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Thermal Stream Effects on Macroinvertebrate Diversity (*Hyalella* spp and *Andesiops* spp) in an Andean Freshwater System (Cachiyacu River Basin, Ecuador)

Proyecto de Investigación

.

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Biología

Trabajo de titulación presentado como requisito
para la obtención del título de Licenciatura en Biología, concentración en
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RESUMEN

La presente investigación determinó la influencia de las aguas termales en la diversidad de especies de ríos andinos. Como sistema de estudio, se trabajó en la cuenca del río Cachiyacu y sus afluentes, los cuales presentan un sistema de experimentación natural con distintos grados de influencia de aguas termales. Se hipotetizó que la variación fisicoquímica de los ríos genera procesos de selección que, potencialmente, generarían comunidades de macroinvertebrados distintas. Bajo este escenario, se espera que ríos termales y no termales, conectados geográficamente, tenga especies diferentes. Metodológicamente, el muestreo se realizó en ríos con influencia termal y ríos control (i.e., sin influencias termales) en la microcuenca del río Cachiyacu, provincia de Napo, Ecuador. Para conocer la diversidad de macroinvertebrados en la zona, se identificaron las especies en base a su morfologías y ADN; también se realizaron árboles filogenéticos de los grupos focales. Se encontró dos especies crípticas del complejo *Andesiops peruvianus* y dos especies del género *Hyalella* spp. Las especies de *Andesiops* no mostraron ninguna diferenciación en cuanto a la ocupación de nichos termales. En contraste, las especies de *Hyalella* si mostraron una diferenciación, con una especie ocupando las aguas con más influencia termal y la otra las aguas con menor influencia. Biogeográficamente, todavía se carece de los datos comparativos necesarios para proponer escenarios de especiación en la cuenca.

Palabras clave: Ecosistemas acuáticos, fuentes termales, diversidad genética, *Hyalella, Andesiops peruvianus.*

ABSTRACT

This research determined the influence of thermal waters on the diversity of Andean river species. As a system of study, we worked on Cachiyacu River basin and its tributaries, which present a natural system of experimentation with different degrees of thermal springs influence. It was hypothesized that the physicochemical variation of the rivers generates selection processes that, potentially, would produce different macroinvertebrate communities. Under this scenario, we expected that geographically connected thermal and non-thermal rivers will have different species. Methodologically, sampling was carried out in streams with thermal influence and control stream (i.e., without thermal influences) in the Cachiyacu river microbasin, Napo province, Ecuador. In order to assess the diversity of macroinvertebrates in the area, the species were identified based on their morphologies and DNA; we also inferred phylogenetic trees of the focal species. We found two cryptic species of the *Andesiops peruvianus* complex and two species of the *Hyalella* genus. The Andesiops species did not show any niche differentiation. In contrast, there was differentiation in *Hyalella*, where one species occupied areas with a marked thermal influence, whereas the other was found in streams with low thermal influence. Biogeographically, we lack enough comparative material to propose speciation scenarios in the basin.

Key words: Aquatic ecosystem, thermal springs, genetic diversity, *Hyalella, Andesiops peruvianus.*

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Freshwater ecosystems are among the most speciose and threatened systems of the world (Abell et al., 2002; Dudgeon et al., 2006), and their species richness is shaped by both environmental conditions and biotic interactions (Dudgeon et al., 2006; Polato et al., 2018). Then, studying the effect of physicochemical influence on diversity is critical for the understanding of community composition, ecosystem management and conservation (e.g., Lessmann et al., 2016).

At different geographic scales, studies have shown that freshwater diversity is affected by latitude, temperature, elevation, chemical compositions, contamination, among others (Jacobsen, 2004, 2008; Vaugh, 2010; Polato et al., 2018). Interestingly, as predicted by Janzen (1967), the intrinsic abiotic variation found in freshwater systems, mainly temperature, has a direct effect in other key traits, such as physiological thermal limits, dispersal, and diversification (Shah et al., 2017; Polato et al., 2018). Elevation has also been shown to explain community composition; for example, Jacobsen (2004) measured the effect of elevation on the patterns of richness of invertebrates along an Andean altitudinal gradient (from sea level to 4000 m a.s.l.), finding that local richness declined linearly as elevation increased, whereas zonal richness remained constant from sea level up to about 1800 m, whereafter it also decreased.

In the Andes, freshwater systems have a fantastic abiotic variation; streams located at very close geographic distances can be produced by groundwater or glacier runoffs. More interestingly, these streams can also be influenced by geothermal activity. Thermal streams are characterized for high temperature and large concentration of metals, which come from geo-volcanic influence (Caissie, 2006). Thus, the climatic, chemical, and geologic complexity of tropical rivers provide an excellent system to study the effect of the abiotic envelope into community composition (Heino, Muotka, Paavola, 2003).

One of the most conspicuous thermal system in Ecuador is the Chacana volcano. The caldera of this volcano is 35 km length and 10–15 km width, being one of the biggest volcanos in Ecuador (Beate et al., 2010; Pilicita Masabanda, 2013). The Chacana has been active through all the Quaternary, with rhyolitic eruptions occurring 240, 180 and 160 ky ago (Hall & Beate, 1991; Hall & Mothes, 2001, 2008). Hot springs, located mainly within the caldera in the Papallacta area, have temperatures between 40 and 67 °C. These springs are near- neutral alkaline chloride waters with high concentrations of boron and arsenic (Beate & Salgado, 2010).

Thermal springs are found at different sites of the Chacana crater, including the Cachiyacu river basin, located in the south of the boiler (Masabanda, 2013). This area has 100 km2 and is the largest rhyolitic eruptive zone in the north of the Andes (Beate et al., 2010). The Cachiyacu area was affected by Younger Dryas Glaciation between 11–10 Ka, provoking erosion in the area and affecting the Cachiyacu basin (Masabanda, 2013). Within the Cachiyacu river basin, there are several thermal streams with high salinity, with temperature of 58,9 ºC to 63.6 ºC in the thermal spring (Pilicita Masabanda, 2013). Thus, in this river basin, we find a very unusual scenario, with streams that have different degrees of thermal influence, creating an ideal scenario to understand the effect of naturally occurring abiotic variation on community composition (Raffaelli, 2004; Woodward et al., 2009) and speciation (Orr & Coyne, 2004; Nevo, 2011; Seehausen & Wagner, 2014).

Our study also allows addressing questions regarding the effect that climatic conditions might have on diversification processes at a microscale. At the Cachiyacu basin, several conditions are basically the same (elevation, latitude, air temperature). Then, if we find differences in the species composition in thermal and non-thermal stream we have two possible explanations: (i) species have been able to speciate in situ and occupy distinctive niches within this microbasin; under this scenario, we expect to find sister species occupying distinctive physicochemical environments within this basin, or (ii) species from distinctive geographic environment have colonized the thermal and non-thermal streams found at the Cachiyacu basin. Under the second scenario, species from the Cachiyacu basin would be more related to taxa from other localities than species from the same basin.

Study organisms

Andesiops spp: The mayfly genus *Andesiops* (Ephemeroptera: Baetidae) is composed by 4 species that are distributed in South America (Ossa-López et al., 2018). *Andesiops peruvianus* is found in the Andes, from Colombia to Argentina, and is commonly used as bioindicator of water quality (Roldán, 1999; Nieto, 2004; Bonada et al., 2006; Menetrey et al., 2008; Zúñiga, 2009). It has been identified as a species complex, with marked geographic variation (Williams et al., 2006; Finn et al., 2014; Múrria et al., 2014; Finn et al., 2016; Gill et al., 2016; Ossa-López et al., 2018). The A. *peruvianus* species complex is commonly found both in thermal and non-thermal streams at the Cachiyacu river basin.

Hyalella spp: This is a genus of freshwater amphipods endemic to the Nearctic and Neotropical regions, with about 70 species (Horton & Lowry, 2013; Alonso & Jaume, 2017). In Ecuador, there are only two known species, H. meinerti (González & Watling, 2003), and *Hyalella* cajasi (Alonso & Jaume, 2017). The first species H. meinerti was collected at 1500 m, whereas H. cajasi is apparently endemic to the Cajas National Park (Alonso & Jaume, 2017). The genus *Hyalella* is commonly found both in thermal and non-thermal streams at the Cachiyacu river basin.

OBJECTIVES

- To determine how freshwater communities vary as a response to abiotic variables in a system where streams are influenced by thermal water.
- To assess if the extreme abiotic variation found in the Cachiyacu river basin can generate speciation processes.

MATERIALS

Sample collection

- Surber net
- Alcohol 99%
- Sample tubes
- Lab tray
- Forceps

Morphological identification

- Optical microscope
- Forceps
- Alcohol 96%
- Alcohol 99%
- Plastic Transfer Pipette
- Identification keys
- Dissecting needle
- Petri dishes
- Sample tubes

DNA extraction

- Lysis Buffer (NaCl 100 mM, Tris-HCl 100 mM pH 8.0, EDTA 25 mM pH 8.0, SDS 0.5%)
- Microscope slides
- Forceps
- Scalpel
- Alcohol 70%
- Proteinase K (Fungal) (20 mg/mL)

- Vortex mix
- Laboratory centrifuge
- Thermo shaker
- RNAsa
- Protein precipitation solution (Tiocianato de guanidina 4M; Tris-HCl 0.1M, pH 7.5)
- Eppendorf tubes 1.5 ml
- Isopropanol
- Etanol 70%
- Tris-HCl 10 mM, pH 8.0
- NaOAc 3M
- 10 mM Tris-Cl, pH 8.0

PCR

- dNTP Mix, 10 mM (Thermo Scientific)
- PCR primers (LCO1490, HCO2195: dgLCO1490, dgHCO2195)
- DNA free water
- 50 mM MgCl2 (Invitrogen)
- 10 X Polymerase reaction buffer
- Platinum Taq DNA Polymerase (Invitrogen)
- Thermo Fisher Thermocycler
- PCR tubes
- ExoSAP-IT (Affymetrix, Inc. Cleveland, Ohio USA)

PCR product analysis

- Electrophoresis equipment
- TBE 1X
- Agarose

- SYBRTM Green I nucleic acid gel stain (Invitrogen)

- 10X BlueJuiceTM Gel Loading Buffer (Invitrogen)

- TrackltTM 100 bp DNA Ladder (Invitrogen)

Water chemical analysis

- 0.45 um filter

- Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Thermo Scientific iCAP 7000 Plus Series ICP-OES)

Psychochemical analysis

- ProDSS Multiparameter Water Quality Meter

METHODOLOGY

Field work

The study was conducted in the Cachiyacu river basin, Napo province, Ecuador. The study area contains two main streams, one with thermal influence, with 3 thermal springs; and the other without thermal influence (Fig 1). These two streams merge, creating a new stream that also has additional input from three thermal springs (Fig. 1). Thermal springs have different sizes, caudal, and distances to the stream. Anthropogenic influence is absent at the study study (Fig. 2).

The thermal springs are very close (50–250 cm) to the thermal stream. Thermal springs have different size, and their water contribution is variable. Thermal water has a high concentration of K, SO4, Ca, Na, Mg, and metals (Pilicita Masabanda, 2013). Once thermal springs merge with non-thermal streams, they produce a change on temperature, pH, and conductivity; the degree of that change depends on the caudal of the thermal springs and the non-thermal stream. Water temperature at the thermal springs is 30–60 ºC.

We sampled a total of 21 sites in February 2017 and in November 2017. The sampling strategy was as described in Figure 3.

Freshwater invertebrates were collected using surber net or a hand net, depending on the width of the streams. All samples were obtained during daytime. The nets were emptied into trays to obtain the visualize invertebrates, which were stored in 99% alcohol, cataloguing them by site. The alcohol of the samples was changed after 24 hours to avoid the DNA degradation (Dominguez and Fernandez, 2009). Invertebrates were collected under the permit MAE-DNB-CM-2015-0017.

Morphological species identification

A total of 276 individuals were identified morphologically, to the lowest possible taxonomic level, using the following keys: Macroinvertebrados bentónicos sudamericanos: sistemática y biología (Dominguez, Fernandez, 2009), The Light and Smith Manual: Intertidal invertebrates from central California to Oregon (Light, 2007), The freshwater amphipods *Hyalella* Smith, 1874 in Chile (Crustacea: Amphipoda) (Gonzáles, 2003). The individuals were identified using an optical microscope and relying on key taxonomic traits (size, coloration, genitalia, pereopods, Antennas, gnathopod, Telson, ocelli, gills). Morphospecies from the genera *Andesiops* and *Hyalella* that were present in thermal and non-thermal streams were used in subsequent molecular identification and phylogenetic analysis (see below).

Molecular species identification

In order to assess cryptic diversity within our sampled sites, we selected morphospecies (*Andesiops* spp, *Hyalella* spp; Table 1) that were found in both thermal and non-thermal streams, using a barcode gene (Cytochrome oxidase I; COI, hereafter). We also sequenced additional species of the two target genera from different geographic regions of Ecuador (Table 1). DNA extraction followed the protocol recommended by Chomkzynski (1993). After extraction, electrophoresis in 2% agarose gel was performed to confirm the presence of DNA, which was quantified using spectrophotometry.

For DNA amplification we used the dgLCO1490 and dgHCO2195 degenerated primers (Folmer et al., 1994; Geller et al., 2013). PCR master mix is as described by Hughes et al. (2003), and the thermocycler was run with the settings defined in Folmer et al. (1994). The resulting amplicon was visualized via electrophoresis with a 2% agarose gel. The successfully amplified samples were cleaned using Exosap and sent to Macrogen for sanger sequencing (Sanger, Coulson, 1975). Forward and reverse sequences were visualized and assembled in Geneious 11.1.5.

Phylogenetic analyses

We generated 73 sequences of *Hyalella* and *Andesiops* from our study site and other geographic regions of Ecuador (Table 1). Additionally, we downloaded publically available sequences from BOLD System (http://www.boldsystems.org/) and GenBank (https://www.ncbi.nlm.nih.gov/genbank/). See Table 1.

We aligned the sequence using MAFTT v. 7 (Katoh et al., 2002) using the strategy Q-INS-i. We examined alignments to find erroneous base calls and gaps using Mesquite 3.51 (Madison, Madison, 2018); also, the data was corroborated using chromatograms of Geneious 11.1.5.

The aligned sequences were analyzed in IQ-TREE v1.6.8 to find the appropriate evolutionary model (Kalyaanamoorthy et al., 2017). Based on this model, a phylogenetic inference was inferred using the maximum likelihood method in the IQ-TREE program v1.6.8 (Nguyen et al., 2015). The reliability and robustness of the tree was assessed using 10000 ultrafast bootstrap approximation (Hoang et al., 2017). The command -bnni was used to reduce the risk of overestimating branch lengths.

Bayesian trees were inferred using Mr Bayes 3.2.6., using the equivalent model found in IQ-Tree v1.6. 8. We run 10 million generations with default options and compared the Baysian results with those obtained under Maximum likelihood criterim. Topologies were visualized in FigTree v1.4 (Rambaut, 2017).

Psychochemical Analysis

To obtain temperature, pH, conductivity and dissolved oxygen a ProDSS Multiparameter Water Quality Meter was used in each site of fig. 3.

To analyze metals, the sample was first filtered through a 0.45 um filter and acidified with concentrated nitric acid (trace metals grade) to a final concentration of 2% of this acid (APHA, 2012).

Quantitative determination of the elements. In an atomic emission spectrometer with inductive plasma coupling (ICP-OES), Thermo Scientific brand, model iCap 7000. The wavelengths for the determination of each element were according to the APHA 3120 method. The samples were measured in triplicate and for quality control a Certified Reference Material (CRM) NIST 1640a, "Trace elements in Natural Water" was used, with the recovery percentages obtained, the data was corrected to obtain a real value of the concentration of the samples (APHA, 2012).

RESULTS

Physicochemical characterization of streams

From each sampled stream (thermal, mixed, and control) I obtained the following physicochemical data: temperature, pH, dissolved oxygen, and conductivity. These data are summarized in Table 2, Table, Figure 4 and Figure 5. Thermal springs have the higher values in temperature, conductivity and an acid pH.

A Tukey's range test shows that temperature, pH, conductivity and dissolved oxygen are significantly different among sites, each site is different from another; except for C1, C2 and C3 that show p-value > 0.05. Also, although thermal variation within each stream is considerable, as shown in Figure 4, our differentiation of streams into control, thermal and mixed categories is appropriate.

Metals concentration also presents a great variation in the Cachiyacu river basin. As expected, in most metals, concentrations within thermal springs are much greater than in other streams (Fig. 5).

Oxygen content ($r= -8.45$; $p < 0.001$) and pH ($r = -2.02$; $p < 0.001$) were negatively correlated with temperature. Conductivity was positively correlated with temperature ($r = 8.83$; $p < 0.001$). Almost all chemical elements were significantly and positively correlated with temperature, only Al wasn’t statistically significant.

Morphological identification of target species

Given that the main goal of our study is to determine if species can adapt and occupy distinctive streams (i.e., with different degrees of thermal influence), we focused on morphologically similar species that were present in all streams. We found two main groups that matched this criterion, the mayfly *Andesiops peruvianus* species complex and the amphipod *Hyalella* sp.

Phylogenetics of *Andesiops peruvianus*

The *Andesiops peruvianus* collected at the Cachiyacu river basin fall into two evolutionary units (Fig. 6). The genetic distance between these two groups is 1.28–1.43% (using the IqTree evolutionary model) or 2.8–3.0% (uncorrected distances in PAUP). The two species are found in syntopy in the thermal and control streams; also, the two species include individuals collected from other nearby Andean localities (i.e., Papallacta).

Phylogenetics of *Hyalella*

The *Hyalella* sp collected at the Cachiyacu river basin fall into two evolutionary units (Fig. 7). One of the molecular species (yellow clade in Fig 7) is found in all streams (thermal, mixed, control). The second species (orange clade in Fig 7) was found in the thermal spring, thermal streams, and thermal mix (absent from control streams). The genetic distance between these two groups is 13.41–13.87% (using the IqTree evolutionary model) or 14.26–14.73% (uncorrected distances in PAUP). It is worth mentioning that the thermal spring has where the species was found has temperature of 24.78 - 30.28 ºC. Relationships among *Hyalella* species are not well supported (Fig. 7).

DISCUSSION

One of the fundamental questions of evolutionary biology is understanding how the abiotic landscape influences community composition and speciation (Janzen, 1967; Coyne & Orr, 2004). The abiotic variation within the Cachiyacu River microbasin, as shown in Tables 2-3 and Figures 4-5, is an excellent example of a dramatic scenario of variation at a microgeographic scale. This variation, in temperature, dissolved oxygen, and heavy metals, imposes strong physiological challenges into the aquatic organisms of the area, but also provides an opportunity for niche specialization and, potentially, speciation (Jacobsen *et al.*, 2003; Coyne & Orr, 2004; Ingram, 2011; Riesch *et al.*, 2018).

The effect of temperature as one of the main evolutionary conditioners for invertebrate biogeography and speciation has been shown in several studies (Burgmer, Hillebrand, & Pfenninger, 2007; Ward & Stanford, 1982; Shah *et al.*, 2007; Polato *et al.*, 2018). One of the overall responses of tropical aquatic species is that they have narrow thermal breaths and limited dispersal, which corresponds to the relatively small environmental variation of tropical ecosystems (Shah *et al.,* 2007; Polato *et al.*, 2018). Also, tropical species. Thus, abrupt changes in temperature and heavy metals, as those seen in streams with thermal influence, are expected to affect the natural history, metabolic processes and presence/absence dynamics of freshwater invertebrates (Dutra *et al.*, 2007; Dutra *et al.*, 2008; Malaj *et al.*, 2012). Also, high concentrations of heavy metals have different effects on invertebrates, with some species evolving tolerance (Tranvik, Bengtsson, Rundgren, 1993; Morgan, Morgan, 1998; Timmermans, 2007; Janssens *et al.*, 2009) and others been negatively affected by these concentrations (Martin & Holdich, 1986; Sola *et al., 2004* Iwasaki *et al.*, 2009).

Since the Cachiyacu River microbasin is located within an Andean area, we expect that species found in the control rivers (i.e., without thermal influence) will be those commonly found in high-elevation ecosystems. We also expected that thermal springs or streams heavily influenced by thermal waters would lack species, or have species that might have adapted to these new conditions (in situ speciation) or colonized the areas from other ecosystems with high temperature (e.g. lowlands) or other basins influenced by volcanic activity.

The results that we obtain show some light in how idiosyncratic can be the response of freshwater invertebrates to the abiotic envelope of the Cachiyacu river basin. The two cryptic species of the *Andesiops peruvianus* complex are present in most of the sampled rivers, except the most extreme environments (i.e., thermal spring). This means that there is not speciation in relation to thermal niches and that the two species coexist in syntopy. It is unclear if the two species have different life cycles to avoid competition or if their abundance differs in the thermal gradient of the river basin. These are questions that should be address in the future. Also, it has been observed that females in the *A. peruvianus* complex may lay eggs on suboptimal areas of the river (Finn *et al.* 2006); thus, it is possible that some individuals that are found in the mixing zone of thermal and non-thermal rivers were in conditions that are far from the optimal niche of the species. Additionally, since the two cryptic species of *A. peruvianus* have also been found in other Andean rivers that lack volcanic influence (Gill *et al.*, 2016), it is likely that they are adapted to the physicochemical conditions of non-thermal rivers (Shan *et al.* 2017). It also important to notice that, since adults of the *A. peruvianus* complex can fly, their dispersal into streams with thermal influence can occur easily form nearby non-thermal streams and rivers.

The scenario for *Hyalella* is very different. There are two species that are found in very different environments. *Hyalella* sp. 2 is present in the thermal stream, mixing zone and thermal spring; in contrast, *Hyalella sp.* 1 is present in the thermal stream, mixing zone, and the control stream. Then, our results show that there is niche specialization of the two species. From a phylogenetic perspective, testing if the species are sister to each other or have geographically distant relatives is challenging given the genetic information available for the genus is scarce and, thus, comparisons are inadequate.

There are some examples of niche partitioning of *Hyalella* in the same geographic area (Casset *et al.,* 2001; Dutra *et al*., 2007, Da Silva and Bond-Buckup, 2008). Also, *Hyalella* has had significant radiation in the Titicaca Lake, where 17 species have been reported, 15 are endemic, and most of these species are concentrated in a small area (Alonso & Jaume, 2017). *Hyalella* at the Titicaca Lake have arrived from several independent dispersal, combined with intralacustrine diversification (Adamowicz *et al.* 2018). It is likely that a similar scenario is at work at the Cachiyacu basin, but more sampling and a better regional comparative dataset are necessary to have a well-defined biogeographic scenario.

RECOMMENDATIONS

-Increase sampling in other Andean river basins with thermal influence, as well as other non-thermal geographical areas of Ecuador (lowlands, montane ecosystems, páramo).

-Increase the number of genes to solve phylogenetic relationships among species.

-Increase the number of invertebrate taxa to test the effect of thermal waters at a broader scale.

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APPENDIX I: FIGURES AND TABLES

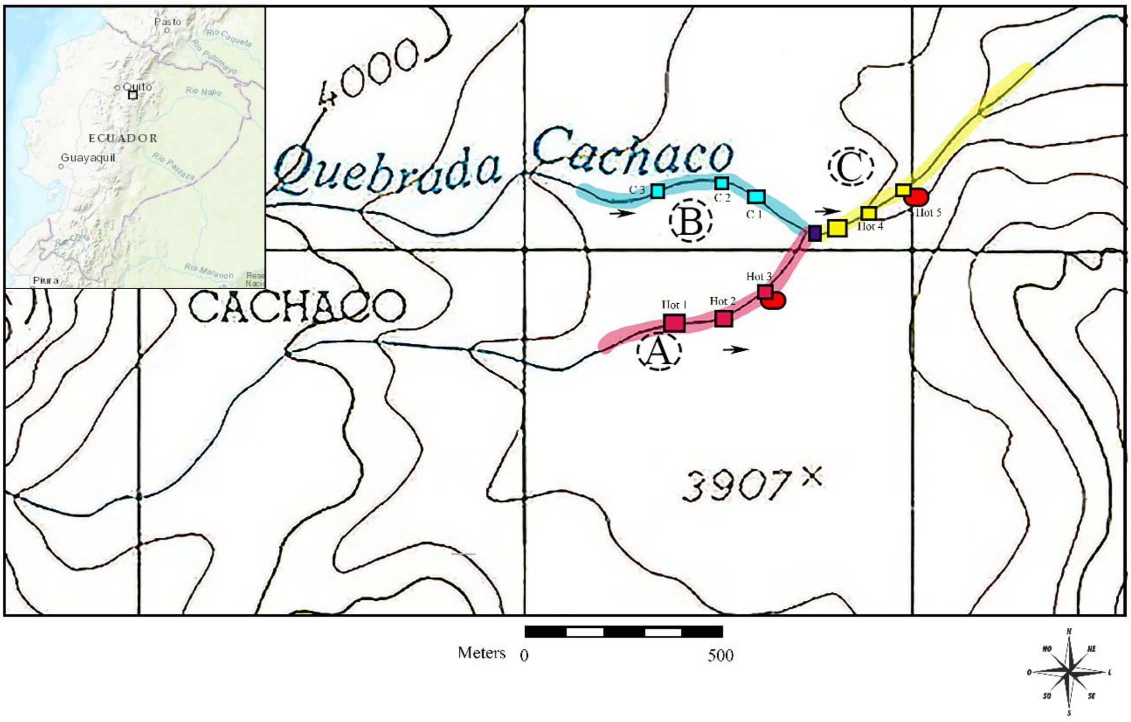

Figure 1. Map of the Cachiyacu river basin. **A** (red) represents the thermal stream; **C** (yellow) is a stream formed by waters from the control and the thermal streams. **B** (blue) represents the control stream, which lacks any thermal influence. Squares represent study sites along each stream.

(a) Low influence site (Thermal stream)

(b) Moderate influence site (Thermal stream)

(c) Moderate influence site (Thermal stream)

(d) Thermal spring

(e) Thermal spring

(f) Non-thermal stream (Control)

Figure 2. Photos of the Cachiyacu river basin showing different degrees of thermal influence.

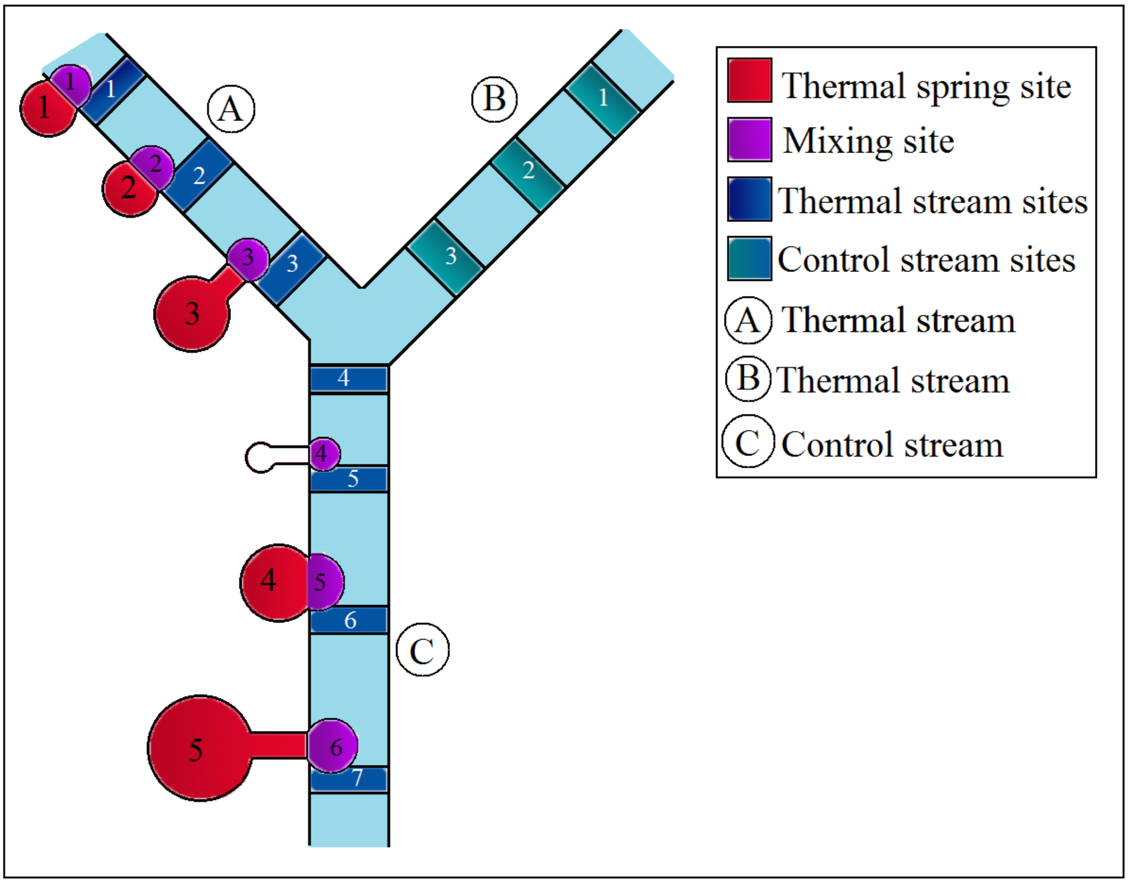

Figure 3. Cachiyacu river basin sampling scheme.

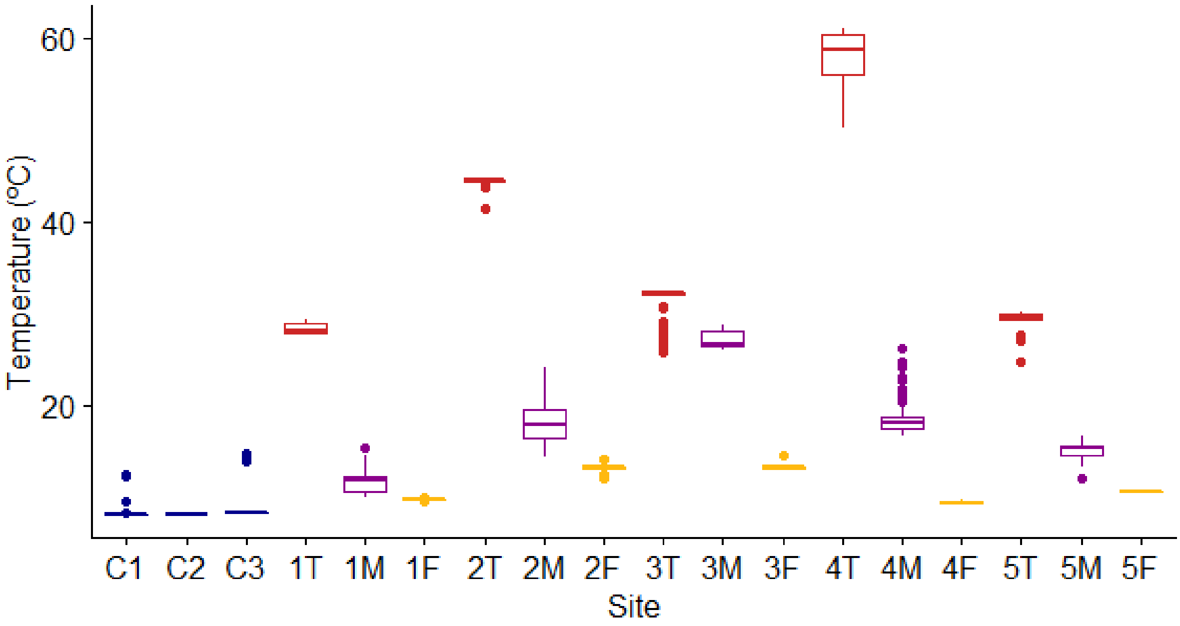

Figure 4. Temperature, pH, conductivity and dissolved oxygen variation in streams at the Cachiyacu river basin. C1, C2, and C3 = Control stream; 1T = Thermal spring 1; 1M = Moderate influence site 1; 1F = Low influence site 1; 2T = Thermal spring 2; 2M=Moderate influence site 2; 2F = Low influence site 2; 3T = Thermal spring 3; 3M = Moderate influence site 3; 3F = Low influence site 3; 4T=Thermal spring 4; 4M=Moderate influence site 4; 4F = Low influence site 4; 5T = Thermal spring 5; 5M = Moderate influence site 5; 5F= Low influence site 5.

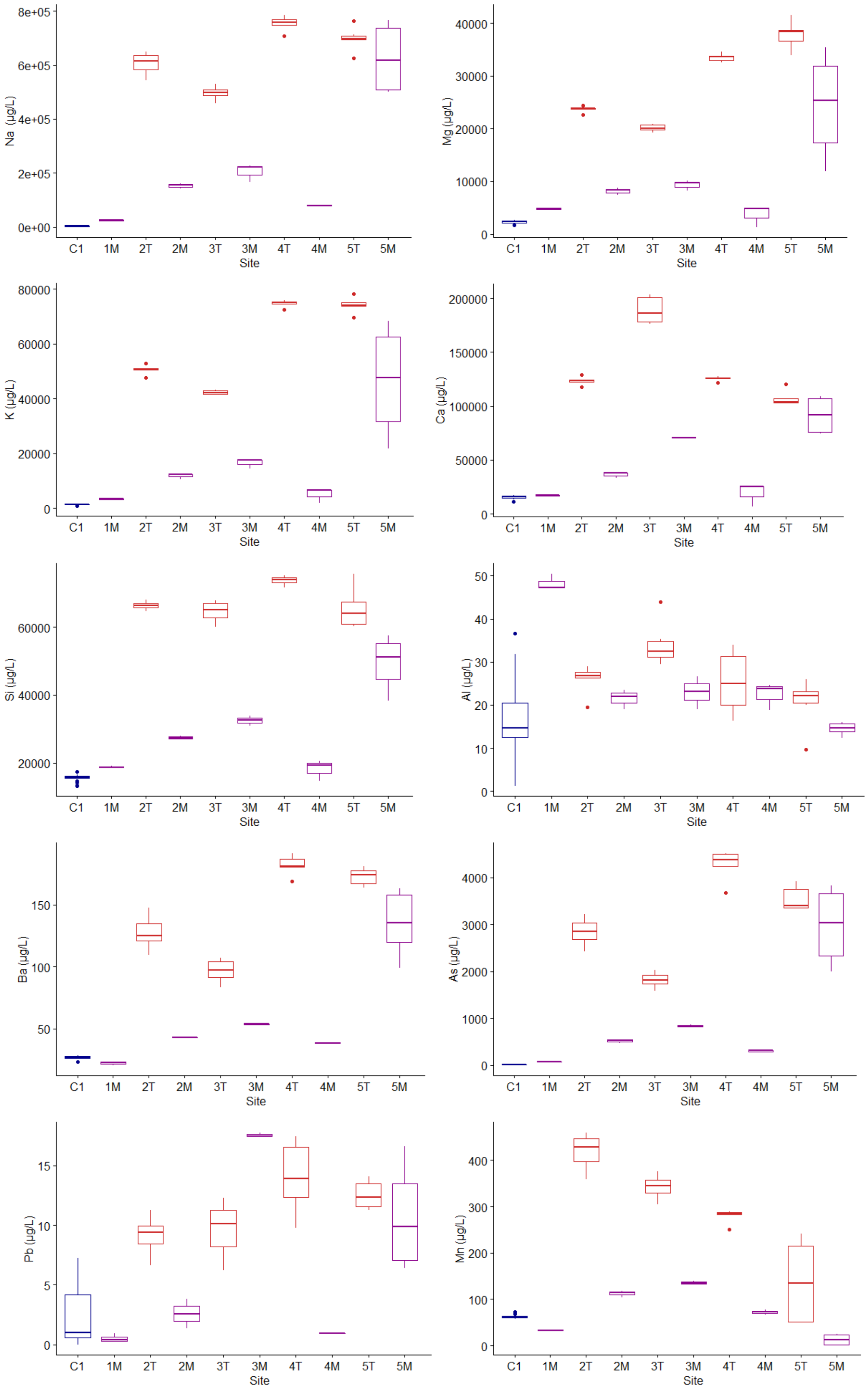

Figure 5. Chemical element (Na, Mg, K, Ca, Si, Al, Ba, As, Pb, Mn) concentrations in streams at the Cachiyacu river basin. C1, C2 and C3=Control streams, 1M=Moderate influence site 1, 2T= Thermal spring 2, 2M= Moderate influence site 2, 3T= Thermal spring 3, 3M= Moderate influence site 3, 4T= Thermal spring 4, 4M= Moderate influence site 4, 5T= Thermal spring 5, 5M= Moderate influence site 5.

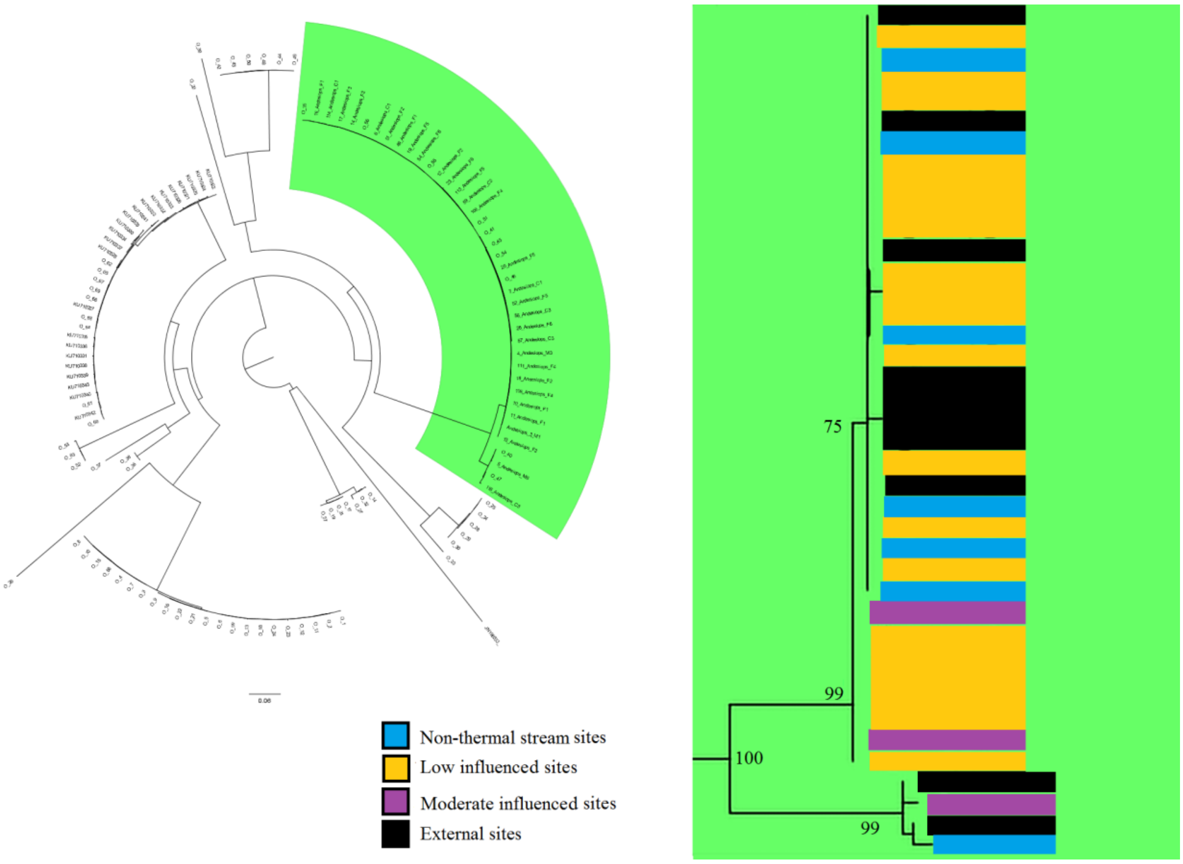

Figure 6. Maximum likelihood tree of *Andesiops peruvianus*, inferred from the mtDNA COI gene. Number on branches represent nodal support values. The boxes show where the individual was collected. The light blue boxes are individuals from non-thermal streams. The yellow boxes are from individuals collected at streams with low thermal influence. Purple boxes represent individuals found at streams with moderate thermal influence. Black boxes are for individuals obtained at other localities. **(a)** Phylogenetic tree with all terminals included in the study (ingroup and outgroup) showing, in green, the sequences from invertebrates obtained in the Cachiyacu basic. **(b)** Close up of the relationships within the *Andesiops peruvianus* complex obtained from streams with distinctive thermal influence.

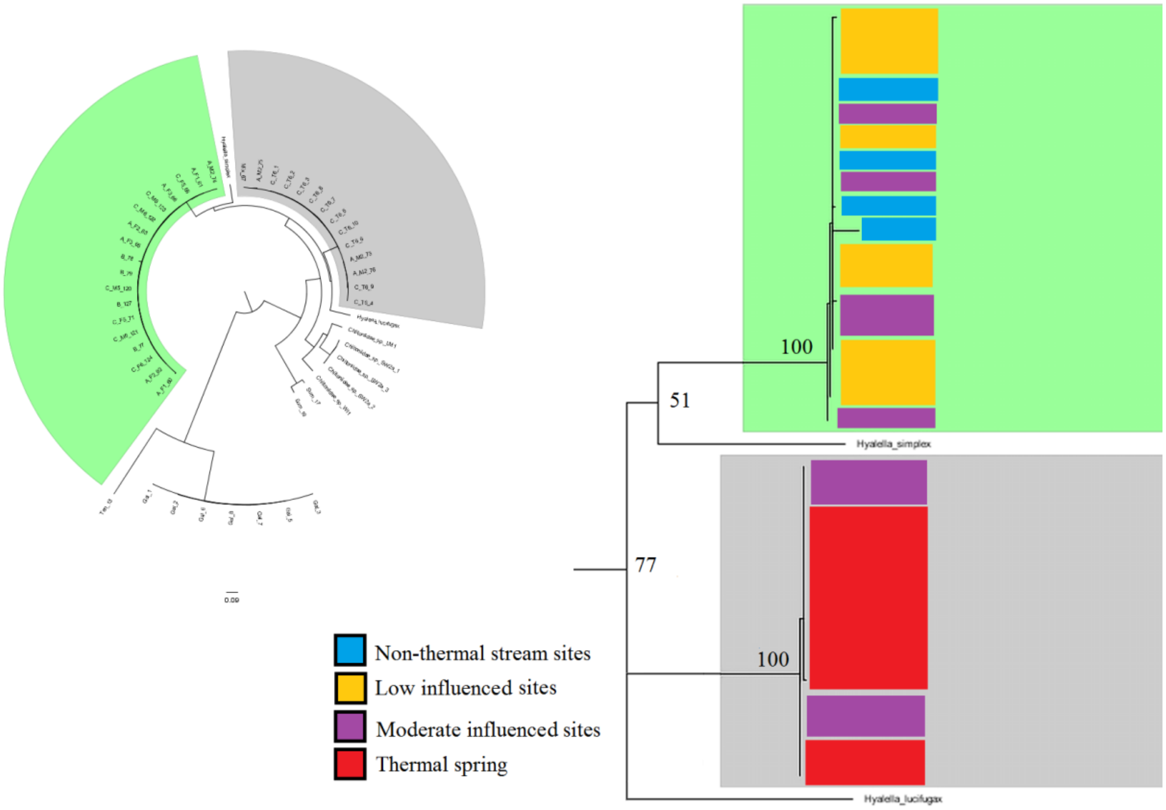

Figure 7. Maximum likelihood tree of *Hyalella* spp inferred from the mtDNA COI gene. Number on branches represent nodal support values, using ultrafast bootstrap approximation (Hoang *et al*., 2017). The light blue boxes represent individuals obtained non-thermal stream, the yellow boxes are from low-influence thermal river sites; purple boxes are from moderate-influence thermal river sites; red boxes are for individual from thermal spring sites. **(a)** Phylogenetic tree with all terminals included in the study (ingroup and outgroup). Gray and green boxes contain all terminals of *Hyalella* spp. collected in Cachiyacu river basin. **(b)** Close up of the relationships of the two *Hyalella* spp, with inner boxes denoting the stream from where each individual was collected.

TABLES

Table 1. Individual sequence information data.

Specie	Field ID	Locality	Latitud	Longitude	Elevation (m)	Genbank/BOLD code
Andesiops sp. K BG	2502	ECA4248	-0.509	-78.182	4248	EVTEC1388-14
Andesiops sp. K BG	2503	ECA4248	-0.509	-78.182	4248	EVTEC1389-14
Andesiops sp. K BG	2504	ECA4248	-0.509	-78.182	4248	EVTEC1390-14
Andesiops sp. K BG	2505	ECA4248	-0.509	-78.182	4248	EVTEC1391-14
Andesiops sp. K BG	2506	ECA4248	-0.509	-78.182	4248	EVTEC1392-14
Andesiops sp. K BG	3642	ECA4248	-0.509	-78.182	4248	EVTEC165-13
Andesiops peruvianus	83859	ECA3991	-0.503419	-78.239	3991	EPHYS100-13
Andesiops peruvianus	83928	ECA3991	-0.503419	-78.239	3991	EPHYS169-13
Andesiops peruvianus	83931	ECA3991	-0.503419	-78.239	3991	EPHYS172-13
Andesiops sp. K BG	2507	ECA3991	-0.503	-78.239	3991	EVTEC1393-14
Andesiops sp. K BG	2508	ECA3991	-0.503	-78.239	3991	EVTEC1394-14
Andesiops sp. K BG	2509	ECA3991	-0.503	-78.239	3991	EVTEC1395-14
Andesiops sp. I BG	2510	ECA3991	-0.503	-78.239	3991	EVTEC1396-14
Andesiops sp. A BG	2481	ECP2001	-0.452	-77.941	2001	EVTEC1418-14
Andesiops sp. A BG	2477	ECP2001	-0.452	-77.941	2001	EVTEC1415-14
Andesiops sp. A BG	2478	ECP2001	-0.452	-77.941	2001	EVTEC1416-14
Andesiops peruvianus	62271	ECP1845	-0.45034	-77.8907	1845	EPHYS1427-14
Andesiops peruvianus	62274	ECP1845	-0.45034	-77.8907	1845	EPHYS1430-14
Andesiops peruvianus	62293	ECP1845	-0.45034	-77.8907	1845	EPHYS1449-14
Andesiops peruvianus	83930	ECP1845	-0.45034	-77.8907	1845	EPHYS171-13
Andesiops sp. A BG	2494	ECP1845	-0.45	-77.891	1845	EVTEC1060-13

Andesiops sp. A BG	2495	ECP1845	-0.45	-77.891	1845	EVTEC1061-13
Andesiops sp. A BG	2496	ECP1845	-0.45	-77.891	1845	EVTEC1062-13
Andesiops peruvianus	62658	ECP2003	-0.44924	-77.943	2003	EPHYS1569-14
Andesiops peruvianus	62659	ECP2003	-0.44924	-77.943	2003	EPHYS1570-14
Andesiops peruvianus	62662	ECP2003	-0.44924	-77.943	2003	EPHYS1573-14
Andesiops sp. C BG	2473	ECP2123	-0.436	-77.959	2123	EVTEC1411-14
Andesiops sp. A BG	2474	ECP2123	-0.436	-77.959	2123	EVTEC1412-14
Andesiops sp. C BG	2475	ECP2123	-0.436	-77.959	2123	EVTEC1413-14
Andesiops peruvianus	3	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	4	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	5	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	6	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	7	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	10	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	11	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	12	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	13	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	14	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	16	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	17	Río Cachiyacu	-0.4083333	-78.2258333	3968	-

Andesiops peruvianus	18	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	19	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	20	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	23	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	26	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	49	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	51	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	52	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	54	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	56	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	57	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	59	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	108	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	109	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	111	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	113	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	114	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Andesiops peruvianus	116	Río Cachiyacu	-0.4083333	-78.2258333	3968	-

Andesiops sp. A BG	2410	ECP2495	-0.404	-78.045	2495	EVTEC1305-14
Andesiops sp. A BG	2412	ECP2495	-0.404	-78.045	2495	EVTEC1307-14
Andesiops sp. N BG	2482	ECP3309	-0.388	-78.148	3309	EVTEC1057-13
Andesiops sp. J BG	3677	ECP3309	-0.388	-78.148	3309	EVTEC1071-13
Andesiops sp. F BG	2459	ECP3691	-0.388	-78.204	3691	EVTEC1400-14
Andesiops sp. F BG	2461	ECP3691	-0.388	-78.204	3691	EVTEC1401-14
Andesiops sp. F BG	3654	ECP3691	-0.388	-78.204	3691	EVTEC176-13
Andesiops sp. L BG	2498	ECP3174	-0.387	-78.139	3174	EVTEC1064-13
Andesiops sp. M BG	2499	ECP3174	-0.387	-78.139	3174	EVTEC1065-13
Andesiops sp. L BG	2500	ECP3174	-0.387	-78.139	3174	EVTEC1066-13
Andesiops sp. C BG	3680	ECP2694	-0.376	-78.075	2694	EVTEC194-13
Andesiops sp. G BG	3682	ECP2694	-0.376	-78.075	2694	EVTEC196-13
Andesiops sp. G BG	2487	ECP2694	-0.376	-78.075	2694	EVTEC1423-14
Andesiops sp. D BG	2467	ECP2957	-0.376	-78.122	2957	EVTEC1406-14
Andesiops sp. G BG	2469	ECP2957	-0.376	-78.122	2957	EVTEC1408-14
Andesiops sp. J BG	2471	ECP2957	-0.376	-78.122	2957	EVTEC1410-14
Andesiops sp. J BG	2407	ECP3898	-0.347	-78.2	3898	EVTEC1375-14
Andesiops sp. J BG	2408	ECP3898	-0.347	-78.2	3898	EVTEC1376-14
Andesiops sp. J BG	2409	ECP3898	-0.347	-78.2	3898	EVTEC1377-14
Andesiops sp. J BG	3659	ECP3498	-0.333	-78.147	3498	EVTEC1070-13
Andesiops sp. J BG	2464	ECP3498	-0.333	-78.147	3498	EVTEC1308-14
Andesiops sp. N BG	3660	ECP3498	-0.333	-78.147	3498	EVTEC1402-14
Andesiops sp. B BG	2381	ECO2212	-0.25	-77.935	2212	EVOTR005-12
Andesiops sp. A BG	2382	ECO2212	-0.25	-77.935	2212	EVTEC1048-13
Andesiops sp. A BG	2383	ECO2212	-0.25	-77.935	2212	EVTEC1049-13
Andesiops sp. C BG	2376	ECO2081	-0.246	-77.908	2081	EVOTR003-12
Andesiops sp. A BG	2378	ECO2081	-0.246	-77.908	2081	EVOTR004-12

Andesiops sp. A BG	2375	ECO2081	-0.246	-77.908	2081	EVTEC1046-13
Andesiops sp. G BG	2384	ECO2604	-0.244	-77.985	2604	EVTEC133-13
Andesiops sp. O BG	3619	ECO2604	-0.244	-77.985	2604	EVTEC147-13
Andesiops sp. J BG	2389	ECO3847	-0.231	-78.15	3847	EVTEC1050-13
Andesiops sp. I BG	2390	ECO3847	-0.231	-78.15	3847	EVTEC1051-13
Andesiops sp. I BG	2391	ECO3847	-0.231	-78.15	3847	EVTEC1382-14
Andesiops sp. G BG	3605	ECO2826	-0.23	-78.006	2826	EVTEC135-13
Andesiops sp. C BG	3606	ECO2826	-0.23	-78.006	2826	EVTEC136-13
Andesiops sp. C BG	2396	ECO2826	-0.23	-78.006	2826	EVTEC1366-14
Andesiops sp. H BG	2394	ECO3201	-0.219	-78.086	3201	EVTEC1385-14
Andesiops sp. F BG	2401	ECO3415	-0.213	-78.113	3415	EVTEC1054-13
Andesiops sp. F BG	2402	ECO3415	-0.213	-78.113	3415	EVTEC1055-13
Andesiops sp. F BG	2400	ECO3415	-0.213	-78.113	3415	EVTEC1370-14
Andesiops peruvianus	-	-	-	-	-	KU710343.1
Andesiops peruvianus	-	-	-	-	-	KU710342.1
Andesiops peruvianus	-	-	-	-	-	KU710341.1
Andesiops peruvianus	-	-	-	-	-	KU710340.1
Andesiops peruvianus	-	-	-	-	-	KU710339.1
Andesiops peruvianus	-	-	-	-	-	KU710338.1
Andesiops peruvianus	-	-	-	-	-	KU710337.1
Andesiops peruvianus	-	-	-	-	-	KU710336.1
Andesiops peruvianus	-	-	-	-	-	KU710335.1
Andesiops peruvianus	-	-	-	-	-	KU710334.1
Andesiops peruvianus	-	-	-	-	-	KU710333.1
Andesiops peruvianus	-	-	-	-	-	KU710332.1
Andesiops peruvianus	-	-	-	-	-	KU710331.1
Andesiops peruvianus	-	-	-	-	-	KU710330.1

Andesiops peruvianus	-	-	-	-	-	KU710329.1
Andesiops peruvianus	-	-	-	-	-	KU710328.1
Andesiops peruvianus	-	-	-	-	-	KU710327.1
Andesiops peruvianus	-	-	-	-	-	KU710326.1
Andesiops peruvianus	-	-	-	-	-	KU710325.1
Andesiops peruvianus	-	-	-	-	-	KU710324.1
Andesiops peruvianus	-	-	-	-	-	KU710323.1
Andesiops peruvianus	-	-	-	-	-	KU710322.1
Andesiops peruvianus	-	-	-	-	-	KU710321.1
Acerpenna sp.	-	-	39.1174	-75.9572	16	JN198533.1
Hyalella sp	60	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	61	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	62	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	63	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	65	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	66	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	67	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	68	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	69	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	71	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	73	Río Cachiyacu	-0.4083333	-78.2258333	3968	-

Hyalella sp	74	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	75	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	76	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	77	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	78	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	79	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	120	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	121	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	122	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	123	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	124	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	127	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	180	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	181	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	182	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	183	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	184	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	185	Río Cachiyacu	-0.4083333	-78.2258333	3968	-

Hyalella sp	186	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	187	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	188	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	189	Río Cachiyacu	-0.4083333	-78.2258333	3968	-
Hyalella sp	190	Galápagos	-0.4083333	-78.2258333	3968	-
Hyalella sp	191	Galápagos	-0.4083333	-78.2258333	3968	-
Hyalella sp	192	Galápagos	-0.4083333	-78.2258333	3968	-
Hyalella sp	193	Galápagos	-0.4083333	-78.2258333	3968	-
Hyalella sp	194	Galápagos	-0.4083333	-78.2258333	3968	-
Hyalella sp	195	Galápagos	-0.4083333	-78.2258333	3968	-
Hyalella sp	196	Galápagos	-0.4083333	-78.2258333	3968	-
Hyalella sp	201	Tena	-0.4083333	-78.2258333	3968	-
Hyalella sp	206	Sumaco	-0.4083333	-78.2258333	3968	-
Hyalella sp	207	Sumaco	-0.4083333	-78.2258333	3968	-
Hyalella simplex	-	Chile	-	-	-	AF520434
Hyalella lucifugax	-	Lago Titicaca	-15.9159111	-69.292812	3815	LT594767
Chiltoniidae sp. LM1	-	Australia	-	-	-	KT958066
Chiltoniidae sp. SW2a_1	-	Australia	-	-	-	KT958054
Chiltoniidae sp. SW2a_2	-	Australia	-	-	-	KT958053
Chiltoniidae sp. SW2a_3	-	Australia	-	-	-	KT958052

Chiltoniidae_sp._ WI1	-	Australia	-	-	-	KT958063

Table 2. Mean and standard deviation of environmental variables: Temperature, pH and conductivity at stream sites in Cachiyacu River basin.

Site	(Mean±SD)	(Mean±SD)	(Mean±SD)	(Mean±SD)
1F	9.73 ± 0.05	185.03 ± 27.48	7.22 ± 0.11	7.36 ± 0.16
1M	11.75 ± 1.19	452.11 ± 208.18	6.66 ± 0.34	6.54 ± 1.06
1T	28.38 ± 0.64	1070.54 ± 1200.5	6.04 ± 0.09	3.27 ± 1.04
2F	13.21 ± 0.31	578.95 ± 43.99	7.03 ± 0.07	6.85 ± 0.10
2M	18.41 ± 2.51	1284.88 ± 420.18	6.91 ± 0.15	5.71 ± 0.82
2T	44.23 ± 0.68	6358.50 ± 1336.37	6.37 ± 0.08	0.82 ± 0.24
3F	13.23 ± 0.29	497.53 ± 195.62	7.75 ± 0.47	6.73 ± 0.35
3M	27.13 ± 1.11	62.78 ± 12.10	7.13 ± 0.27	4.78 ± 0.28
3T	31.8 ± 1.09	4533.22 ± 1099.47	6.84 ± 0.23	4.10 ± 0.33
4F	9.42 ± 0.16	129.89 ± 55.77	7.50 ± 0.13	7.69 ± 0.30
4M	18.67 ± 1.86	1763.62 ± 425.26	7.77 ± 0.68	6.42 ± 0.61
4T	57.98 ± 3.05	8110.65 ± 5177.26	7.15 ± 0.10	2.13 ± 0.46
5F	10.61 ± 0.10	228.34 ± 104.58	8.02 ± 0.25	7.92 ± 0.03
5M	14.89 ± 1.36	1136.94 ± 585.35	8.52 ± 0.24	7.39 ± 0.18
5T	29.50 ± 0.87	5370.26 ± 2051.60	8.19 ± 0.12	7.40 ± 0.85
C1	8.45 ± 1.01	65.1 ± 19.76	7.17 ± 0.43	7.75 ± 0.31
C2	8.18 ± 0.03	71.05 ± 0.08	7.22 ± 0.01	7.88 ± 0.003
C3	9.39 ± 2.51	75.35 ± 38.90	7.18 ± 0.11	7.82 ± 0.48

Table 3. Maximum and minimum values for the following environment traits: Temperature, pH and conductivity at stream sites in the Cachiyacu River basin.

Site	Temperature Max-Min	Conductivity Max-Min	pH Max-Min	Dissolved oxygen Max-Min
1F	9.61 - 9.94	4.9 - 313.80	6.9 - 7.37	6.72 - 7.68
1M	10 - 15.33	15.3 - 706.00	6.36 - 7.61	4.26 - 11.99
1T	27.67 - 29.44	112.8 - 3038.10	5.93 - 6.16	1.57 - 4.55
2F	12.17 - 14.06	502.2 - 717.90	6.95 - 7.27	6.53 - 7.14
2M	14.28 - 24.11	17.9 - 2284.40	6.64 - 7.26	2.89 - 6.75
2T	41.39 - 44.5	383.3 - 6684.50	6.21 - 6.63	0.69 - 1.73
3F	12.83 - 14.56	9.4 - 600.90	7.17 - 8.87	4.97 - 6.92
3M	25.94 - 28.83	48.2 - 82.80	6.83 - 7.47	4.17 - 5.01
3T	25.83 - 32.33	48.9 - 4831.00	6.67 - 7.71	2.83 - 5.00
4F	9.33 - 9.78	4 - 219.80	7.09 - 7.76	6.57 - 7.87
4M	16.72 - 26.17	1416.7 - 3232.00	7.26 - 10.14	3.95 - 6.90
4T	50.22 - 61.00	206 - -11582.90	7.05 - 7.40	1.63 - 3.08
5F	10.5 - 10.83	8 - 292.30	7.63 - 8.55	7.87 - 7.97
5M	12.17 - 16.56	52.5 - 1903.60	8.26 - 8.96	7.2 - 7.81
5T	24.78 - 30.28	102.7 - 6294.50	7.93 - 8.33	4.61 - 8.09

C1	8.17 - 12.44	1.3 - 71.50	5.42 - 7.43	6.56 - 7.87
C2	8.17 - 8.22	70.9 - 71.20	7.21 - 7.23	7.87 - 7.88
C3	8.22 - 14.83	1.5 - 151.60	6.94 - 7.23	6.91 - 9.62