodes correspond to bootstrap support values. The tree was rooted using three *Limnophyes* species. Sequences of seven species of *Cricotopus* was also included to test the paraphyly of *Cricotopus* species

(2013), and there are at least four different species of the subgenus *Oliveiriella* in the study area.

Cricotopus (Oliveiriella) rieradevallae *Prat & Paggi new species*

Type material. Holotype, male adult from Ecuador, Saltana river 00°19′1.80″S 78°13′8.8″W, 14.i.2010 leg. Blanca Ríos-Touma, Malaise trap. Allotype, female 14.i.2010 same data as Holotype. Paratypes: same data as Holotype except for the following data: two adult males Pichincha, Res. Paluguillo, Queb. Saltana 00°19′1.80″S 78°13′8.8″W 3869 m a.s.l. 8.iv.2010; one male adult 11.iii.2010, leg. B. Ríos-Touma & F. González, Malaise trap; one adult female, 14.i.2010; two larvae and pupal exuviae 14.i.2010 (GY04N3334, GY04N3338) and 03.v.2010; two pupae 07.iv.2010 (GY04N4318) and 05.v.2010.

Table 4 Uncorrected p-distances of *cox1* partial sequences between species analyzed in this study.

	GY04	NA02	MO08 & VR-6	*O. almeidai*	*C. glacialis*	*C. annulator*	*C. trifascia*	*C. glacialis_2*	*C. ornatus*	*C. sylvestris*
GUAY04										
NA02	0.144									
MO08 & VR-6	0.119	0.150								
C. (O.) almeidai	0.139	0.151	0.125							
C. (I.) glacialis	0.162	0.162	0.129	0.139						
C.(C.) annulator	0.174	0.183	0.153	0.155	0.155					
C.(C.) trifascia	0.178	0.178	0.150	0.182	0.175	0.154				
C. (I.) glacialis_2	0.165	0.162	0.125	0.144	0.014	0.151	0.171			
C. (I.) ornatus	0.162	0.167	0.144	0.158	0.130	0.155	0.168	0.130		
C. (I.) sylvestris	0.160	0.167	0.131	0.128	0.049	0.132	0.175	0.044	0.116	
C. (I.) tricinctus	0.158	0.169	0.142	0.144	0.132	0.146	0.189	0.132	0.102	0.123

Diagnostic characters. Male, female, and preimaginal stages of *C.* (*O.*) *rieradevallae* sp. n. are differentiated from *C.* (*O.*) *almeidai* n. comb. and *C.* (*O.*) *sanjavieri* n. comb. by the following characters:

Adults. The male can be distinguished from the other two species of subgenus *Oliveiriella* described until now by having a total length of 3.60 mm; 8–9 clypeus setae; 45–60 setae in the scutellum and 7–10 squamal setae (Table 5). Alar spot in the anal cell absent (in *C.* (*O.*) *sanjavieri* is present, Fig 2b). Legs with 67–71 comb teeth in hind tibia; fore and mid tibia white band ratio (BR) 0.35 and 0.27, respectively (Fig 2c, e); hind leg completely dark (Fig 2g). This color pattern in the legs is different compared with *C.* (*O.*) *sanjavieri* (Fig 2d, f). The female can be distinguished from the other two species by having a total length of 3.42 mm; 11–14 clypeal setae, 20 acrostichal setae; 60–68 scutellar setae; the band ratio on fore and mid tibia and the wings with the same characteristic as in males (Table 5).

Pupae. C. (*O.*) *rieradevallae* from river Saltana is larger than the other two species (Tables 5 and 8). Length of the respiratory organ (OR) 240–273 µm and with a clear terminal notch and its entire surface serrated (Fig 3a). The medial precorneal setae are more than 10 times longer than the anterior and posterior (Fig 3a, b) compared with *C.* (*O.*) *sanjavieri*, of which the length of the median precorneal setae is at most three times longer than the other two setae, whereas *C.* (*O.*) *almeidai* (Brazil) of which all the setae are of similar size (Table 5). This is also true with the first and third dorsocentral setae of the cephalothorax which are much longer than the second one (Fig 3b) (in *C.* (*O.*) *sanjavieri* are of similar length). The abdomen (Fig 3c–g) has much longer lateral setae than those of *C.* (*O.*) *almeidai* but shorter than those of *C.* (*O.*) *sanjavieri*. *Cricotopus* (*Oliveiriella*) *rieradevallae* has characteristic spines in the sternites II and III that lack in the other two species found until now (Fig 3d, e).

Larvae. The only characters that seem to differentiate *C.* (*O.*) *rieradevallae* from the other species are the higher AR, major length of the antennal blade, and a higher MR (Tables 5 and 9). The presence of transversal marks in the mentum is characteristic of all the Andean high-altitude river species (Fig 4b). The pattern of small dots of the head capsule is also very conspicuous and both (marks and the pattern) facilitated the distinction of the *Oliveiriella* larvae from the Andean high-altitude rivers from the other subgenera in the *Cricotopus* group and especially from Genus 1 sensu Roback & Coffman (1983).

Description (Fig 2)

Male imago. (n = 4, except when otherwise stated). Total length 3.42–3.78, 3.60; wing length 2.00–2.49, 2.44; total length/wing length 1.43–1.52, 1.47; wing length/length of profemur 2.68–2.87, 2.78. General coloration dark brown, almost black. Thorax and abdomen dark brown. Wing with brown areas almost reaching M_{3+4} and without a spot in the

Table 5 Distinctive characteristics of the three species of *Cricotopus* (*Oliveiriella*) known from Neotropical region.

	C. (*O.*) *almeidai*	*C.* (*O.*) *sanjavieri*	*C.* (*O.*) *rieradevallae*
Male			
Wing length (mm)	1.09	1.9–2.43	2.0–2.49
Clipeus setae	12	14–16	8–9
Scutellum setae	12	18	45–60
Squamal setae	5	7–9	7–10
Comb teeth in the hind tibia		57–60	67–71
Fore and mid tibia with band ratio	<1/2	<1/2	>1/2
Hind leg	Brown to yellow	Brown to yellow	Dark
Alar spot in the anal cell	Absent	Present	Absent
Pupae			
Body size (mm)	2.2–2.8	2.41–3.19	3.22–3.82
RO length (µm)	136–242	220–280	240–273
RO notch	Absent	Absent	Present
Medial/anterior precorneal seta size	1	3	>8
1st and 3rd/2nd dorsocentral setae	1	1	>7
Spines in sternites II and III	Absent	Absent	Present
Larvae			
Size	2.7–3.2	3.4–4.02	3.1–3.73
AR	1.4–1.8	1.16–1.88	1.83–1.89

RO respiratory organ, *AR* antennal ratio.

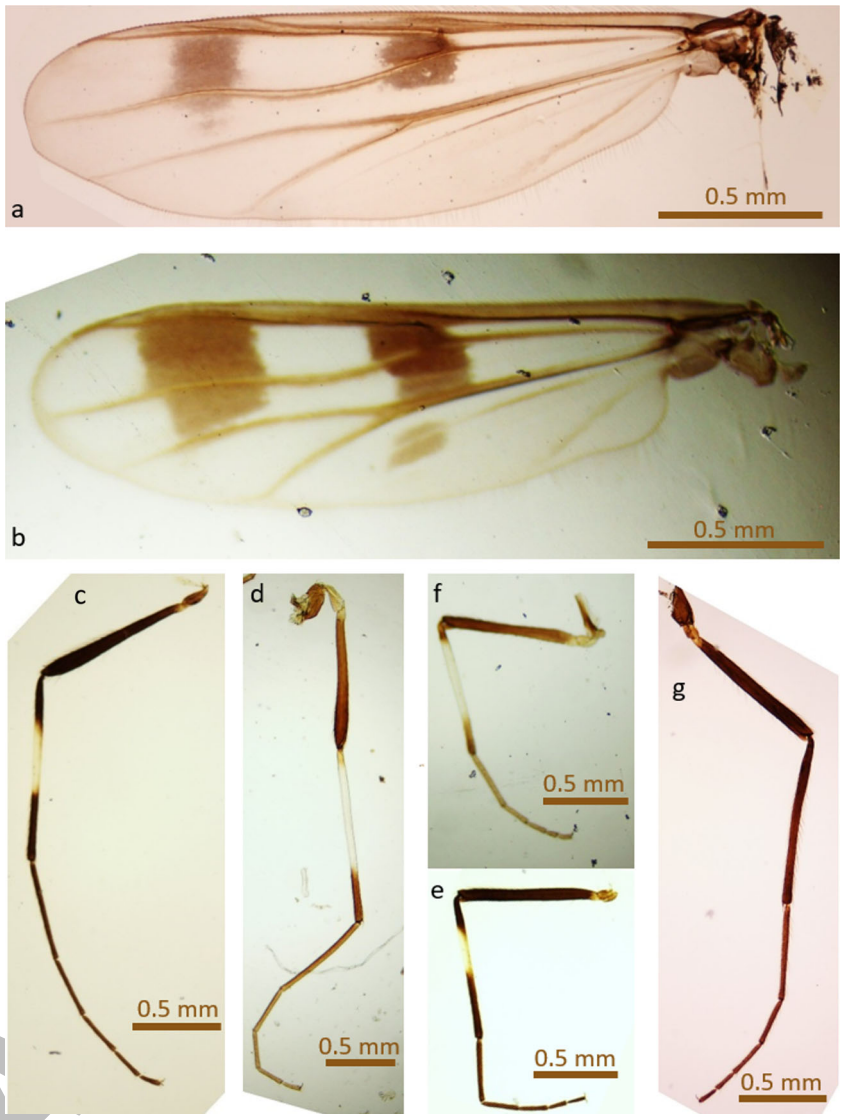

Fig 2 *Cricotopus* (*Oliveiriella*) *rieradevallae* Prat & Paggi sp. n. adult. **a** Wing. **c** PI. **e** PII. **g** PIII. *Cricotopus* (*Oliveiriella*) *sanjavieri* adult. **b** Wing. **d** PI. **f** PII.

anal cell (Fig 2a), abdomen and leg coloration pattern as generic diagnosis.

Head. Temporal setae 4–5, 5. Clypeus with 8–9, 8 (3) setae. Tentorium 136–152, 141 long; palpal segment length (1–4): 20, 25, 45, 60, fifth segment not measurable (1).

Thorax. Acrostichals 16–18 (2); dorsocentrals 20 (1); prealars 3; scutellars 45–60, 60 (3).

Wing. VR 1.14–1.20, 1.17; C/WL 0.90–0.92, 0.91; Cu/WL 0.43–0.44, 0.43. Costa extended beyond R_{4+5} 75–83, 81 Cu slightly curved. Squama with 7–10, 9 setae.

Legs. With a white band (WB) on fore and mid tibiae covering less than half of each tibia in a ratio of (WB/Ti_1) 0.40–0.29, 0.35 (3) and (WB/Ti_2) 0.23–0.32, 0.27 (3) (Fig 2c, e, g). Tibial spur of fore leg 45–51, 48 long; spurs of mid tibiae 20–22 and 16 long; spurs of hind tibia: 67–71, 70 and 20–22, 22 long, comb with 13–15, 14 teeth. Sensilla chaetica 5 (1) on hind Ta_1. Pulvilli absent. Length and proportions of legs as in Table 6.

Abdomen. Chaetotaxy: TI with an anterolateral group of about 12 setae and distal group of 3–4 dorsal setae, TII–IV with 2 median setae and a transverse row of about 10 dorsal setae; TV–VI with a median longitudinal row of 10–12 setae; TVII–VIII with a central patch of 20–30 irregular distributed setae. TII–VIII each with 5 lateral setae.

Genitalia. Hypopygium. Gonocoxite 207–224, 207 long; gonostylus 75–83, 77 long. Tergite IX with 6–10 dorsal setae. Laterosternite 3–4 setae. Megaseta 8–10, 9 long. HR: 2.56–2.78, 2.70. HV: 4.30–5.07, 4.69.

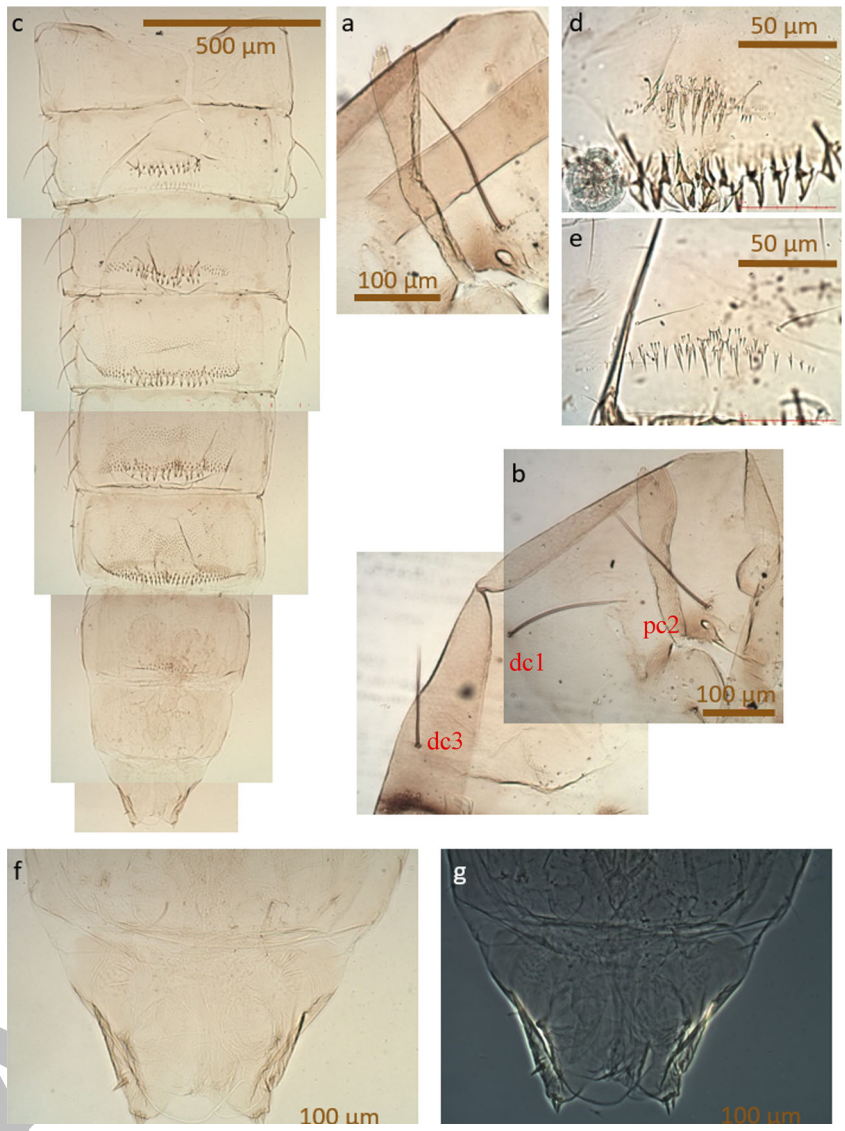

Fig 3 *Cricotopus* (*Oliveiriella*) *rieradevallae* Prat & Paggi sp. n. Pupae: **a** Respiratory organ; **b** Thorax with long dorsocentral and precorneal setae (dc1, dc3 and pc2); **c** Abdomen; **d** Spines of sternite II; **e** Spines of sternite III; **f** Abdominal segment IX; g) Abdominal segment IX (phase contrast).

Female imago. (*n* = 2 except when otherwise stated). Total length: 3.42; wing length 2.57–2.74; total length/wing length 1.24–1.33; wing length/length of profemur 3. Color pattern as in male.

Head. AR = 0.33–0.48. Temporal setae 5–7; clypeals 11–14; tentorium 142–152 long; palpal segment length (1–5): (15–20), (27–33), (41–46), (52–65), (92).

Thorax. Antepronotals 1; acrostichals 20; prealars 3; dorsocentrals 17–19; scutellars 60–68.

Wing. VR 1.23; C/WL 0.93–0.94; Cu/WL 0.42–0.44. Costa extended beyond R_{4+5} 108–166. Cu slightly curved; 11 R and 7 R_{4+5} setae; squama with 7–9 setae.

Legs. With a white ring on fore and mid tibiae covering less than half of each tibia, in a ratio of (WB/Ti_1) 0.31 and (WB/Ti_2) 0.27. Tibial spur of fore leg 34–38 long; spurs of mid tibiae 20 and 14 long; spurs of hind tibia: 61–75 and 22–24 long, comb with 13–14 teeth. Pulvilli absent. Sensilla chaetica 10 on middle Ta_1 and 8–10 on hind Ta_1. Length and proportions of legs listed in Table 7.

Abdomen. Distribution of setae as in male. S VI–VII with a central patch of about 20–22 setae.

Genitalia. Gonocoxite IX with 6–8 setae; tergite IX with 7 setae; sternite VIII with 11–16 setae. Seminal capsule ovate, light brown, 112–165 long, 83–122 wide, with long ducts, at least with one loop. Notum 190 long. Cerci 69–81 long.

Pupa. (*n* = 4, except when otherwise stated, measurements of additional material are in Table 8). Total length 3.22–3.82, 3.48.

Table 6 Lengths (in µm) and proportions of legs of *Cricotopus* (*Oliveiriella*) *rieradevallae* Prat & Paggi sp. n. (males *n* = 4).

	Fe	Ti	Ta_1	Ta_2	Ta_3	Ta_4	Ta_5	LR	BV	SV
P_1	851–913.882	996–1079.1048	560–581.576	332–353.342	249–291.265	187–208.192	104–125.114	0.54–0.56. 0.55	2.70–2.90. 2.75	3.30–3.43. 3.55
P_2	809–851.835	809–872.851	353–394.374 (3)	187–228.201 (3)	187–145.159 (3)	104 (3)	104 (3)	0.44–0.45. 0.44 (3)	3.40–3.81. 3.62 (3)	4.37–4.59. 4.49 (3)
P_3	830–892.872	934–1017.991	540 (3)	270–291.277 (3)	228 (3)	104–125.118 (3)	104–125.111(3)	0.53–0.58. 0.55 (3)	3.25–3.26. 3.25 (3)	3.27–3.50. 3.42 (3)

P1 fore leg, *P2* mid leg, *P3* hind leg, *Fe* femur, *Ti* tibia, *Ta* tarsus, *LR* leg ratio (length of Ta_1/length of tibia), *BV* length of (femur + tibia + Ta_1)/length of (Ta_2 + Ta_3 + Ta_4 + Ta_5), *SV* length of (femur + tibia)/length of Ta.

Cephalothorax. (Fig 3a, b). Precorneals: anterior 22–24, median 193–207, posterior 18–24 µm long. Dorsocentrals first and third 149–157 long, second and fourth approximately 20 long (2) (Fig 3b). Thoracic horn 240–273 (2) long apically truncated with a median notch and surface serrated as in Fig 3a.

Abdomen. (Fig 3c–g). Tergite II with two posterior rows of each 12–15, 13 hooklets. TIII with two rows of each 11–15, 14 hooklets and small spinules on either side of these rows connected to a central shagreen. TIV–V with 1 posterior row of 9–13, 12 hooklets, and small spines anteriorly also connected to shagreen (Fig 3c). TVI with a posteromedian group of small spinules and a row of moderately 12 strong spines. TVII–VIII with a posteromedian patch of spinules. TVI–VII with posteromedian fine shagreen. Abdominal segments: I with one strong lateral setae 121 long; II–V with two strong lateral setae 124–141 long on small tubercles. Tergites II–VIII with a pair of strong D_5 near hind margin. Sternites with ventromedian patches of spinules (Fig 3d, e). Anal lobe with 3 very short spine-like anal macrosetae (Fig 3f, g) length and distance between spines in Table 8).

Larva. (*n* = 2, except when otherwise stated, measurements of additional material are in Table 9). Total length 3.31–3.73. Body color (live) with intense blue and white bands, dark brown when fixed (Fig 4c).

Head. (Fig 4a, b). 415–519 long; 519 wide when mounted on slides. Postmentum 207 long. Head dark brown rugulose with pale spots around the eyes, and the gula more dark, with a pattern of small dots and several transversal marks between and below the mentum.

Antenna. (Fig 4c, d). Basal antennal segment 53–55 long; segments (2–5): 14–16; 6; 4; 4 long. AR 1.83–1.89. Blade 41 long. Ring organ at 6.09 from base of basal antennal segment.

Mentum. (Fig 4b). Median tooth twice as wide as the first lateral tooth (Table 9). Second lateral tooth smaller and narrower than the rest of the lateral teeth, sixth lateral tooth very small. Seta submenti simple and lateral, close to the base of the mentum.

Mandible. (Fig 4c). Dark brown with 4 inner teeth, with rugose outer margin, 142–148 long. Premandible simple.

Abdomen. (Fig 4e). Long and simple setae (at least half of the segment wide). Posterior parapod 207 long, 116–133 wide. Procerci 14–20 long; 24–28 wide. Anal tubules 331–373 long, longer than posterior parapods.

Etymology. This species is named in memory of the late Maria Rieradevall, a distinguished ecologist and

Table 7 Lengths (in µm) and proportions of legs of *Cricotopus* (*Oliveiriella*) *rieradevallae* Prat & Paggi sp. n. (females *n* = 2).

	Fe	Ti	Ta_1	Ta_2	Ta_3	Ta_4	Ta_5	LR	BV	SV
P_1	851–913	955–1038	498–560	311–353	228–249	166–187	104–125	0.52–0.54	2.75–2.85	3.48–3.63
P_2	768–851	789–872	332–394	187–208	145–165	104	104	0.42–0.45	3.5–3.64	4.37–4.69
P_3	809–913	934–1017	498–540	249–270	208–228	104–125	104–125	0.53	3.31–3.38	3.50–3.58

P1 fore leg, *P2* mid leg, *P3* hind leg, *Fe* femur, *Ti* tibia, *Ta* tarsus, *LR* leg ratio (length of Ta_1/length of tibia), *BV* length of (femur + tibia + Ta_1)/length of (Ta_2 + Ta_3 + Ta_4 + Ta_5), *SV* length of (femur + tibia)/length of Ta.

chironomidologist, and a founding member of the F.E.M. research group.

Distribution of Cricotopus *(*Oliveiriella*)* spp. *in the high Andean tropical rivers*

Larvae, pupae, or pupal exuviae of *Oliveiriella* were found in a total of nine sites from southern Peru to northern Colombia (Table 1). The number of larvae and/or pupae found in each site was always low (1 to 20 individuals, Table 1) in streams of low (1 m wide) to medium (8 m) size and in a range of altitudes from 2308 to 4431 m a.s.l. Stones are the dominant substrate of many of the rivers and some of them have a considerable slope (up to 16%).

Conductivity of the streams was low from 10 to 100 µS/cm temperature between 5, 6, and 14°C and pH mostly neutral

Table 8 Morphometric characteristics of the pupae of *Cricotopus* (*Oliveiriella*) found in the high tropical Andean rivers. Unless indicated, data are in microns and correspond to the length of the character. Site characteristics are in Tables 1 and 2.

Site	SA10	SA12	AGU14	CHIN02	GUAY04
n	7	1	1	3	5
Project	CERA	CERA	CERA	BIQURA	BICURA/FUCARA
	Mean			Mean	Mean
Th L	929 (889–945)	863	863	943 (917–992)	1053 (1030–1099)
Ab L	2199 (2173–2274)	2267	2292	1946 (1853–2014)	2663 (2552–2801)
L Dc setae 1	175 (160–194)	201	202	50 (41–62)	178 (160–194)
L Dc setae 2	34 (22–58)	63	18	49 (42–59)	34 (14–52)
L Dc setae 3	159 (143–189)	165	183	41 (34–46)	161 (149–172)
L Dc setae 4	23 (18–28)		33	27 (21–35)	21 (13–39)
L Prec setae 1	49 (41–57)	57	51	69 (59–74)	43 (35–54)
L Prec setae 2	196 (187–207)	209	200	80 (77–83)	216 (184–244)
L Prec setae 3	45 (35–59)	39	42	49 (46–52)	26 (14–39)
RO L	226 (220–232)	193	133	249 (233–275)	296 (273–314)
RO W	43 (26–53)	57	20	42 (24–62)	65 (55–84)
HR T-II (n)	10 (8–11)	6	10	14 (14–15)	13 (12–15)
HR T-III (n)	12 (10–14)	8	10	14 (14–15)	14 (11–15)
HR T-IV (n)	11 (8–14)	9	8	12 (11–13)	13 (9–17)
HR T-V (n)	10 (8–13)	9	11	11 (10–11)	12 (9–13)
L Ab setae S-II	128 (112–137)	138	146	106 (95–112)	147 (138–156)
L Ab setae S-III	122 (114–133)	132	145	118 (115–121)	149 (132–159)
L Ab setae S-IV	127 (112–138)	133	136	118 (122–118)	137 (124–153)
Sp S-II (n)	No/small	Small	8	No	8 (4–13)
Sp S-III (n)	No/small	Small	6	No	9 (8–13)
L AS setae 1	20 (18–22)	12	18	17 (15–20)	16 (14–23)
L AS setae 2	19 (15–21)	11	19	13 (11–16)	20 (18–23)
L AS setae 3	19 (15–21)	11	18	21 (19–25)	18 (16–23)
AS distance 2–3	12 (8–15)	7	11	22 (15–27)	9 (8–11)
AS distance 1–2	26 (17–40)	16	31	36 (29–44)	45 (33–73)

L length, *W* wide, *n* number, *Th* thorax, *Ab* abdomen, *Dc* dorsocentral, *Prec* precorneal, *Sp* spine, *T* tergite, *S* sternite, *RO* respiratory organ, *HR* hook row, *AS* anal segment.

Table 9 Morphometric characteristics of the larvae of *Cricotopus* (*Oliveiriella*) in the high tropical Andean rivers. Unless indicated, data are in microns and correspond to the length of the character. Site characteristics are in Tables 1 and 2.

Site		SA10	MO16	MO08	NAPO02	GUAY04
Project		CERA	CERA	BIQURA	BIQURA	BIQURA/FUCARA
n		8	1		1	
Head cap	L	406 (391–412)	368	397 (373–418) (*n* = 4)	390	461 (430–493) (*n* = 16)
	W	311 (293–320)	306	335 (307–359) (*n* = 4)	291	379 (349–403) (*n* = 16)
Postmentum		186 (165–198)	184	185 (174–198) (*n* = 7)	185	220 (201–230) (*n* = 29)
Antenna	Basal L	44 (41–48)	42	46 (44–50) (*n* = 7)	43	56 (49–63) (*n* = 28)
	II L	13 (11–16)	15	14 (12–16) (*n* = 7)	12	15 (12–16) (*n* = 28)
	III L	6 (5–7)	7	5 (5–7) (*n* = 6)	5	6 (4–8) (*n* = 25)
	IV L	5 (3–5)	5	5 (4–5) (*n* = 6)	5	4 (3–6) (*n* = 24)
	V L	3 (2–4)	3	3 (2–4) (*n* = 4	2	3 (2–4) (*n* = 10)
	AR	2 (1–2)	1	2 (1–2) (*n* = 4)	2	2 (2–3) (*n* = 24)
	AB L			27 (22–32) (*n* = 4)		25 (19–33) (*n* = 10)
	LO L			9 (9–9) (*n* = 2)		8 (7–10) (*n* = 5)
Mentum	W	110 (106–125)	106	112 (103–115) (*n* = 6)	108	118 (104–137) (*n* = 25)
M1	W	16 (13–18)	17	17 (17–20) (*n* = 6)	15	21 (18–29) (*n* = 26)
M2	W	8 (7–9)	8	8 (8–9) (*n* = 7)	8	8 (7–11) (*n* = 26)
MR (M1/mentum)		0.14 (0.12–0.17)	0.16	0.157 (0.146–0.162) (*n* = 5)	0.14	0.17 (0.14–0.25) (*n* = 25)

L length, *W* wide, *AR* antennal ratio, *AB* antennal blade, *LO* lauterborn organs.

(Table 2). Oxygen levels were always over 60% saturation with values over 7.91 mg/l. All the streams are oligotrophic with low concentrations of pollutants, including heavy metals. For more details of the physicochemical characteristics of these rivers, see also Villamarín *et al* (2014). Project BIQUIRA samples were collected from reference areas, sites without or with low human disturbance; therefore, although some parameters were not measured in BIQUIRA, the water quality of these streams was good. Sometimes the heterogeneity of substrates in the river may be low (as indicated by the values of IHF lower than 50 in Table 2), and the riparian area may be impacted (values of QBR-And lower than 75, Table 2). Values of QBR-And may be lower than expected for a reference site, due to the absence of a true riparian forest in the high-altitude streams (Acosta *et al* 2009, Acosta & Prat 2010).

Morphological variability of larvae and pupae of high-altitude Andean Cricotopus *(*Oliveiriella*) species*

Larvae. Live larvae of *Oliveiriella* of river Saltana were quite unique for its body color (blue and white), the intense black head capsule and the very long anal tubules (up to 395) that are more than double the length of the posterior parapods (Fig 4e). In living samples, these two characteristics made *Oliveiriella* easily recognizable under the stereoscope at low (×10) magnification. When fixed, the larvae lost the color but still the long anal tubules and the presence of body setae, with a length close to half of the segment wide, made the larvae distinctive in the stereoscope. However, the larvae may lose the anal tubules when treated with KOH, which may create some confusion if only slide material is studied. Some of the larval head characteristics are similar to larvae of individuals of the genera comprised in the *Cricotopus* group (including the bifid SII, the crenulations in the mandible, or the general form of the mentum), but the head capsule of *Oliveiriella* offers some characteristic features that allow us to distinguish these larvae from the rest of the *Cricotopus* group. The color of the head is brown with a pattern of small dots. In the mentum and postmentum, there several line marks, which are characteristic (Fig 4b). Distinction of the larvae of different members of the *Cricotopus* complex in the Andes may be found in Prat *et al* (2012).

According to Andersen *et al* (2013), larvae of *Cricotopus* (*Oliveiriella*) have yellow to light brown head capsules, which is not the case of the *C.* (*Oliveiriella*) larvae of the Andes that have a dark brown head. The same authors include *C.* (*Oliveiriella*) in the group of species with setal tufs and the outer margin of the larvae completely smooth, which is not true in the larvae from the Andes (that wear simple setae and have a rugose outer margin of the mandible) and that otherwise have transversal marks in the gula that are characteristic as well as are the long anal tubules. In fact, in the paper of Krestian *et al* (2009), there is a figure of the mentum of *Oliveiriella* (Fig 3a) where it can be seen that the

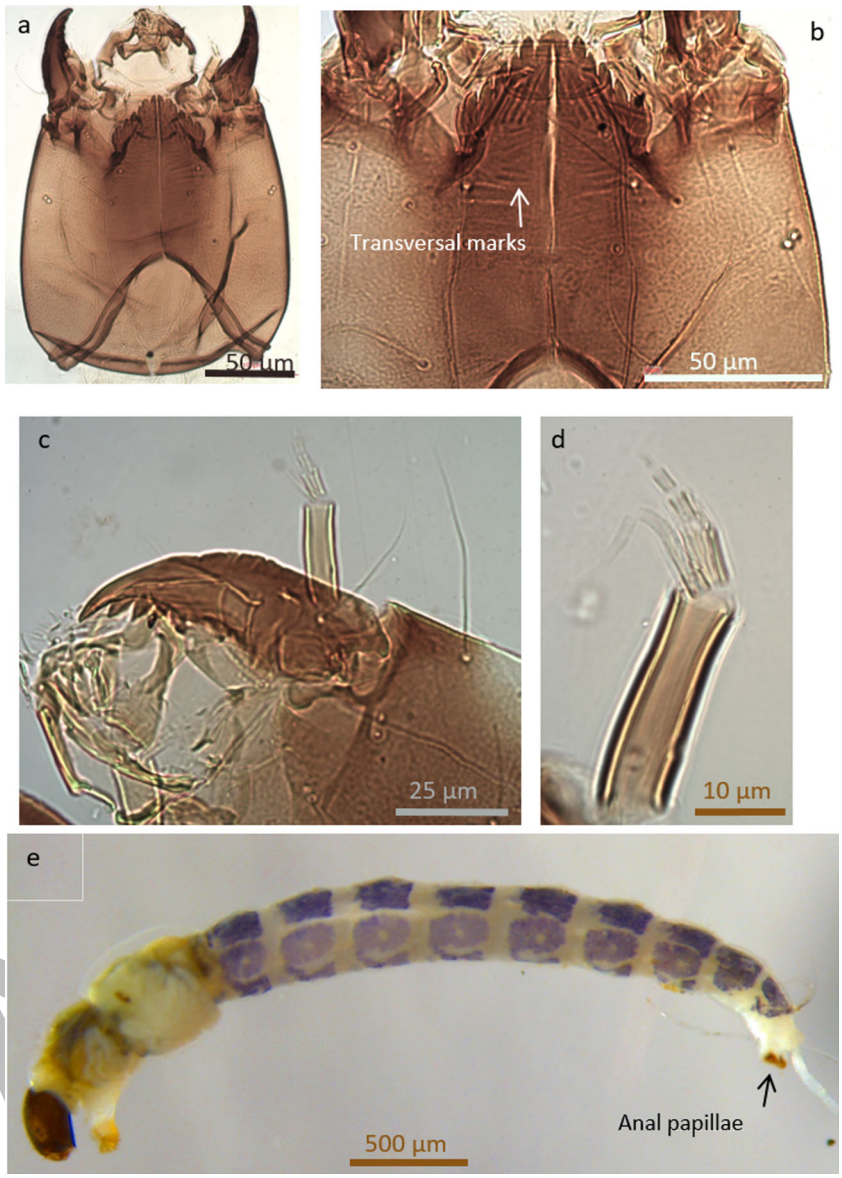

Fig 4 *Cricotopus* (*Oliveiriella*) *rieradevallae* Prat & Paggi sp. n. larvae. **a** Head capsule. **b** Mentum. **c** Mandible. **d** Antennae. **e** Larvae showing the long papillae (picture: Paula Ossa).

mentum is dark and with striae similar to those of ours Fig 4b. The key of Andersen *et al* (2013) should be consequently changed to allow the distinction of the *Oliveiriella* subgenus members from the genus *Cricotopus*.

After measuring a total of 50 larvae from five sites, some differences appear between the larvae of site GUAY04 (Saltana) and the other sites. Larvae of the GUAY04 site are larger, with a higher AR and MR than the other larvae, which are very similar. We use the measure of postmentum as a measure of size of the larvae (see Table 9) because in the slides, the head capsule larval length and width may depend on the position of the larvae and the pressure applied during the preparation of the slide.

Pupae. The pupae of *Oliveiriella* are very characteristic, as described by Wiedenbrug & Fittkau (1997) and Tejerina & Paggi (2009) and very different from any species of the group *Cricotopus* as can be seen in the key of pupae or Prat *et al* (2014). The presence of hook rows (HR) in the segments II to V is characteristic, as well as the size and shape of the three setae of the anal segment that are transformed in small spines (Fig 3f, g). In Table 8, we compare the characteristics of the *Cricotopus* (*Oliveiriella*) pupae and pupal exuviae found in the

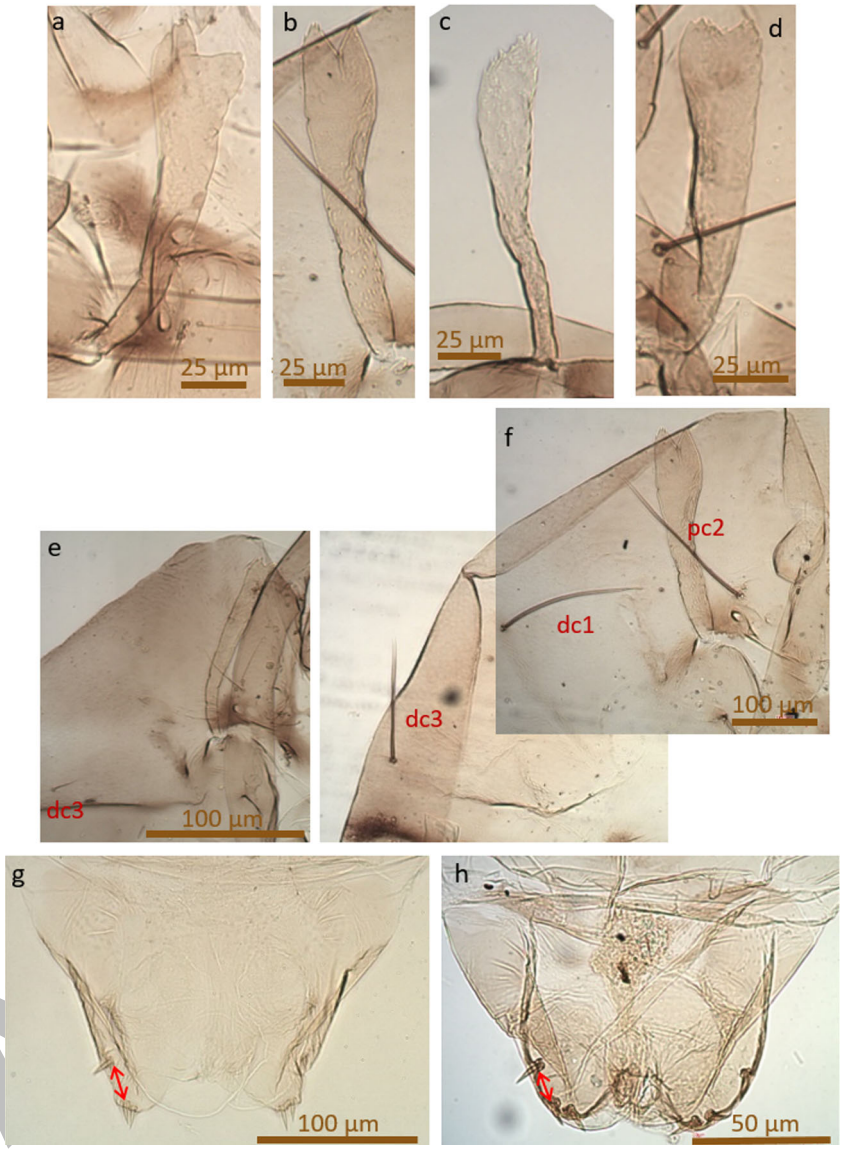

Fig 5 *Cricotopus* (*Oliveiriella*) spp. pupae. Respiratory organ: **a** CHIN02 (Colombia), **b** GUAY04 (Ecuador), **c** AGU14 (Ecuador), **d** SA10 (Peru). Thorax: **e** CHIN02 (short dorsocentral and precorneal setae), **f** GUAY04. Abdominal segment IX: **g** GUAY04, **h** SA10. In *red* the distance between the AS1 and AS2 (see Table 8).

tropical Andean high-altitude streams (5 sites, 19 individuals). There are two different groups of species easily recognizable by the difference of length of precorneal medium setae and the length of dorsal setae 1 and 3 with respect to the dorsal seta 2 (Tables 5 and 8). In the first group, only one specimen appeared in the CHIN02 site (from Chinchiná River). Pupae at this site have both the precorneals and dorsal setae of similar length as in *C.* (*O.*) *almeidai* and *C.* (*O.*) *sanjavieri*, although the setae are shorter (41 to 49 in CHIN02 vs 109 in *C.* (*O.*) *sanjavieri*, Tejerina & Paggi 2009). The second group contains specimens with long dorsal setae 1 and 3 and very long medium precorneal setae (Table 5). In between this large group, two subgroups can also be distinguished, the GUAY04 samples, with spines in the sternites II and III longer than 5 µm, which correspond to *C.* (*O.*) *rieradevallae* sp. n. described in this paper, and the others (*C.* (*O.*) sp. Santa) that do not have such spines (or only very small ones) and according to the molecular analysis belong to undescribed species. Also, the distance between the AS1 and AS2 in these two subgroups is different (Fig 5g, h). The respiratory organ (RO) is of similar length among all the pupae studied except for the sole pupal exuviae found in the Aguarico stream (AGU14 in Table 8; Fig 5c) which is shorter and without final notch, while in all the other pupae, the RO has a distinctive notch in the terminal part that is serrated (Figs 3a and 5a–b, d). The RO of *C.* (*O.*) *almeidai* and *C.* (*O.*) *sanjavieri* do not have this notch. The length of the RO is similar in the pupae that we have examined and similar to the length of the other two species described until now. The mean number of hooklets in the tergites is lower than those found in the two other described species (Table 8) but this number is very variable.

The pupal characteristics facilitated the description of a preliminary key of the morphotypes known at present, which most likely belong to different species.

Preliminary key of the pupae of Cricotopus (Oliveiriella)

1. All precorneal and dorsocentral setae short (max 120 µm) (Fig 5e) 2
- One precorneal setae much longer (over 180 µm) than the other two (Fig 5f). Dorsocentral setae 1 and 3 longer (over 140 µm) compared with 2 and 4 (Fig 3b) 4
2. Respiratory organ with a conspicuous notch (Fig 5a). Less than 20 HR spines in abdominal segment III ..... *C.* (*O.*) sp. Chinchiná
- Respiratory organ without a notch. More than 20 HR spines in abdominal segment III 3
3. RO simple apex, blunt. Length: 181 µm (136–242) ..... *C.* (*O.*) *almeidai* (Oliveira)
- RO serrated at tip: Length: 246 µm (220–280) ..... *C.* (*O.*) *sanjavieri* Tejerina & Paggi
4. Without spines, or with only very small ones, in the abdominal sternites II and III. RO with a distal notch (Fig 5d) ..... *C.* (*O.*) sp. Santa
- With apparent spines (between 6 and 12 µm) in the abdominal sternites II and III (Fig 3d, e). RO with or without a distal notch 5
5. Large species, RO over 3.5 mm and with a notch in the tip (Fig 5b) ..... *C.* (*O.*) *rieradevallae* sp. n.
- Smaller species, RO below 3 mm and without a notch (Fig 5c) ..... *C.* (*O.*) sp. Aguarico

Discussion

In this paper, we describe a new species of *Cricotopus* (*Oliveiriella*) from all their instars and we include the molecular characteristics. The use of molecular analysis confirms the statement of Andersen *et al* (2013), based on morphological characteristics, that *Oliveiriella* must be included in the genus *Cricotopus* as a subgenus. Our molecular data, thus, support the inclusion of *Oliveiriella* as a subgenus of *Cricotopus*.

Cricotopus (*Oliveiriella*) *rieradevallae* sp. n. may be distinguished from the other described species of these subgenera in all three instars. The adult has a different pattern of spots on the wing and different color patterns in the legs and abdomen, as well as a different number of bristles in the squama and clypeus. The pupae may be distinguished by the size and form of the respiratory organ (RO) and the length of the medium setae of precorneals and the length of dorsocentral setae 1 and 3, and the presence of spines in the sternites II and III. Larvae are larger than those of the other species.

In the pupae examined in the sites studied (see Tables 1 and 8), we found very different combinations of the size and form of the RO, in the size of the precorneal and dorsocentral setae, and in the presence or not of spines of sternites II and III. This suggests the possible presence of at least four new species, which is supported by the molecular analysis. All the *Oliveiriella* converge among themselves before converging with other *Cricotopus*. The already known species *C.* (*O.*) *almeidai* (from Argentina) is clearly distinguished from the other species and is more similar to our material from Peru and Colombia than those of Ecuador.

There are four distinct populations of the Andean central area: Ecuador (NA02 and GUAY04), Peru (MO08), and Colombia (VR-6). Interestingly, the two late populations seem to be identical despite the fact that they are more distant than the NA and GUAY materials. Note that all the material was collected at high altitude.

In this paper, we enlarged considerably the distribution of the subgenus *Cricotopus* (*Oliveiriella*) to the north and to the high-altitude rivers. We have found larvae and pupae of *C.* (*Oliveiriella*) at high-altitude Andean streams between 2457 and 3845 m a.s.l., in eight of the 186 sites we studied. Although we found larvae in the Saltana river throughout the year and the adults flying most of the year at this site, the abundance was never as high as other *Cricotopus*. The two species known to be present, i.e., *C.* (*O.*) *sanjavieri* and *C.* (*O.*) *almeidai*, were found in Argentina and Brazil (Paggi & Donato 2007, Tejerina & Paggi 2009, Tejerina & Malizia 2012, Wiedenbrug & Fittkau 1997). Larvae and pupae of an unknown species have been found in North America (Krestian *et al* 2009) which most likely means that the subgenus has a wide distribution in South and Central America. Although we have described only one species in this paper (*C.* (*O.*) *rieradevallae*), the differences found in the pupae and pupal exuviae collected in other high-altitude Andean streams indicate that many species are likely to be present, which is corroborated by the molecular analysis that differentiates *C.* (*O.*) *almeidai* of the Andean species while up to three species may be distinguished from the material collected in the central Andes.

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CAPÍTULO 3

Genotoxic effects and gene expression in *Danio rerio* (Hamilton 1822) (Cypriniformes: Cyprinidae) exposed to mining-impacted tributaries in Manizales, Colombia

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Abstract

The zebrafish (*Danio rerio*) is one of the most studied aquatic organisms for water biomonitoring, due to its sensitivity to environmental degradation and resistance to toxic substances. This study determined the presence of micronuclei and nuclear abnormalities in peripheral blood erythrocytes, and assessed the gene expression of caspase-3 (*CASP-3*) and metallothionein 1 (*MT-1*) in gills and liver of *D. rerio*. The study fish (*n*=45) were exposed to water collected from two stations with mining impact (E2 and E3) and a reference station without evident mining contamination (E1), all located in La Elvira stream (Manizales-Colombia). In addition, a positive control (PC) with $HgCl_2$ (50 µg/L) and negative control (NC) with tap water were included. The fish from the PC and E2 and E3 treatments displayed genotoxic effects and changes in gene expression, with significant differences in micronuclei formation and the presence of blebbed nuclei. The cytochrome oxidase subunit I (*COI*) gene was used as reference and proved to be stable compared to the β-actin and 28S ribosomal RNA (*28S*) genes. In gills, *CASP-3* expression was higher in the PC, and *MT-1* expression was higher in the PC and E3 treatment. In liver, *CASP-3* was expressed in the E2 treatment, and *MT-1* expression was low. These results show that the genotoxic effects and differential gene expression observed in fish exposed to water from La Elvira stream could also be affecting the organisms present in this habitat.

Keywords: Biomonitoring, erythrocytes, gene, genotoxic.

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Introduction

Countries located in the Neotropical region, such as Colombia, present a high water richness associated with an exuberant aquatic biodiversity, which together provide diverse ecosystem services (water availability, food production, climate regulation) and translate into life quality benefits for human settlements (Acosta et al. 2009). However, in recent years, growing urbanization and industrialization processes have generated a strong pressure on these resources as a result of anthropogenic activities associated with hydrographic basics, leading to changes in terrestrial and aquatic ecosystems (Ramírez et al. 2008).

Mining is among the main economic activities of the Andean region in Colombia. The *Eje Cafetero* or Coffee-growing region (departments of Caldas, Quindío, and Risaralda) contains a high percentage of the non-legalized mining in the country (Defensoría del pueblo Colombia 2010). The main impacts generated by mining are basin deforestation and discharge of extremely toxic compounds, which can impact water resources and have negative effects on the biodiversity (Etter and Wyngaarden 2000; Defensoría del pueblo Colombia 2010).

Industrialization and urbanization have led to the development of water biomonitoring methods. Fish can serve as water quality bioindicators, due to their sensitivity to environmental degradation and resistance to heavy metals. Consequently, genotoxic and mutagen bioassays allow evaluating the exposure level to toxic chemical substances of anthropogenic origin present in aquatic ecosystems, and improving environmental monitoring (Al-Sabti and Metcalfe 1995; Kilgour et al. 2005; Clifton et al. 2010; Walia et al. 2013). Among the most used laboratory species for biomonitoring, is the zebrafish, *Danio rerio* Hamilton (1822), originating from Southeast Asia. Its ease of study in the laboratory is due to a number of favorable attributes, including its small size, rapid development, sexual dimorphism, and genome knowledge. These characteristics have long endeared it to investigators in numerous areas, as a model of human disease and development and other disciplines, including animal behavior, fish physiology, and aquatic toxicology, positioning it as an excellent biological model (Fishman, 2001; Lamason et al. 2005).

D. rerio has been previously used in morphological and ontogenic studies, as well as histochemical and gene expression assays in *in vivo* toxicology (Martínez and Espinosa, 2002; Clifton et al., 2010; Dou and Zhang, 2011), and is sensible to environmental degradation, which makes it an excellent bioindicator (Matsumoto and Cólus 2000; Clifton et al. 2010). Fish can absorb toxic substances (for example, heavy metals) through their gills, and bioaccumulate them in organs such as the liver, which leads to effects that include nuclear vacuolation, cytoplasmic collapse, blood vessel congestion, mitochondrial changes, irregularities in hematopoietic tissues, and excessive mucus production in gills that restricts oxygen uptake (Tang et al. 2007; Reis-Henriques et al. 2009; Abdul et al. 2013). In addition, toxic substances accumulation can cause damage to biomolecules such as DNA, RNA, and proteins, leading to carcinogenic and mutagenic effects (Maioli et al. 2011; Walia et al. 2013).

The effects on organisms can be observed through genotoxic and gene expression assays. Genotoxic assays can be studied through the presence of micronuclei and nuclear abnormalities. Micronuclei (MN) are cytoplasmic bodies that contain chromatin and can form during the metaphase-anaphase transition of mitosis, due to the inhibition of chromosome displacement in anaphase (aneugenic event) or the inhibition of mitotic spindle fibers fixation to the kinetochore, which generates acentric chromosomal fragments (clastogenic event) that do not integrate into the daughter cell nucleus following anaphase (Carrasco et al. 1990; Miller et al. 1998; Souza and Fontanetti 2006; Corredor-Santamaría et al. 2012).

Nuclear alterations (NA) can be induced through exposure to toxic substances that impact organisms' natural environments (Ngan et al. 2007; Palhares and Grisolia 2009). Nuclear morphological alterations occur as a result of abnormalities during chromatid segregation in cell division or during gene amplification (Shimizu et al. 1998). Nuclear abnormalities (microcytes, binucleated cells, and notched, lobed and blebbed nuclei) have been identified in peripheral erythrocytes of fish as a consequence of genotoxic or cytotoxic aggressions (Carrasco et al. 1990; Souza and Fontanetti 2006; Palhares and Grisolia 2009; Corredor-Santamaría et al. 2012).

Molecular markers development allows studying gene expression, which can be assessed as the response of organisms to certain environmental conditions. Genes such as

metallothionein 1 (*MT-1*), Caspase-3 (*CASP-3*), Caspase-9 *(CASP-9*), and Cytochrome p450 1A (*CYP 1A*) have been studied in expression studies induced under exposure to heavy metals (Swain et al. 2004; Quirós et al. 2007; Deng et al. 2009; Jin et al. 2010; Zhang et al. 2015).

In this regard, and considering that industrialization and mining in Manizales (Caldas, Colombia) generates effluents that affect tributaries such as the Chinchiná River basin, this work aimed to study the possible effects of mercury-contaminated waters on *D. rerio*, through the assessment of micronuclei (MN) and other nuclear alterations (NA) in erythrocytes, as well as gene expression in the gills and liver.

Materials and methods

Study area and collection of water samples

The study area included three stations in La Elvira stream that belongs to the eleven tributaries of Manizales creek, and is one of four streams that are currently of great importance due to their contaminating load, as it collects waste water from gold mining exploration processes (Corporación Autonoma Regional de Caldas Corpocaldas 2015). La Elvira stream is located in the Chinchiná River basin in the municipality of Manizales, department of Caldas (Colombia). The three sampling stations were established under the framework of the project: Evaluation of the impact of mining, agriculture and livestock farming in the ecological and genetic responses of aquatic macroinvertebrates.

Despite records of contaminating load in La Elvira, this stream is not currently monitored (Corporación Autonoma Regional de Caldas Corpocaldas 2015). Therefore, in this study, we conducted three sampling events, where we measured physical, chemical, and hydrobiological variables in low, moderate, and high rains seasons. The high rains sampling event was done on November 04 of 2014. Additionally, 6 L-water samples were collected from the water column of each station, which were subsequently used for the genotoxic assays. Since water samples were collected for the biological assays, the stabilization methods normally used for chemical analyses, such as pH adjustment through addition of specific chemicals, could not be done, since these could lead to sample toxicity. Furthermore, the ISO 5667-16 normativity recommends taking into account storage period duration and conservation mode efficiency (Castillo 2004); thus, once collected, the

samples for the bioassays were processed as soon as possible, in order to avoid changes in their original composition, as a result of physical or chemical reactions and/or biological processes. The maximum storage period did not exceed five hours at room temperature, under darkness and in styrofoam freezer boxes.

The E1 sampling station (05°03'10.9"N 75°24'33.6"O) was chosen as reference, due to a wide river-margin vegetation (≥ 10m) and no evident anthropogenic impact (lack of any evident mining impact) (Gutiérrez-López et al. 2016). The other two sampling stations (E2 and E3) (05°03'4.4"N 75°24'33.1"O; 5°1'53"N 75°24'43.8"O) are impacted by effluents generated from gold mines (Bastidas and Ramírez 2007 unpublished work). The E1, E2, and E3 sampling stations are located at 2766, 2725, and 2611 meters of altitude, respectively, and they share a similar physical habitat characterization, with an undulating topography, the presence of riparian vegetation, and open canopy coverage (0-25% of the stream was shaded).

Physicochemical water analysis

On February 12, July 08 and November 04 of 2014, the following physical water variables were measured *in situ*: temperature, pH, conductivity, dissolved oxygen, and oxygen saturation (multi-parameter Lutron™). Additionally, 8 L-water samples were collected from the water column from each station, and the following chemical variables were measured at the laboratory: chemical oxygen demand (COD), biological oxygen demand (BOD), total suspended solids (TSS), total solids (TS), cyanide (CN), boron (B), lead (PB), mercury (Hg), ammoniacal nitrogen (NH_3-N), phosphates (PO_4), sulfates (SO_4), iron (Fe), chloride (Cl^-), lipids and oils, nitrates (NO_3), nitrites (NO_2) and aluminum (Al). The analysis of these variables was performed by ACUATEST S.A. (Online Resource Table A 1), and IDEAM-certified laboratory, under norm ISO-17025.

Test organism and genotoxic assays

Adult wild-type zebrafish (*D. rerio*) (4-5 cm) were obtained from a commercial provider in Manizales, Caldas (Colombia). The fish were kept in 20 L glass aquariums, acclimated during 15 days with aeration, pH 7.0, constant temperature (21°C), 12 h-light/12

h-dark photoperiod, and fed once a day with TetraMin® (Corredor-Santamaría et al. 2012; Marín et al. 2014).

Following the acclimation period, the fish were randomly assigned to groups of three individuals per aquarium (2 L capacity) and were exposed for 12 hours to water collected from the sampling stations. The control groups included a negative control (NC) with tap water, and a positive control (PC) with mercuric chloride ($HgCl_2$) 50 µg/L (Hassaninezhad et al. 2014). HgCl concentration was chosen according to previous studies in other fish species, with treatments from 10 µg/L to 100 µg/L (Hassaninezhad et al. 2014; Naranjo-Gómez et al. 2014). Each treatment included three replicates, for a total of 45 fish. Erythrocyte and gene expression assays were conducted at the Molecular Biology Laboratory of the Department of Biological Sciences at the Universidad de Caldas in Manizales (Caldas-Manizales).

Micronuclei and nuclear alterations test

Following 12 hours of exposure, fish were anesthetized by cold shock in order to obtain blood samples through ventral punctures with heparinized needles. Three blood smears per organism were done in order to determine normal cell frequency (N) compared to micronuclei (MN) and nuclear alterations (NA) frequencies. The blood smears were stained for 10 minutes with Wright's stain.

Slide reading was carried out by counting 2000 erythrocytes per slide (Corredor-Santamaría et al. 2012). Only blood cells with intact nuclear and cytoplasmic membranes were counted, while those with overlapped or damaged membranes were discarded (Al-Sabti and Metcalfe 1995). The following criteria were considered for MN identification: MN diameter inferior to 1/3 of the main nucleus, clear separation from the nucleus, same color and intensity, and presence in the cell cytoplasm (Grisolia 2002). MN count was expressed as a percentage (CMN %), calculated by the number of micronuclei observed per 2000 erythrocytes. In addition, the Proliferation Index (PI) was calculated, which is based on the CMN % of the negative control compared to the CMN % of the experimental samples.

Morphological nuclear abnormalities (NA) in erythrocytes were classified according to the criteria of Carrasco et al. (1990); Souza and Fontanetti (2006). These corresponded to

blebbed nuclei (BL) that show a small invagination in the nuclear membrane and containing euchromatin; binucleated cells (BN); lobed nuclei (LB) with characteristic micronuclei connected to the nucleus through a stem of nucleoplasmic material; notched nuclei (NT) that show a marked notch with nuclear material; and, finally, microcytes (MC), which are micronuclei eliminated by the cell. Each NA was counted according to its type and expressed as a percentage (for example, NA: LB %), calculated from the number of micronucleated erythrocytes observed per 2000 cells.

MN and NA observations were carried out under a Leica DM500 optical microscope with 1000X magnification. For each individual, the frequency of micronuclei and other nuclear abnormalities was calculated, and significant differences in the distribution of normal cells and cells with MN and NA were determined between the treatments and controls, using the Kruskal-Wallis non-parametric test and Dunn's multiple comparisons test. All of the statistical analyses were carried out in R version 2.15.3 (R Development Core Team 2014).

RNA extraction and cDNA synthesis

Forty five organisms were dissected, their liver and gills were labeled and conserved separately in 350 µl of RNA*later* (Qiagen®) at (-86°C). Messenger RNA (mRNA) was isolated using the ZR Tissue and Insect RNA MicroPrep™ kit (Zimo Research®), and genomic DNA was eliminated through *DNase* digestion with the *RNase-free DNase* kit (Qiagen®), according to the manufacturers' instructions. Finally, the RNA was resuspended in 10 µl *RNase*-free water and stored at (-86°C).

RNA quality was assessed through electrophoresis in 1% agarose gels, as well as a NanoVue™ Plus spectrophotometer. RNA quantification was carried out on a Qubit® 2.0 fluorometer, using the RNA HS Assay kit (Invitrogen®), according to the manufacturer's instructions. First strand cDNA synthesis from gill and liver RNA (1 ng/µl and 5 ng/µl, respectively) was done using the SuperScript® III First-Strand Synthesis SuperMix kit (Invitrogen®), with oligo$(dT)_{20}$ (50µM), according to the manufacturer's instructions.

Molecular confirmation of *D. rerio* and quantitative real-time polymerase chain reaction (qRT-PCR)

The molecular confirmation of *D. rerio* was done by amplifying a segment of the mitochondrial cytochrome oxidase subunit I (*COI*) gene with primers LCO1490 (F) 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 (R) 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). The final polymerase chain reaction (PCR) volume was 40 µL, containing 21.4 µL ultrapure water, 8 µL 5X PCR buffer, 2.4 µL $MgCl_2$ (25 mM), 3.2 µL dNTPs mix (10 mM), 0.8 µL of each primer (25 µM), 2 U GoTaq® Flexi DNA Polymerasa (Promega®), and 3 µL cDNA. The amplifications were carried out on a Techne TC-PLUS thermocycler with the following conditions: Initial denaturation at 94°C for 5 min, followed by 5 cycles at 94°C for 5 min, 46°C for 1 min 30 s and 72°C for 1min 30 s, followed by 35 cycles at 94°C for 1 min, 53°C for 1 min and 72°C for 1 min, completing the reaction with a final extension at 72°C for 5 min.

The following genes were used as candidate endogenous or reference controls: β-actin (Deng et al. 2009; Zhang et al. 2015), 28S ribosomal RNA (*28S*) (Ossa et al. 2012), and cytochrome oxidase I (*COI*) gene. The following genes were used as targets: Metallothionein 1 (*MT-1*) (Quirós et al. 2007), Caspase-3 (*CASP-3*) (Deng et al. 2009; Jin et al. 2010; Zhang et al. 2015); Caspase-9 (*CASP-9*) (Jin et al. 2010), and cytochrome p450 1A (*CYP 1A*) (Quirós et al. 2007). The primers that amplified the *COI* gene segment were designed from *D. rerio* sequences (Genbank accession JQ667531.1), using the Primer-BLAST tool (Table 1).

Gene amplification specificity was assessed through a classic PCR, which consisted of a final PCR reaction volume of 40 µL, containing 21.8 µL ultrapure water, 8 µL 5X PCR buffer, 2.4 µL $MgCl_2$ (25 mM), 3.2 µL dNTPs mix (10 mM), 0.6 µL of each primer (25 µM), 2 U GoTaq® Flexi DNA Polymerasa (Promega®), and 3 µL of cDNA. The amplifications were carried out on a Techne TC-PLUS thermocycler under the following conditions: Initial denaturation at 95°C for 10 min, followed by 41 cycles at 95°C for 30 s, 50°C for 20 s and 72°C for 1 min, completing the reaction with a final extension at 72°C for 7 min.

The PCR products were visualized on horizontal 1% agarose gels with 1X TBE pH 8.0 running buffer at 110 v/50 mA, stained with SYBR Safe® dye and visualized on a GelDoc-It®2 310 Imager (UVP). The PCR products were purified with the QIAquick PCR

purification kit (Qiagen®), according to the manufacturer's instructions, and were sequenced at Macrogen Inc. Korea and SSigMol of the Institute of Genetics of the Universidad Nacional de Colombia. The sequenced fragments were evaluated and edited using Geneious Trial v8.14 (Drummond et al. 2009) and Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). In addition, the sequences were search by MegaBlast against the public databases in order to confirm the gene sequence, and were deposited in Genbank accessions.

The qRT-PCR reactions were carried out on a StepOnePlus thermocycler (Applied Biosystem®), with a final reaction volume of 20 μL and a 10% excess to avoid volume variations. The qRT-PCR reaction contained 11 μL SYBR® Select Master Mix with ROX (Applied Biosystem®), 0.374 μL of each primer (10 μM), 8.052 μL of DEPC-treated water (DEPC; Invitrogen®), and 2.2 μL of cDNA.

The qRT-PCR efficiency ($E= 10^{-1/m}$) was determined for each reference and target gene primers (Pfaffl 2001), based on a pool of 2 μL of each cDNA (control and treatment samples) from the liver and gills separately. Four serial dilutions and a control reaction were run, with three replicates each, and a relative standard curve was constructed for each primer pair. PCR efficiencies were used to convert the threshold cycle values (*Ct*) into net data (relative quantities).

Based on the PCR efficiency, a dilution of each treatment sample (E1, E2, and E3) and control (NC and PC) were run, with three replicates each, together with negative qRT-PCR control reactions in which cDNA was replaced by DEPC-treated water.

The PCR program (Standard ≤60°C) consisted of an initial incubation step for 2 min at 50°C and denaturation for 2 min at 95°C; followed by 40 cycles of denaturation for 15s at 95°C, annealing for 15s at 58°C, and extension for 1 min at 72°C. Finally, a melting curve was run from 58°C to 95°C with a temperature increase of 1°C every 10 s. The melting curves allowed checking that the amplification corresponded to a single specific product, observed by a single peak.

The *Ct* values for each of the three candidate reference genes were analyzed with GeNorm (Vandesompele et al. 2002); NormFinder (Andersen et al. 2004); BestKeeper v. 1 (Pfaffl et al. 2004), and the delta-Ct (Δ*Ct*) comparative method (Silver et al. 2006). Relative gene expression, or relative quantification (RQ), was calculated according to the formula

($\Delta\Delta Ct$): Relative expression = $2^{-[\Delta Ct\ sample-\Delta Ct\ control]}$. The standard deviation (SD) value was also calculated for each relative gene expression value as a measure of the amount of variation in the data.

Results and discussion

Micronuclei and nuclear alterations assay

At 12 hours post-treatment, no mortality or macroscopic lesions were observed in any of the fish exposed to the treatments (E1-E3) and controls (NC and PC). Most genotoxicity studies use fish exposed to heavy metals during 24h-96h periods in order to induce MN and NA in erythrocytes (Heddle et al. 1991; Hassaninezhad et al. 2014; Naranjo-Gómez et al. 2014). However, for *D. rerio*, as early as 12 h of exposure induces the presence of MN and NA (Fig. 1a-1l). Significant differences were found between the number of normal cells (N) (H=22.60, gl=4, p<0.001; Fig. 2a) and cells with MN (H=29.23, gl=4, p<0.001; Fig. 2b) (Online Resource Table A 2).

In total, NA, which included blebbed (BL), lobed (LB) and notched (NT) nuclei, binucleated cells (BN) and microcytes (MC), did not show significant differences between treatments and controls (H=9.33, gl=4, p=0.053; Fig. 2c). This can be possibly associated with the experiment's exposure time, in which abnormalities increase beginning from 24 h and generally decrease after 96 h (Walia et al. 2013). However, blebbed nuclei (BL) showed significant differences (H=10.21, gl=4, p=0.040) (Fig. 2d) (Online Resource Table A 2). Shimizu et al. (1998) report that budding of the nuclear membrane during interphase can cause blebbed (BL) and lobed (LB) nuclei during the S phase and these buds can contain genetic information related with oncogenes. The results obtained could indicate that blebbed nuclei (BL), with significant differences, could be associated to cancerigenous cells due to anthropic contamination, as has been reported for other organisms (Beyersmann and Hartwig 2008). Furthermore, abnormalities formation is a way to eliminate any extra genetic material from the nucleus and maintain cellular integrity. In addition to this, heavy metals interfere with chromosomal segregation during cell division, due to inhibition of actin tubules polymerization and interference with mitotic spindle formation (Miura and Imura 1987; Kosai et al. 2011).

The MN assay indicated the putative presence of contaminants capable of affecting the stream's water quality in the E2 and E3 stations. Data from literature has shown a relationship between genomic instability and the presence of abnormalities arising from industrial contaminants related to heavy metal effluents (Serrano-Garcia and Montero-Montaya 2001; Fenech and Crott 2002). Although MN and NA formation can also occur spontaneously in fish erythrocytes (Al-Sabti and Metcalfe 1995), variations in their occurrence frequency could be possibly related to the organism's inherent characteristics associated with ingesting, accumulating, metabolizing, and/or efficiently excreting substances (Jha 2004; Souza and Fontanetti 2006). This could explain the presence of MN and NA in the NC and E1 treatment. Likewise, other factors, such as DNA repair efficiency and cellular elimination kinetics could also be involved in MN and NA variation (Bolognesi and Hayashi 2011; Walia et al. 2013; Furnus et al. 2014).

The Proliferation Index (PI), obtained from the comparison between CMN % and its respective control, showed a value of 7.27 for the PC, 2.2 for E1 treatment, 3.23 for E2 treatment, and 18.1 for E3. The highest PI values were observed for treatments with water from the mining-exposed stations (E2 and E3) and the PC. These results confirm that the presence of heavy metals in aquatic environments can have serious consequences in the well-being of organisms exposed to these substances. However, the physicochemical variables of E1are found within the range established by Decree 1594 of 1984 of the Ministry of Agriculture of Colombia, which dictates water uses and delimitates the water characteristics for each use (Online Resource Table A 1).

RNA extraction and cDNA synthesis

RNA integrity observed through electrophoresis showed RNA bands corresponding to the 18S and 28S ribosomal subunits (Fig. 3a). The spectrophotometry reading showed an RNA purity ≥ 1.8 at an absorbance ratio of 260/280 nm (Sambrook et al. 1989; Wilfinger et al. 1997). The fluorometry reading indicated an RNA concentration ranging from 50.5 ng/mL and 860 ng/mL, which is found within the optimal range for cDNA synthesis (Fleige et al. 2006; Gallagher and Desjardins 2007).

Molecular confirmation of *D. rerio* and quantitative Real-Time PCR (qRT-PCR)

The identification tool (IDS) from the Barcode of Life Data Systems (BOLD) showed a 100% similarity to the species *D. rerio* for the four sequences obtained (Genbank accessions KY684196-KY684199; MF685020- MF685023). Furthermore, the results retrieved from MegaBlast confirmed the identity and similarity to the β-actin, 28S ribosomal RNA (*28S*), Metallothionein 1 (*MT-1*), Caspase-3 (*CASP-3*), and Cytochrome oxidase subunit I (*COI*) genes (Table 1).

The genes that showed optimal expression levels under the following amplification parameters were used to evaluate gene expression in the gills and liver from *D. rerio*: Ct >8 and <35, amplification efficiencies between 90-120%, correlation coefficients above 0.95, standard deviations (SD) <0.38, and no evidence of primer dimers (for example, Fig. 3b-3d) (Applied Biosystems 2010). These parameters agree with Ridgeway and Timm (2015), and the genes that did not meet them were excluded from the analyses (Table 1).

The assessment of the potential reference genes, *COI* and *28S*, reported in other organisms (Tang et al. 2007; Xue and Cheng 2010), and β-actin, previously reported for *D. rerio* (Deng et al. 2009; Zhang et al. 2015), showed that the *COI* gene is more stable for normalization, so it was chosen as the reference gene (Fig. 3b-3d), followed by β-actin. This tendency is the same for both tissues and is observed with all of the software used: GeNorm, NormFinder, and BestKeeper, as well as the ΔCt comparative method (Table 2). The *COI* gene has been reported as a normalizer that can potentially vary between tissues and taxonomic groups (Ridgeway and Timm 2015). This has also been observed for β-actin, which, to date, has been the most commonly used reference gene for qRT-PCR data normalization in zebrafish (*D. rerio*), due to its stability attributed to its role in cellular motility. On the contrary, literature reports indicate that the expression of this gene is not stable in terms of analyzing tissue groups. β-actin has been found differentially expressed between tissue samples (Gutala and Reddy 2004; Yperman et al. 2004; Olsvik et al. 2005; Ingerslev et al. 2006; Xue and Cheng 2010). Although the *28S* gene has been reported as an adequate reference gene in other organisms (Xue and Cheng 2010) and the primers used here have performed adequately in classic PCRs in several taxonomic groups, including *D. rerio*, the qRT-PCR results indicated that it is not a good reference gene for the study (Table 2). These results prove the importance of testing the selection of the reference gene under the specific conditions of the experiment (Pfaff et al. 2004).

The following results were found based on the *CASP-3* and *MT-1* target genes assessed in gills and liver tissues: *CASP-3* gene expression in gills is higher for the PC, *MT-1* gene expression in gills for the PC and E3 treatment was the highest (Fig. 4a). In liver, *CASP-3* gene expression was highest in the E2 treatment, and *MT-1* gene expression was low in the PC and E3 treatment (Fig. 4b).

CASP-3 and *MT-1* gene transcription is related to metallic and organic xenobiotic contamination, which can cause oxidative stress and induce MN formation, as well as result in apoptotic (*CASP-3*) or antiapoptotic (*MT-1*) processes through a series of signaling cascades that do not depend solely on these genes, although their regulation and expression can be influenced by several factors, such as temperature, concentration, and exposure time (Decordier et al. 2005; Pedersen et al. 2009; Gao et al. 2013).

In fish, exposure to mining effluents can affect organs such as the gills and liver, due to the absorption and bioaccumulation of these wastes in the organism, thereby, affecting its metabolism and detoxification (Walia et al. 2013; Furnus et al. 2014).

Conclusions

D. rerio is not a native species of the study area, although their great potential for *ex situ* studies, their sensitivity to environmental degradation and resistance to heavy metals, which makes it an excellent water quality bioindicator. The results show that the fish exposed to the treatments of water collected from stations with evidence of mining impact, presented changes in the cell nucleus (micronuclei, blebbed nuclei) and changes in the gene expression of metallothionein 1 (*MT-1*) and caspase-3 (*CASP-3*).

In this regard, the results obtained display the effects caused in fish as a consequence of mining-contaminated-water conditions in the Upper Chinchiná River basin, which could also be responsible for genotoxic effects in the organisms that inhabit the study area.

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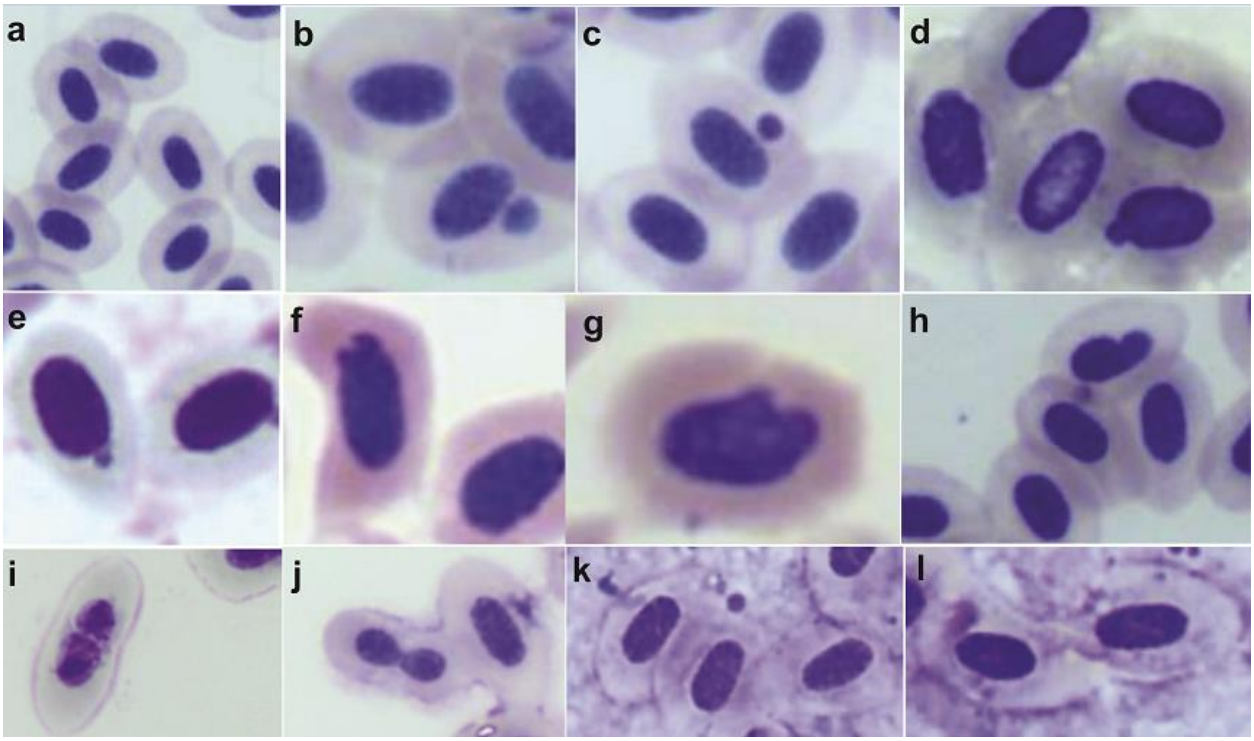

Fig. 1 Erythrocytes assessed in *Danio rerio*. **a** Normal cells (N); **b-c** Cells with micronuclei (MN), Various nuclear abnormalities. **d-e** Cells with blebbed nuclei (BL); **f-g** Cells with lobed nuclei (LB); **h** Cells with notched nuclei (NT); **i-j** Binucleated cells (BN); **k-l** Microcytes (MC) (Light microscopy – LM, Leica DM500).

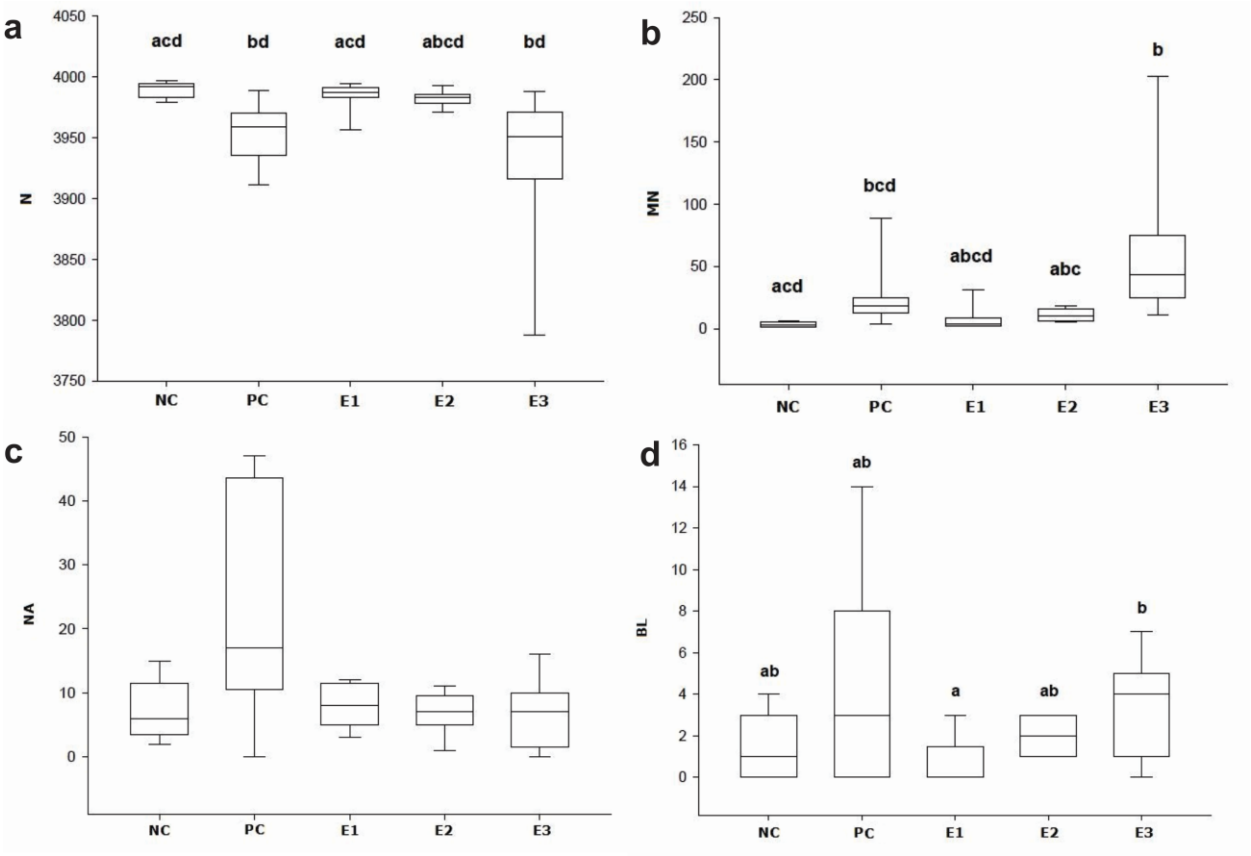

Fig. 2 Erythrocyte frequency in *Danio rerio* following 12 h of exposure: Reference station (E1): Mining-impacted stations (E2 and E3): Negative control, tap water (NC): Positive control, $HgCl_2$ 50 µg/L (PC). Different letters between the bars indicate significant differences. **a** Normal cells (N); **b** Cells with micronuclei (MN); **c** Cells with nuclear abnormalities (NA); **d** Cells with blebbed nuclei (BL) (R version 2.15.3).

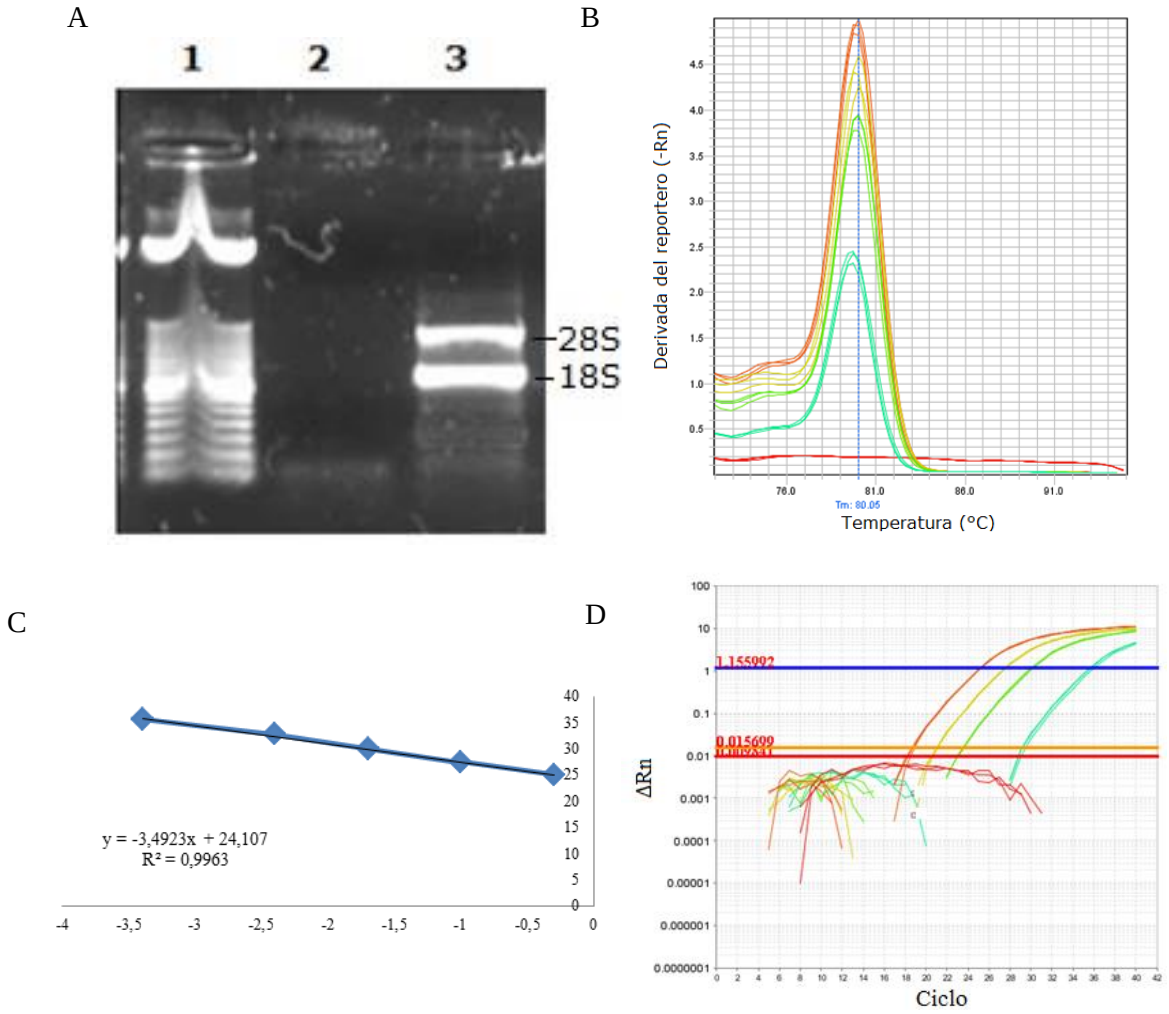

Fig. 3 Molecular analyses in *Danio rerio*. **a** Electrophoretic profile of total RNA in a 1% agarose gel with 1X TBE. Lanes: 1, molecular weight marker (1Kb plus Ladder); 2, negative control; 2, total RNA sample from *D. rerio* in the reference station (E1). Stain: SYBR Safe®, separation conditions at 80V for 45 min (GelDoc-It®2 310 Imager-UVP). **b** Melting curve for the cytochrome oxidase I (*COI*) reference gene, -Rn: derivative of the normalized fluorescence (StepOnePlus thermocycler - Applied Biosystem®); **c** Linear regression for the *COI* gene (Microsoft Excel 2010); **d** Fluorescence curve for the *COI* gene, ΔRn: fluorescence of the different normalized fluorochromes (StepOnePlus thermocycler - Applied Biosystem®).

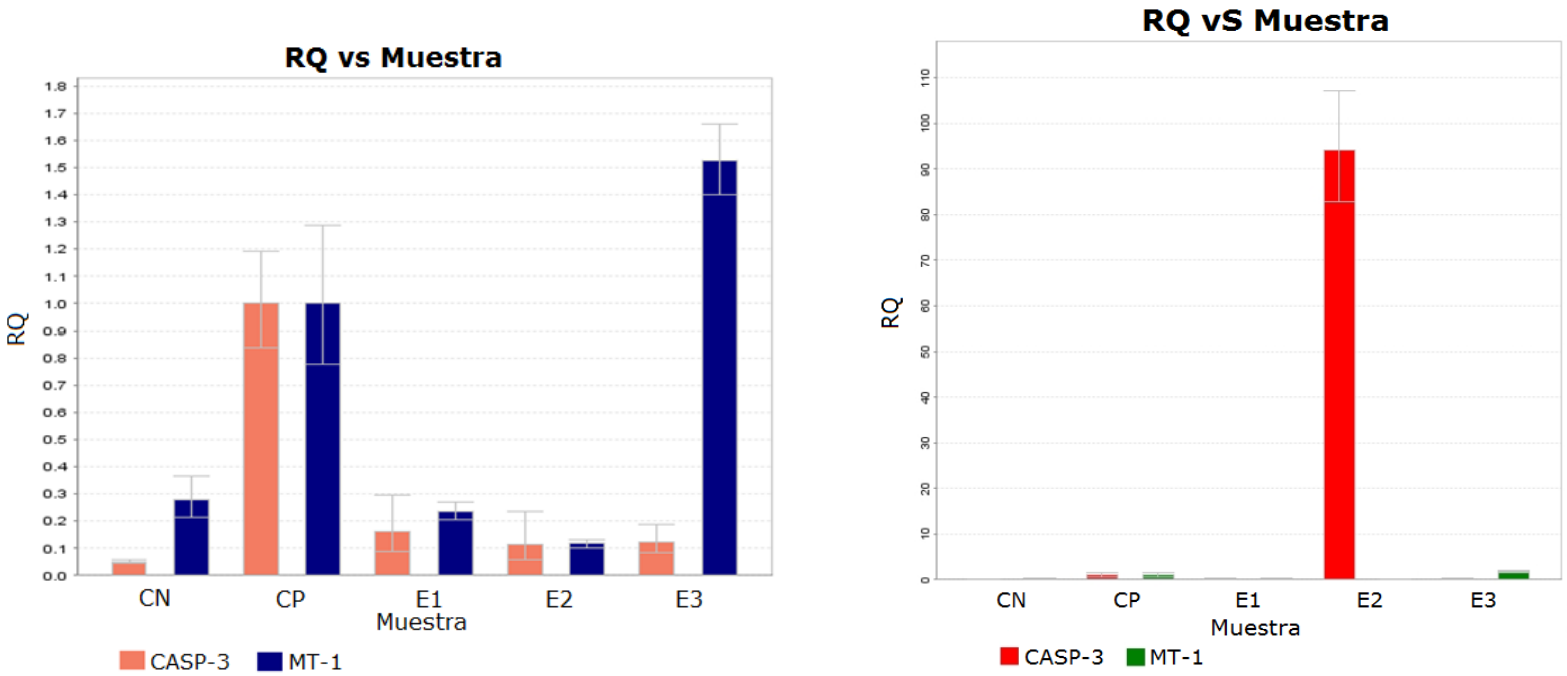

Fig. 4 Relative quantification (RQ) in *D. rerio* following 12 h of exposure: Reference station (E1); mining-impacted stations (E2 and E3); negative control, tap water (NC); Positive control $HgCl_2$ 50 µg/L (PC); Target genes: caspase-3 (*CASP-3*) and metallothionein 1 (*MT-1*); Reference (normalizer) gene, Cytochrome oxidase subunit I (*COI*). **a** In gills; **b** In liver (StepOnePlus thermocycler - Applied Biosystem®)

Table 1. Information of the qPCR primers used.

Gene	Primers	Sequence 5´-3´	%Ident.[a]	E-1*100 (%)[b]	R^{2}[c]
β-actin	BActF	CGAGCAGGAGATGGGAACC	99.7	93	0.99
	BActR	AACGGAAACGCTCATTGC			
28S	28SForward	CGGTAACGCGACCGATCCCG	100	93	0.99
	28SReverse	CCTCTCTCGGGGCGAACCCA			
Metallothionein -1	MT1F	AATTGTGGTGCCACCTGCAAGTGC	100	96	0.99
	MT1R	GACCTCCTCACTGACAGCAGCTGG			
Caspase - 3	Caspase-3F	CCGCTGCCCATCACTA	100	93.4	0.99
	Caspase-3R	ATCCTTTCACGACCATCT			
Caspase - 9	Caspase-9F	AAATACATAGCAAGGCAACC	n.d.	n.d.	n.d.
	Caspase-9R	CACAGGGAATCAAGAAAGG			
Cytochrome p450 1A	CYP1A-F	CACTGACTCCCTCATTGACCAC	n.d.	n.d.	n.d.
	CYP1A-R	ACAGATCATTGACAATGCCCAC			
Cytochrome oxidase I	COIF	TATTTGGTGCTTGAGCCGGA	100	93.3	0.99
(COI)	COIR	AGTGCTCCTGGTTGGCTAAG			

[a]Sequence identity percentage (Ident.) between the amplicon and the Genbank reference sequence.

[b]Measurement of the qPCR reaction efficiency (E) (calculated through the relative standard curve method).

[c]qPCR reaction reproducibility (R^{2}).

n.d. = no data; the gene was excluded from the study.

Table 2. Stability analysis of the candidate reference genes for gills and liver in *D. rerio.*

Gene	Tissue	Stability value			
		GeNorm	**NormFinder**	**BestKeeper[a]**	**Delta Ct**
COI	Gills	0.945	0.472	0.32	1.162
	Liver	0.447	0.145	0.32	0.713
β-actin	Gills	0.945	1.198	0.765	1.457
	Liver	0.447	0.423	0.45	0.752
28S	Gills	1.432	1.564	1.12	1.675
	Liver	0.827	0.968	0.76	1.018

[a] Significant differences per gene: COI p-value<0.010; β-actin p-value <0.036; 28S p-value <0.003.

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Table A 1. Physical, hydrobiological, and chemical characteristics of the sampling stations. Reference station (E1). Mining-impacted stations (E2 and E3).

Variables	Parameter	Measurement units	Sampling station		
			E1	E2	E3
Physical	Water temperature	°C	11.7	14.7	14.6
	pH		7.28	7.42	7.56
	Conductivity	µS	117	225	182
	Dissolved oxygen	mg/L	12.6	4.77	4.37
	Oxygen saturation	%	162.4	60.7	59.1
Chemical	Chemical oxygen demand	mg/L	15	15	176
	Biological oxygen demand	mg/L	3.21	3.21	15.3
	Total suspended solids	mg/L	8	551	1619
	Total solids	mg/L	48	604	1716
	Cyanide	mg/L	0.1	0.1	0.1
	Boron	mg/L	0.8	0.8	0.8
	Lead	mg/L	0.01	0.01	0.01
	Mercury	mg/L	0.001	0.001	0.024
	Ammoniacal nitrogen	mg/L	0.1	0.1	0.1

	Phosphate	mg/L	0.5	1.7	5.7
	Sulfate	mg/L	21	86	147
	Iron	mg/L	0.21	1.5	5.88
	Chloride	mg/L	2.5	3.6	5.9
	Lipids and oils	mg/L	0.5	1.7	0.9
	Nitrates	mg/L	1	1	1.5
	Nitrites	mg/L	0.07	0.58	1
	Aluminum	mg/L	0.2	1.68	20.28

Environmental Monitoring and Assessment

Electronic Supplementary Material

Table A 2. Frequency of normal erythrocytes (N), micronuclei (MN), and nuclear abnormalities (NA) in *D. rerio*, following 12 h of exposure: Reference station (E1). Mining-impacted stations (E2 and E3). Negative control, tap water (NC). Positive control, $HgCl_2$ 50 µg/L (PC).

						NA											
	Evaluated	N		MN		BN		LB		NT		BL		MC		TOTAL	
Condition	Cells	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
NC	36000	**35901**	99,725	**30**	0,083	**20**	0,056	**15**	0,042	**22**	0,061	**12**	0,033	**0**	0	69	0,136
PC	36000	**35577**	98,825	**218**	0,605	**29**	0,081	**39**	0,108	**90**	0,250	**40**	0,111	**7**	0,019	205	0,469
E1	36000	**35862**	99,622	**66**	0,183	**11**	0,031	**22**	0,061	**29**	0,078	**6**	0,017	**4**	0,011	72	0,156
E2	36000	**35842**	99,561	**97**	0,269	**9**	0,025	**10**	0,028	**20**	0,056	**17**	0,047	**5**	0,014	61	0,131
E3	36000	**35395**	98,319	**543**	1,508	**4**	0,011	**15**	0,042	**12**	0,033	**29**	0,081	**2**	0,006	62	0,156

n= Number of cells.

CONSIDERACIONES FINALES

- *Andesiops peruvianus* es un complejo de especies por lo que se hace necesario continuar con su clarificación taxonómica, y así realizar posteriores investigaciones que permitan evidenciar si puede ser considerada(s) como una especie bioindicadora de la calidad de los ecosistemas acuáticos. Con el fin de contribuir a este proceso, se realizó adicionalmente la amplificación de los genes citocromo oxidasa subunidad I (COI), 28S, metalotioneína 1 (MT-1) y caspasa 3 (CASP-3), a partir del ADNc de la(s) especie(s) *Andesiops peruvianus*, secuencias que serán sometidas al *Genbank*, y que se pueden a futuro aportar en investigaciones integrales de monitoreo hídrico.

- Las herramientas morfológicas y moleculares permiten posicionar al *Género 1* sp. *2* como una especie bioindicadora de la calidad del agua, variaciones morfométricas en el área dorsal de la cabeza (DHAr) y la presencia de deformidades y abundancias diferenciales en las estaciones de muestreo evaluadas, permiten potenciar su aplicación en estudios de biomonitoreo hídrico.

- Es necesario continuar con trabajos taxonómicos en *Cricotopus (Oliveiriella) rieradevallae* sp. Sin embargo se destaca la abundancia de sus larvas en IV estado larvario (*n*=24) en la estación ubicada en la quebrada Romerales del Municipio de Villamaría, Caldas, frente a las larvas capturadas (*n*=1) en la estación de la quebrada Californa (con impacto evidente por minería) ubicada en el mismo municipio. Destacando la importancia que puede tener esta especie en los estudios de biomonitoreo hídrico, por ser una especie susceptible a la perturbación antrópica.

- *Danio rerio* sin ser una especie nativa de la zona de estudio, pero su gran potencial para estudios *ex situ*, su sensibilidad ante la degradación ambiental y resistencia a metales pesados; permitieron evidenciar que los peces expuestos a los tratamientos de aguas recolectadas de las estaciones con registro de impacto minero, presentaban cambios en el núcleo celular (micronúcleos, núcleos *blebbed*) y en la expresión de los genes metalotioneína

1 (MT-1) y caspasa 3 (CASP-3), concordantes con las observaciones de deformidades y la variación morfométrica de *Género 1* sp. *2*.

- El empleo de herramientas integradoras permitio contribuir al estudio de la calidad de agua en las quebradas estudiadas. Sin embargo se hace necesario complementar los resultados obtenidos, con otros estudios ecológicos y genéticos, *in situ*, un modelo experimental con un mayor tamaño de muestra y una mayor medición en el tiempo, que den un panorama mas sólido del real estado de la Cuenca alta del Río Chinchiná.

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