Distribution, Abundance and Molecular Analysis of Genus Barbadocladius Cranston & Krosch (Diptera, Chironomidae) in Tropical, High Altitude Andean Streams and Rivers

N Prat, C Ribera, M Rieradevall, C Villamarín & R Acosta

Neotropical
Entomology

ONLINE FIRST

Springer

Springer

Springer

SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Distribution, Abundance and Molecular Analysis of Genus *Barbadocladius* Cranston & Krosch (Diptera, Chironomidae) in Tropical, High Altitude Andean Streams and Rivers
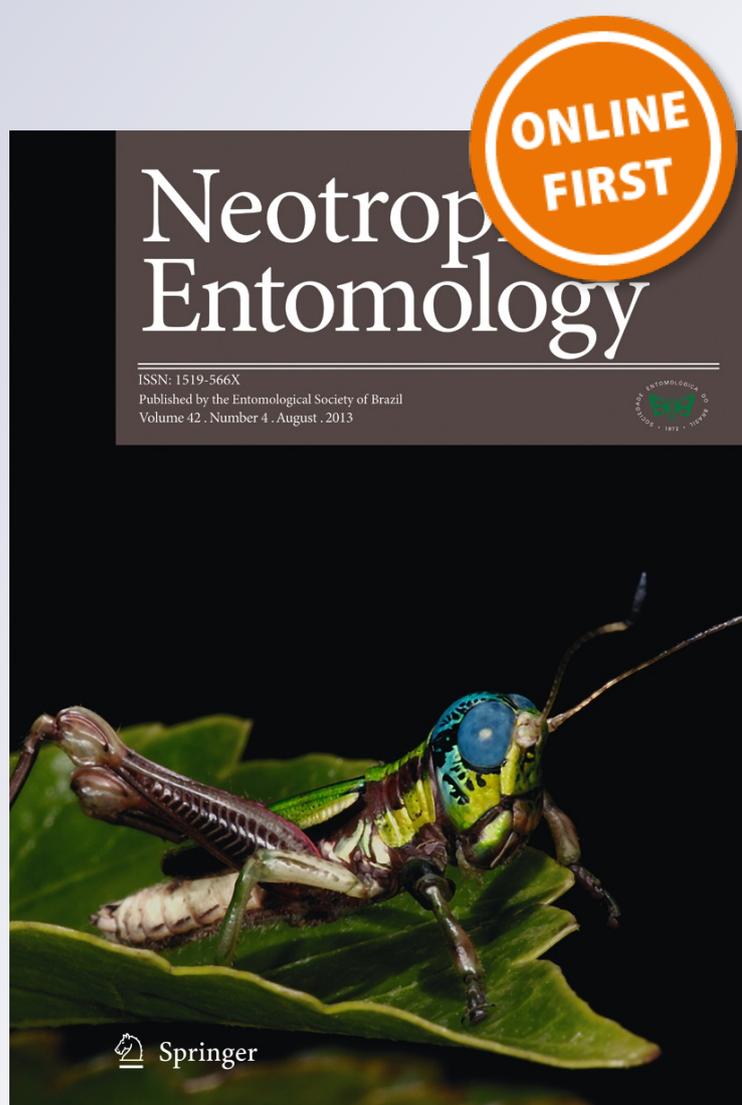
N Prat[1], C Ribera[2,3], M Rieradevall[1,3], C Villamarín[1], R Acosta[1]

[1]Grupo de investigación Freshwater Ecology and Management (F.E.M.), Depto d'Ecologia, Univ de Barcelona, Barcelona, Spain
[2]Depto de Biologia Animal, Univ de Barcelona, Barcelona, Spain
[3]Institut de Recerca de Biodiversitat (IRBio), Univ de Barcelona, Barcelona, Spain

Abstract

The distribution of the genus *Barbadocladius* Cranston & Krosch (Diptera: Chironomidae), previously reported from Chile to Bolivia, has extended northwards. Larvae, pupae and pupal exuviae of this genus have been found in the high mountain tropical streams of Peru to 9°22′56″, but are restricted to very high altitude streams (altitudes over 3,278 m asl) compared to the lower altitude streams (below 1,100 m asl) in which the genus is reported in Chile and Argentina. Based on morphological studies, both described species in the genus, *Barbadocladius andinus* Cranston & Krosch and *Barbadocladius limay* Cranston & Krosch, have been found in Peru as pupae or pupal exuviae. Morphological analysis of the larvae and pupae revealed no differences between the two described species from Patagonia and Peru, which are of similar size and with a similar armament of hooklets and spines in pupal tergites and sternites. However, molecular analysis of larvae and pupae revealed that in Peru, there are at least two different evolutionary lines, one distributed widely and another restricted to one site. Phylogenetic analysis (using *cox1* mitochondrial sequences) of all available sequences of *Barbadocladius* shows that the Chilean and Argentinean material differs from that of Peru. Therefore, a total of four molecular segregates are identified, although morphologically, neither larvae nor the pupae may be differentiated.

Introduction

The Chironomidae (Diptera) genus *Barbadocladius* was established by Cranston & Krosch (2011), and its name refers to the long and dense beard of setae located at the lateral to posterior mentum of the larval head. This distinctive larva was first described in 1983 by S.H. Roback & P. Coffmann as "Genus 9", possessing an unknown combination of characters for a chironomid larva (Roback & Coffmann 1983). In the same paper, these authors described a very unusual pupa as "Genus 4". These two unnamed genera have since been associated with each other, named as genus *Barbadocladius*, and described in all morphological stages by Cranston & Krosch (loc.cit.). Two species were described by

these authors, *Barbadocladius andinus* Cranston & Krosch and *Barbadocladius limay* Cranston & Krosch, including molecular data. In this paper, two specimens of larvae were analysed using partial sequences of mtDNA COI and two nuclear protein-coding genes (CAD1 and CAD3). In another paper (Krosh *et al* 2011), the molecular phylogenetic analysis of two larval specimens (not associated with either species described morphologically) placed the genus as a sister group to a large and diverse clade estimated to comprise taxa largely of "Gondwanan" distribution.

The original material of Roback & Coffmann (1983) for Genus 4 and Genus 9 specimens was collected in Bolivia ca. 55 km East of La Paz in 1977 at 4,200 m asl, while Cranston & Krosch (2011) collected additional material of *Barbadocladius*

in Chile and Argentina in 1997 and 2006, further south from the original finding. Keys to distinguish the adults and the pupae were provided in the paper describing the genera and the two species mentioned above; the larvae are indistinguishable. According to the authors' description, *Barbadocladius* is a trans-Andean genus located between 39° and 41° S, and living from the lowlands to altitudes up to 1,100 m asl. Nevertheless, *Barbadocladius* distribution has not been studied in the tropical high Andes.

In our previous studies of Chironomidae biodiversity in streams in the highland Andes from Peru to Colombia, we have occasionally found larvae and pupae of *Barbadocladius*, but never in great abundance (Acosta & Prat 2010, Villamarín 2012). Recently, in a project specifically dealing with Chironomidae distribution and molecular variability in the Andes over 2,000 m asl from the central part of Colombia to southern Peru (project BIQURA, =*BI*odiversidad de *QU*ironómidos de *R*íos *A*ltoandinos, http://www.ub.edu/riosandes/index.php/biqura.html), we collected more specimens of genus *Barbadocladius*. In this paper, we report new findings of larvae, pupae and pupal exuviae of this genus and its phylogenetic relationships using *cox1* mitochondrial sequences.

Study area

The study area consisted of 193 sites from central Colombia to southern Peru, between the latitudes 5° North and 16° South (Fig 1 and Table 1). We have included data from our present and past research projects, including the Ph.D. thesis of R. Acosta (Acosta & Prat 2010) and C. Villamarín (unpublished data). Of the 17 basins listed in Table 1, a selection of 8 were sampled for molecular analysis as part of the BIQURA project: (1) 4 tributaries of the Amazon river that eventually drains into Atlantic Ocean (eastern range) and (2) 4 rivers delivering their water to the Pacific Ocean (western range). In each basin of the BIQURA project, a number of sampling sites were visited for collection, some at the highest altitudes (>3,500 m asl) where rivers and basins lack riparian vegetation (the so-called Páramo or Puna areas), and others between 2,000 and 3,500 m asl, where usually both the basin and the riparian zone are covered by trees. All sites were located in areas not or scarcely disturbed by man (close to reference conditions) and without any important source of water pollution (including the absence of mining).

Of all the sites sampled, *Barbadocladius* was found in 13 (in 5 of the 17 investigated basins; Fig 1) a total of 14 times (in one site, it was found on two different dates). The exact locations and general environmental characteristics of the sampling sites, together with the material found at each site, are listed in Table 2.
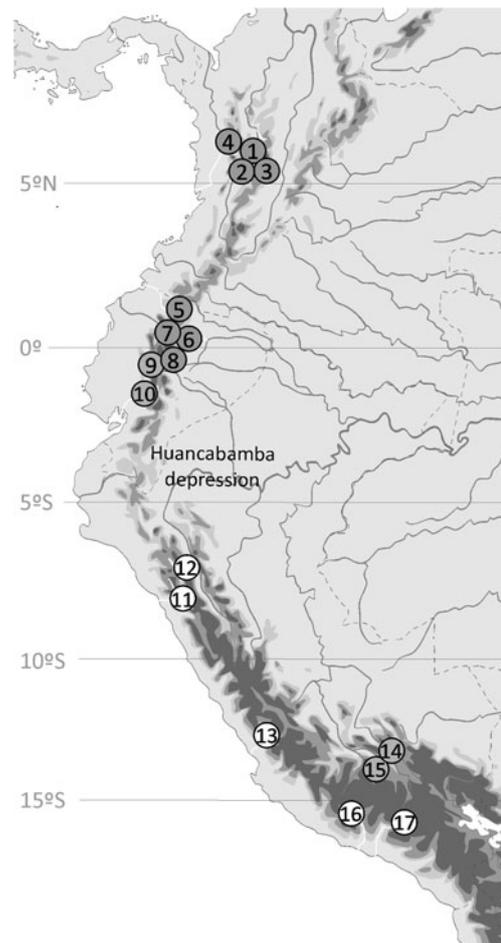
Fig 1 Map showing the localization of the different river basins studied in the tropical high Andean area. For details, see Table 1. The individuals of genus *Barbadocladius* were found only in basins 11, 12, 13, 16 and 17.

Material and Methods

Sampling and processing of samples for morphological and molecular analysis

The collection of macroinvertebrates for BIQURA samples was conducted in 2011 (exact dates in Table 2). The sampling strategy in the BIQURA project was designed to maximize Chironomidae diversity. Surber samples and Drift samples were taken simultaneously. Surber samples were taken in different substrates following the protocol described by Núñez & Prat (2010) and Villamarín *et al* (2013). At each site, samples were examined in the field, and at least 100 larval midges were picked out from each sample and preserved in 96% ethanol for DNA analysis. At the same time, at least two drift samples were collected at each site while the sampling and sorting of benthos from the Surber samples took place (2–3 h of exposure). The main purpose of collecting drift samples was to collect pupae and pupal exuviae. The sorting and classification of drift samples took place at the laboratory. All specimens used for molecular analysis were preserved in 96% ethanol.

Table 1 Basins sampled for the study of the Chironomidae biodiversity of the tropical, high altitude, Andean streams and rivers.

Code in Fig 1	Country	Basin	Side	No. of sites 2,000–3,000 m asl	No. of sites >3,000 m asl	Research project
1	Colombia	Chinchiná-Cauca-Magdalena	Atlantic-Caribean	4	2	BIQURA
2	Colombia	Campoalegre-Cauca-Magdalena	Atlantic-Caribean	2	0	BIQURA
3	Colombia	Gualí-Magdalena	Atlantic-Caribean	0	2	BIQURA
4	Colombia	San Juan	Pacific	2	0	BIQURA
5	Ecuador	Mira	Pacific	6	10	CERA
6	Ecuador	Aguarico-Napo	Atlantic-Amazon	8	6	CERA
7	Ecuador	Guayllabamba-Esmeraldas	Pacific	2	5	BIQURA
8	Ecuador	Papallacta, Tambo-Napo	Atlantic-Amazon	3	3	BIQURA
9	Ecuador	Pastaza-Marañón	Atlantic-Amazon	8	8	CERA
10	Ecuador	Guayas	Pacific	12	3	CERA
11	Peru	Santa	Pacific	1	15	CERA, BIQURA
12	Peru	Mosna-Marañón	Atlantic-Amazon	4	12	CERA, BIQURA
13	Peru	Cañete	Pacific	5	30	Acosta Ph.D. thesis
14	Peru	Urubamba	Atlantic-Amazon	0	15	CERA
15	Peru	Ampay-Apurimac	Atlantic-Amazon	3	4	BIQURA
16	Peru	Cotahuasi-Ocoña	Pacific	0	7	BIQURA
17	Peru	Colca-Camaná	Pacific	2	13	CERA

Information reported here refers to country, river basin name, topographical orientation of the basin (side) and the number of sampling sites grouped in two altitudinal ranges (between 2,000 and 3,000 m a.s.l. and those above 3,000 m a.s.l.).

*BIQURA BI*odiversidad de *QU*ironómidos en *R*íos *A*ltoandinos (Sampling in 2011); *CERA C*alidad *E*cológica de *R*íos *A*ltoandinos (Sampling in 2007, from Villamarín Ph. D. thesis). Samples from Acosta Ph.D. thesis were taken in 2005.

Table 2 Sites where *Barbadocladius* specimens have been found.

Sites Code	Date	Basin	Side	River name	Altitude (m asl)	Longitude	Latitude	Temperature (°C)	Conductivity µS/cm	Flow (l/s)	Individuals examined
BIQURA											
COT01	12/July/2011	Ocoña	Pacific	Huancarama	3,278	72° 54′ 51″	15° 7′ 45″	7.9	35.2	16.1	6 L
COT02	13/July/2011	Ocoña	Pacific	Sumana	3,470	72° 40′ 50″	15° 3′ 42″	6.6	82.4	1184.2	13 L +3P
COT03	13/July/2011	Ocoña	Pacific	Manantial S/N	3,854	72° 42′ 4″	15° 1′ 14″	11.8	29.1	40.8	4 L
COT04	13/July/2011	Ocoña	Pacific	S/N	3,640	72° 42′ 43″	14° 59′ 10″	9.8	15.0		6 L
COT05	14/July/2011	Ocoña	Pacific	Chinche	4,486	72° 38′ 32″	14° 54′ 15″	7.4	13.7	632.0	2 L
COT07	14/July/2011	Ocoña	Pacific	Ajoruro	4,670	72° 38′ 20″	14° 55′ 28″	3.6	130.7	42.5	1 L
SA15Der	03/July/2011	Santa	Pacific	Yanayacu	3,582	77° 24′ 54″	9° 46′ 31″	8.0	33.6	748.0	10Pex
SA05Der	01/July/2011	Santa	Pacific	Ishinca	3,364	77° 31′ 19″	9° 22′ 56″	10.0	26.0	799.7	5Pex
Other											
CA01	09/September/2004	Cañete	Pacific	Cañete	4,396	76° 0′ 54″	12° 9′ 6″	7.0	70.0	257.5	3 L
CA06	01/March/2005	Cañete	Pacific	Estanza	3,913	75° 51′ 49″	12° 5′ 29″	2.0	420.0	455.0	10 L
CA06	24/May/2005	Cañete	Pacific	Estanza	3,913	75° 51′ 49″	12° 5′ 29″	2.0	420.0	455.0	1Pex
CO10	06/October/2008	Colca	Pacific	Escalera	3,778	71° 35′ 21″	15° 38′ 33″	2.0	19.6	63.0	1Pex
MO3	21/October/2007	Mosna	Atlantic	Mosna	3,282	77° 11′ 44″'	9°37′ 42″	11.7	91.6		1 L+2Pex
MO10	22/October/2007	Mosna	Atlantic	Garguanga	3,485	77° 25′ 33″	9°10′ 53″	11.7	35.3		1Pex

BIQURA sites are the ones used for molecular analysis (*COT* Cotahuasi basin; *SA* Santa river basin). Only larvae (L) and pupae (P) were prepared for molecular analysis (Pupal exuviae, Pex, were examined only morphologically). For other sites than those of BIQURA project, data are from Acosta Ph. D. thesis (*CA* Cañete river) and Villamarín Ph. D. thesis. (*CO* Colca river basin; *MO* Mosna river basin). For the location of the basins, see Fig 1 and Table 1. *S/N* means no known name of the river is available (from the Spanish 'Sin Nombre').

At the laboratory, the larvae of Chironomidae were grouped into different morphotypes under a stereo microscope, and identified using the key of Prat *et al* (2012). If different genera have similar morphotypes, we grouped the animals under the name "morphotype #1". For each morphotype, up to five to ten individuals were prepared in the following manner: The head was dissected from the thorax, 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). The body was then used for destructive DNA extraction. A total of 32 *Barbadocladius* larvae were found at six different BIQURA sites (Table 2). Only three pupae were found in all the BIQURA sites, and before DNA extraction, they were classified as *B. andinus* according to Cranston & Krosch (2011). One pupa was used for DNA extraction, and two were prepared for examination under the microscope using the techniques of Cranston & Krosch (2011). None of the three pupae were mature; vouchers of the two pupae are in the collection of the authors. Moreover, some pupal exuviae (*n* =15) from two BIQURA sites where the larvae was not found were examined (Table 2). Unfortunately, we were not able to make any extraction of the pupal exuviae before we prepared them in Euparal. All the vouchers of these exuviae are in the author's collection.

Additional material from *Barbadocladius* was present in benthic and drift samples from three different basins in Peru from our previous studies, two sites in the Cañete river (Acosta & Prat 2010), two sites in the Mosna river and one site in the Colca river (Villamarín 2012, Ph.D. thesis). The samples were taken in 2005 and 2007 (Table 2). In the first case, benthic samples were taken with a D net, and in the second case with a Surber sample (following the same methodology of the BIQURA project described earlier). In both cases, samples were fixed in the field with formaldehyde and examined in the laboratory under a stereo microscope. Slides of 14 larvae and 5 pupal exuviae from these sites were prepared following the same methodology described above. It was not possible to extract the DNA of this material.

DNA extraction, PCR amplification and sequencing

Samples for DNA molecular analysis consisted of 31 larvae and 1 pupa (Table 3). 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 list of individuals and their corresponding localities can be found in Table 3. In addition to the *Barbadocladius* larvae, we used one larva of *Limnophyes* sp. from COT-7 (nº 339) as an outgroup. The two sequences of *Barbadocladius* existing in GenBank, corresponding to the individuals sequenced by Cranston & Krosch (2011), were also included in the analysis.

A total of 670 bp of the cytochrome oxidase I gene (*cox1*) was amplified from each individual using C1-J-1718/C1-N-2191 (mt6/Nancy) primers. As the two *Barbadocladius* sequences available from GenBank (Cranston & Krosch 2011) used the C1-J-2183/C1-N-2776 (Jerry/Maggie) primers, we also amplified this gene fragment from 20 individual representatives of each clade to include in the analysis the two existing sequences. Primers and conditions used are listed in Table 4. PCR reactions were carried out in a final volume of 25 μL, using either Taq polymerase (Promega) or Biotools Pfu DNA Polymerase (Biotools). PCR products were cycle-sequenced in both directions using the same PCR primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed in an ABI 3700 automated sequencer at the CCiTUB (Centres Científics i Tecnològics de la Universitat de Barcelona). Raw sequences were edited and assembled with GENEIOUS v. 4.6.5 (Drummond *et al* 2009). The new sequences used in this study have been deposited in GenBank with accession numbers KF386113–KF386145 (see Table 3).

Alignment and phylogenetic analysis

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.

Maximum Likelihood (ML) analyses were conducted using the online version of RAxML (Stamatakis *et al* 2008). One thousand iterations of the random addition of taxa were performed applying the parameters of Gamma model of rate heterogeneity. The online version obtained bootstrap support values from 100 pseudoreplicates. The uncorrected genetic distances between the taxa were assessed using MEGA v.5.0 (Tamura *et al* 2011).

Results

Distribution of the genus Barbadocladius in high altitude Andean tropical streams

From the samples taken in the basins shown in Fig 1, we have collected data on the taxa composition of Chironomidae from 193 sites in the study area, with more than 6,000 larvae examined (more than 4,000 have been prepared for molecular analysis) and more than 2,000 pupal exuviae. As shown in Fig 1 and Tables 1 and 2, we have found larvae, pupae or pupal exuviae of *Barbadocladius* in only 13 sites in five different basins, all of them exclusively in Peru and mostly on the Pacific side, except for one sample collected on the Atlantic side (Mosna river). *Barbadocladius* densities were always low (<13 ind m^{-2}), and a total of 46 larvae, 3 pupae and 15 pupal exuviae were collected. The pupal exuviae from the BIQURA project were found only in drift samples from the Santa River

Table 3 List of specimens included in the molecular analysis, with their identification code in Fig 2, locality and GenBank accession numbers.

Code	Genus	Locality	Mt6/Nancy	Jerry/Maggie	GenBank accession no.
40Def	*Barbadocladius sp2*	COT05	X	X	KF386118
45a	*Barbadocladius sp2*	COT05		X	KF386119
176	*Barbadocladius sp1*	COT01	X		KF386139
183	*Barbadocladius sp1*	COT01	X		KF386142
182	*Barbadocladius sp1*	COT01	X	X	KF386117
177	*Barbadocladius sp1*	COT01	X	X	KF386114
340	*Barbadocladius sp1*	COT02	X	X	KF386132
283	*Barbadocladius sp1*	COT03	X	X	KF386125
344	*Barbadocladius sp1*	COT02	X	X	KF386131
348	*Barbadocladius sp1*	COT02	X	X	KF386127
341	*Barbadocladius sp1*	COT02	X	X	KF386115
446	*Barbadocladius sp1*	COT02	X	X	KF386129
342	*Barbadocladius sp1*	COT02	X	X	KF386128
447	*Barbadocladius sp1*	COT02	X	X	KF386123
347	*Barbadocladius sp1*	COT02	X	X	KF386124
346	*Barbadocladius sp1*	COT02	X	X	KF386116
349	*Barbadocladius sp1*	COT02	X		KF386135
111	*Barbadocladius sp1*	COT04	X		KF386134
633	*Barbadocladius sp1*	COT02	X		KF386145
345	*Barbadocladius sp1*	COT02	X		KF386136
282	*Barbadocladius sp1*	COT03	X	X	KF386130
136a	*Barbadocladius sp1*	COT04	X	X	KF386122
186a	*Barbadocladius sp1*	COT01	X	X	KF386120
185	*Barbadocladius sp1*	COT01	X	X	KF386121
343	*Barbadocladius sp1*	COT02	X	X	KF386126
109	*Barbadocladius sp1*	COT04	X		KF386133
281	*Barbadocladius sp1*	COT03	X		KF386143
313	*Barbadocladius sp1*	COT07	X		KF386144
110	*Barbadocladius sp1*	COT04	X		KF386137
112	*Barbadocladius sp1*	COT04	X		KF386138
113	*Barbadocladius sp1*	COT04	X		KF386141
284	*Barbadocladius sp1*	COT03	X		KF386140
HQ872983	*Barbadocladius*	Chile		X	HQ872983
HQ872984	*Barbadocladius*	Argentina		X	HQ872984
339	*Limnophyes*	COT07	X	X	KF386113

Basin, at two sites where larvae were not found not in the drift or in the benthos samples (Table 2). We only found larvae and pupae at the same time in one site (COT02, both analysed for molecular analysis). Individuals were always found at very high altitude (between 3,278 and 4,670 m asl), in cold water streams in Páramo or Puna areas (with scarce or no vegetation on the river shores or in the basin), in both small- and medium-sized streams with variable flow and low mineralization (Table 2).

Table 4 List of primers used in this study.

Gene fragment	Primer	Or.[a]	Sequence (5'-3')	Reference
cox1	MT6	F	GGAGGATTTGGAAATTGATTAGTTCC	Simon *et al* (1994)
	NANCY	R	CCCGGTAAAATTAAAATATAAACTTC	Simon *et al* (1994)
	JERRY	F	CAACATTTATTTTGATTTTTTGG	Simon *et al* (1994)
	MAGGIE	R	GGATAATCAGAATATCGTCGAGG	Hedin & Maddison (2001)

[a] Primer orientation.

Morphological analysis: pupae

Cranston & Krosch (2011) described the two species of *Barbadocladius* (*B*. *andinus* and *B*. *limay*) based on morphological characteristics of pupae and pharate male adults (using two male imagoes and ten pupae of the former species, and one male and several pupae of *B*. *limay*). The two species differ in their size in the original description: the *B*. *andinus* pupa is 2–2.8 mm (*n* =10) long, and the *B*. *limay* is 1.5 to 1.9 mm long (*n* =8). The distribution of hooklets and spines on the abdomen shows conspicuous differences between both species (compare Tables 5 and 6). While *B*. *andinus* has hooklets in conjunctives TIII, TIV and SV and SVI, *B*. *limay* has hooklets in conjunctives TII to IV and SIV to SVI (T=Tergite; S=Sternite). The abdominal spines in *B*. *andinus* are long and narrow, while in *B*. *limay*, they are short and stout. The two pupae prepared for morphological examination (from site COT 02) and most of the pupal exuviae found at the two sites on the Santa River (SA15Der and SA05Der) were very similar to *B*. *andinus*, except for four smaller pupal exuviae found at site SA05 that were similar to *B*. *limay*.

As seen in Table 5, the pupae and pupal exuviae of Peruvian material of *B*. *andinus* pupal types are at the lower range of size given in the original description. The thoracic horn (TH) is difficult to observe and measure; in the few individuals where we have observed it, its length is close to 60 μm, and it is oval like in Fig 3c in Cranston & Krosch (2011). The number of hooklets and spines in tergites and sternites was very similar to the numbers given in the original description, except that the SV hooklets were never present in our examined material. We found no clear relationship between exuviae size and the number of hooklets or spines in the tergites and sternites. The length of the anal setae may be smaller than the original data of Cranston & Krosch (2011), especially in the pupal exuviae taken from the Santa River. On the basis of these measurements, no clear differences were found between the pupal characteristics of our specimens and those of Cranston and Krosch (loc cit.).

The four pupal exuviae of *B*. *limay* that we found in Peru at 3,364 m a.s.l. are very similar to those described for this species in Cranston & Krosch (2011) in terms of size and tergite and sternite ornamentation (Table 6). The most striking difference is that our specimens never had a hook row in sternite IV.

Morphometric analysis of larvae

According to Cranston & Krosch (2011), only the larvae of *B*. *andinus* are known with certainty (because they found three larval exuviae attached to mature pupae). More larvae were found in Chile and Argentina by these authors, but they failed to find differences in the size of the larvae they collected, despite the clear differences in size between the pupa and adults of the two species they described. We have tried to find differences in the size of our larvae compared to the morphological characteristics provided by Cranston & Krosch (loc. cit; Table 7).

We could not measure the entire size of the larvae because we used the larval body for DNA extraction and because the body length varies depending on the developmental stage. We therefore used the postmentum length as a proxy for larval size to compare our data with those of Cranston & Krosch (2011). We also measured the head length, but the size

Table 5 Morphometric characteristics of pupae and pupal exuviae of *Barbadocladius andinus* (Cranston & Krosch 2011).

	n =10 Cranston	*n* =2 COT02 Range	*n* =11 SA15Der Range	*n* =9 SA05Der Range
Total length (mm)	2–2.8		2.09–2.44	1.99–2.37
TH length (μm)			ca 60	ca 60
TIII hooklets (*n*)	7–17	9–13	8–12	5–11
TIII spines (*n*)	22–30	25–37	17–42	15–24
TIV hooklets (*n*)	8–16	12–13	5–13	7–11
TIV spines (*n*)	19–36	27–37	27–52	21–36
SV hooklets	0–6			
TV spines (*n*)	23–35	47–52	26–51	24–39
TVI spines (*n*)	19–38	35–42	26–45	24–40
SVI hooklets (*n*)	6–16	5	3–7	4–11
TVII spines (*n*)	17–29	39–42	21–45	18–33
SVII spines (*n*)	10–15	17–18	9–18	9–15
TVIII spines (*n*)	10–18	20	10–18	9–20
Anal lobe length (μm)		130–154	95–147	105–146
Anal setae length (μm)	70–98	83–97	50–98	43–96

The original data from Cranston & Krosch (2011) are provided for comparison. The number (*n*) of hooklets or spines in Tergites (T) and Sternites (S) is indicated. Measurements are in millimeters or micrometers.

TH thoracic corn.

Table 6 Morphometric characteristics of the pupal exuviae of *Barbadocladius limay* (Cranston & Krosch 2011).

	Cranston n =8 Range	SA15 Der n =4 Range
Total length (mm)	1.5–1.9	1.75–1.93
Abdomen length (mm)		1.12–1.2
TII hooklets (*n*)	18–20	16–23
TIII hooklets (*n*)	17–22	20–23
TIII spines (*n*)	19–28	16–36
TIV hooklets (*n*)	15–21	12–22
TIV spines (*n*)	20–32	30–36
SIV hooklets (*n*)	0–2	0
TV Spines (*n*)	23–41	30–40
SV hooklets (*n*)	10–20	17–24
TVI spines (*n*)	20–39	25–39
SVI hooklets (*n*)	18–23	13–23
TVII spines (*n*)	21–33	29–41
SVIII spines (*n*)	3–4	10–12
TVIII spines (*n*)	14–18	10–15
Anal lobe length (µm)		72–111
Anal setae length (µm)		
1		15–24
2		30–32
3		42

As in the precedent table, we provide the original data from Cranston & Krosch (2011) to compare with our measurements.

of the head on the slides may be heavily modified due to cover slip pressure, while the postmentum length is less variable. We measured 42 larvae, and the range of postmentum lengths was greater than the measures provided by Cranston & Krosch (2011) (99–131 µm compared to 102–110 µm; Table 7). The same is true for other measures (mentum and mandible length and the length of each antennal segment). The AR, which was close to 1.52 in. Cranston & Krosch (2011), varied from 0.95 to 1.65 in our larvae. There was no statistical relationship between any size measure and the AR using all available data. Note that the two individuals found in COT05 (which, according to molecular data, are different) were larger, but the values for the length of the antennal segments or the AR were in the same range as the others.

Molecular data

Molecular analysis was possible at 6 of the 14 sites were *Barbadocladius* was found in Peru, all concentrated in only the Cotahuasi basin in southern Peru (Fig 1, Tables 1–3). Because the pupal skins collected from some basins (Santa, Mosna, Colca and Cañete) were prepared in Euparal, or the larvae were from vouchers from previous studies (Cañete and Mosna rivers), we could not extract DNA from these samples. After DNA extraction, some individuals gave no positive results for molecular analysis, which left a total of 31 larvae and 1 pupa with good DNA extraction and amplification.

The final dataset used for the phylogenetic analysis included 32 sequences of *Barbadocladius* from the Cotahuasi basin, the two *Barbadocladius* sequences from Gene Bank (HQ872983 from Chile and HQ872084 from Argentina) by Cranston & Krosch (2011) and one *Limnophyes* sp. larvae to root the tree, yielding 35 terminals and 1,280 aligned characters (see Table 3 for dataset information). The tree obtained by the ML analysis is shown in Fig 2.

Table 7 Morphometric characteristics of the *Barbadocladius* larvae found in streams of Peru.

	n =3 Cranston	n =6 COT01	n =10 COT02	n =4 COT03	n =5 COT04	n =2 COT05	n =1 COT07	N =3 CA01	n =10 CA06	n =1 MO03
Head length (µm)	295	296–330	325–392	266–326	304–322	363–380	363	286–299	307–339	283
Postmentum length (µm)	102–110	103–119	108–122	101–114	109–116	130–131	126	116–119	105–122	99
Mandible length (µm)	76–85	76–87	86–96.6	69.4–80.2	76.7–84.9	81–94	84	78–83	82–97	77.86
Mentum width (µm)	53–60	58–65.6	64.3–78.3	58.7–68.8	54.2–69.9	67.7–76.5	81	75–79	62–78	68.23
Seta submentum length (µm)	31–31	23–32.4	26.3–40.6	30.8–35.1	26.7–35.8	34.5–46.9	23.89	29–31	29.6–37	25.92
AS1 (µm)	32–33	27–31	27.9–32.8	26.5–32.0	26.9–31.4	33.2–35.0	34.19	30.4–33.4	27.3–34.1	25.17
AS2 (µm)	10–11	7.30–9.94	8.5–10.3	8.1–10	8.7–11.8	10.1–11	9.71	8.5–10.1	6.8–10.3	10.39
AS3 (µm)	5	5–7	5.1–7.7	6.2–7.4	6.3–8.7	7.5–7.7	7.62	7.6–8.6	6.6–9.5	8.93
AS4 (µm)	3	2.0–3.3	2.7–3.8	2.7–3.4	2.1–4.0	2.4–2.8	3.43	2.6–4.1	2.1–4.1	3.50
AS5 (µm)	3	1.0–2.5	2.4–3.9	2.3–2.9	1.5–3.3	2.4–3.2	2.91	2.5–3.7	2.2–3.9	3.60
AR (µm)	1.5–1.52	1.36–1.65	1.22–1.58	1.24–1.31	1.2–1.48	1.34–1.55	1.44	1.26–1.39	1.16–1.42	0.95

Sampling site codes in Table 2. The values of Cranston and Krosch (2011) are given for comparison. Only COT samples were available for molecular analysis.

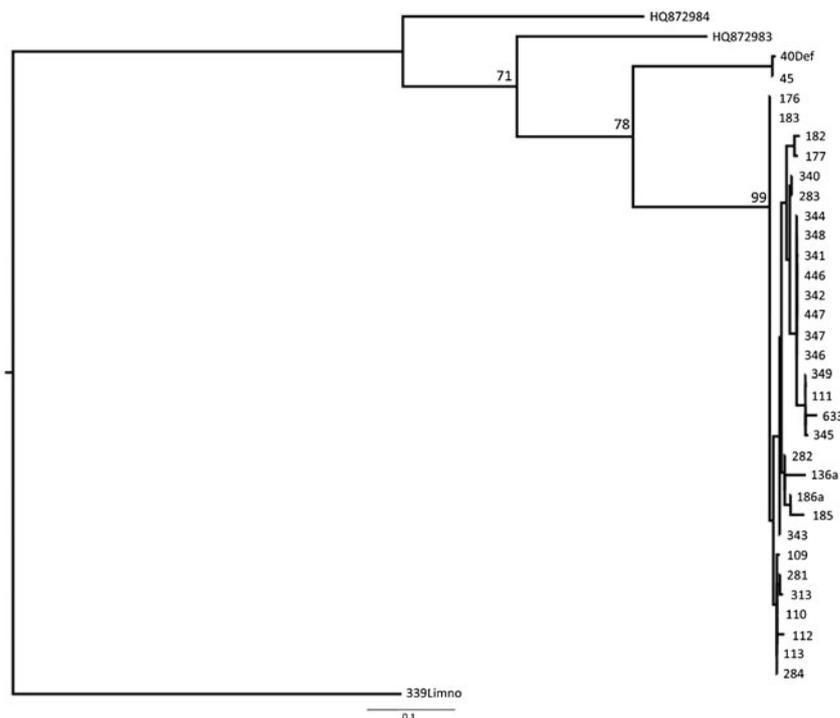*AS* antennal segment (length), *AR* antennal ratio.

Fig 2 ML tree of the *Barbadocladius* samples using partial *cox1* gene sequences. *Numbers in nodes* correspond to bootstrap support values. The tree was rooted using a *Limnophyes* sp, from the COT07 site. *Numbers on terminals* correspond to the code of the specimens listed in Table 3. The sequences deposited in Gene Bank corresponding to the paper of Cranston and Krosch (2011) were included.

Most of the samples analysed were grouped in the same clade with high bootstrap support (99) and low internal uncorrected genetic distance (1.03%), suggesting that the species is widely distributed throughout the basin sampled (sites COT01; COT02, COT03, COT04; COT07). The two larvae from COT05 clustered in a separate clade with moderate support (78) and with a high uncorrected genetic distance (10.56%) between both clades, suggesting that they constitute different evolutionary lines. As indicated previously, the two larval individuals of COT05 were slightly larger than the others (Table 7).

The two *Barbadocladius* sequences from Gene Bank included in the analysis are located in a basal position. The individual HQ872983, from Chile, constitutes the sister evolutionary line of the two Peruvian representatives; and the sequence HQ872984, from Argentina, is located in the basal position on the tree. The average uncorrected genetic distance between these four clades ranges from 10.56% to 14.44% (see Table 8). Because of the geographical separation involved, this clear genetic differentiation between these four evolutionary lines indicates the existence of separate populations of the same species forming a cline from south to north, or even the presence of four putative new genetically distinct species. Unfortunately, in the absence of mature pupae or adults, we cannot discriminate between these two hypotheses.

As the two sequences deposited in Gene Bank are identified as *B*. *andinus*, and our pupa or pupal exuviae show similar morphological characteristics to the species description in Cranston & Krosch (2011), at this moment it is not possible to identify the four molecular evolutionary lines using morphological characteristics.

Discussion

Barbadocladius was first reported by Cranston & Krosch in 2011 while examining the evolution of Gondwanan Orthocladiinae. In addition to the material originally collected by Roback & Coffmann (1983) in Bolivia (as larval taxon Genus 9 and pupal taxon Genus 4), Cranston collected more material in the Patagonian Andes. As a result, he characterized

Table 8 Mean uncorrected *p* distances of *cox1* between (lower left) and within clusters (upper right).

	HQ872983	HQ872984	*Barbadocladius sp1*	*Barbadocladius sp2*
HQ872983			0.010328	0.001698
HQ872984	0.134892			
Barbadocladius sp. 1	0.138984	0.144433		
Barbadocladius sp. 2	0.115179	0.129496	0.105689	
Limnophyes sp.	0.139286	0.133094	0.157212	0.158823

Barbadocladius as a trans-Andean genus distributed between 39° and 41° S and from 260 to 1,185 m asl (Cranston & Krosch 2011). In this paper, we extend the distribution further north (to 9° S, table 2). However, the distribution is restricted in this area to very high altitude rivers and streams (above 3,278 m). The reason for these altitudinal differences in *Barbadocladius* distribution along the latitudinal gradient in the Andes is likely because cold stenothermic conditions in lower latitudes (Peru, Ecuador) occur at higher altitudes compared to more southerly latitudes (Patagonian Andes) where the same conditions occur at lower altitudes (Illies 1969, Jacobsen *et al* 1997). This means that a species' altitudinal range is highly dependent upon geographical latitude. The inverse relationship between altitude and latitude with temperature has been described for the Andes in several papers (Jacobsen *et al* 1997, Jacobsen 2003, Jacobsen & Marín 2007). All of the river sites where the larvae, pupae or pupal exuvia were found are located in the Páramo or Puna, an area of cold waters without specific riparian vegetation and with only scattered shrubs in the catchment (García & Beck 2006, Mena & Hofstede 2006).

From our extensive sampling of 17 basins in Páramo and Puna areas ranging from 5°N to 16°S, we conclude that the distribution of *Barbadocladius* does not extend further north than 9°S in the Peruvian Andes, as it has not been found in the highlands of Ecuador and Colombia. In this context, it is interesting to note that the Huancabamba Depression is situated at 6°S latitude (Fig 1), and constitutes a major structural and physiographic break in the Andes where continuity is partially interrupted by the confluence of the Chamana-Marañón rivers, separating the Cordillera Central from the Cordillera Oriental. The Huancabamba Depression is the lowest pass (2,145 m asl) between Colombia and southern Chile (Duellman & Pramuk 1999). The present elevations and drainage patterns in the Huancabamba Depression most likely were established in the Pleistocene (Gansser 1973, Harrington 1956). During this period, climatic fluctuations included cooler, drier conditions during glacial phases and warmer and higher moisture conditions during interglacial phases. These climatic fluctuations, together with the complex topography, presumably resulted in isolation of highland areas that favored high levels of endemism (Henning *et al* 2011, Rivera *et al* 2011). Furthermore, these conditions provided geographical barriers to dispersion for many taxa, such that the Huancabamba Depression corresponds to the northern or southern limit of modern distribution of many species including frogs (Duellman & Pramuk 1999), mantids (Rivera *et al* 2011) and plants (Cosacov *et al* 2009, Weigend *et al* 2010). This could also be the case for *Barbadocladius* in our study.

The pupae of the two species of *Barbadocladius* are very different in size; *B. limay* is no longer than 2 mm in length, while *B. andinus* always is larger than 2 mm. The Peruvian material confirms the differences in size of the pupal exuviae. Our study of the armament characteristics of the tergites and

sternites showed no clear differences between the individuals of each species described by Cranston & Krosch (2011) and our material. According to the pupal morphology, there seem to be only two species of the genus *Barbadocladius* in the Andes. The morphology of the pupa found at the COT02 site was very similar to *B. andinus* of Chile and Argentina, but the molecular analysis indicated that it may be a different species (the same species as the larvae found at the same site, which is the species more widely distributed in the Cotahuasi basin, Fig 2). As most molecular analyses have been made with larvae, the situation is even more complicated when the morphology of the larvae is compared with the molecular results because no clear differences exist between larvae of the different species obtained from the clade. The morphometric characteristics of the larvae in the paper by Cranston & Krosch (Table 7) are those of the three larvae of *B. andinus* that they found associated with pupae.

In their notes on larvae, Cranston & Krosch (2011) explained that all the larvae they found do not segregate into two different size classes, despite the fact that the pupae are of very different size. In addition, Cranston & Krosch (2011) indicated that the two molecular voucher larvae deposited in Gene Bank are unreared larvae that are even larger (potsmentum length 125–126) than the *B. andinus* larvae associated with pupae (102–110), so they suggested that the two vouchers cannot be considered representative of *B. andinus* with certainty. Although we found a larger range of sizes in our material than Cranston & Krosch (2011), with large variability between sampling sites (Table 7), we cannot differentiate two groups of larvae by size. However, at the molecular level, we found two different clades in the Cotahuasi basin, one widely distributed (five sites, between 3,278 and 4,670 m asl) and the other present at only one site (COT05) at 4,486 m asl. The larvae of the COT05 site are the largest that we found. Finally, the phylogenetic analysis (based on larvae) shows that the molecular arrangement of the two larvae from Chile and Argentina deposited at Gene Bank is different from those of Peru and even different between them, suggesting that four populations of the same species, genetically quite differentiated, are present in the high altitude tropical Andes, which could even be considered four different species.

Krosch *et al* (2011), in studying the evolutionary relationships of putative Gondwanan Orthocladiinae using molecular techniques, included the genus *Barbadocladius* as a sister group of a large and diverse clade estimated to comprise taxa largely of Gondwanan distribution. According to Krosch *et al* (2011), the pupal structures of this genus resemble those of the genus *Eukiefferiella* (absent from South America), indicating that this taxa may be an ecological vicariant of that taxa, although is not as frequent and abundant as *Eukieferiella* is in European and North American streams and rivers. As indicated by Cranston & Krosch (2011), the presence of hooklets in

pupae seems to be a homoplasious feature, existing in other distant genera. The larva is totally different from other larvae of Orthocladiinae, with some similarities to those of the genus *Synorthocladius*. The morphological analysis of the Peruvian *Barbadocladius* does not change the assumption of Cranston & Krosch that this is a sister clade to a large clade that comprises taxa largely of Gondwanan distribution (Krosch *et al* 2011). However, the molecular analysis reveals more specific taxonomic variability than what was found in Argentina and Chile, indicating the possible presence of a speciation in process from south to north along the Andes. Additionally, climatic fluctuations during the Pleistocene may also have contributed to speciation through isolation of many cold stenothermic taxa (such as *Barbadocladius*) in high altitude refuges (Jacobsen *et al* 1997, Rivera *et al* 2011). We hypothesize that several cryptic species are present along the latitudinal gradient from 41° to 9°S. In the future, new samples from the original site in Bolivia, where the species was first caught by Roback & Coffmann (1983), could confirm if there is a cline between the taxa we have collected in Peru and those from Patagonia collected by Cranston & Krosch (2011).

In conclusion, we have extended the distribution of the genus *Barbadocladius* further north, but also restricted its distribution to south of latitude 6°. In its present northern distribution, it is restricted to very high altitude streams, confirming that this is a trans-Andean taxa (present in both the Pacific and Atlantic rims) with a Gondwanan distribution. Although from the morphology of larvae and pupae no more than the two species may be differentiated, the molecular analysis suggested that several cryptic species may exist in the area between 9 and 41°S. As for the moment few materials have been studied, we cannot establish how many species are in this large area with certainty. We suspect that a clear cline effect exists. Given its distribution, the altitudinal gradient (inversely proportional to the latitude), and the high altitude where the populations are found in Peru, the *Barbadocladius* species complex could be considered in the high part of the Andes as a set of isolated Gondwanan species located in mountain refuges where the ecological conditions allow their survival.

References

Acosta R, Prat N (2010) Chironomidae assemblages in high altitude streams of the Andean region of Peru. Fundam Appl Limnol 177:57–79

Cosacov A, Sérsic AN, Sosa V, De-Nova JA, Nylinder S, Cocucci AA (2009) New insights into the phylogenetic relationships, character evolution, and phytogeographic patterns of *Calceolaria* (CALCEOLARIACEAE). Am J Bot 96:2240–2255

Cranston PS, Krosch M (2011) *Barbadocladius* Cranston & Krosch, a new genus of Orthocladiinae (Diptera: Chironomidae) from South America. Neotrop Entomol 40:560–567

Drummond AJ, Ashton B, Cheung M, Heled J, Kearse M, Moir R, Stones-Havas S, Thierer T, Wilson A (2009) Geneious v.4.6.5. created by Biomatters

Duellman WE, Pramuk JB (1999) Frogs of the Genus *Eleutherodactylus* (Anura: Leptodactylidae) in the Andes of Northern Peru. Scientific Papers. Natural History Museum The University of Kansas 13: 1–78

Epler J (2001) Identification manual for the lñarval Chironomidae (Diptera) of North and South Carolina. A guide to the taxonomy of the midges of southeastern United States, including Florida. North Carolina Department of Environment and Natural Resources, Raleigh, NC and St. Johns River Water Management District, Plalatka, FL., Special Publication SJ2011-SP13, pp 526

Gansser A (1973) Facts and theories on the Andes. J Geol Soc (London UK) 129:93–131

García E, Beck S (2006) Puna, p. 51–76. In: Moraes M, Øllgaard B, Kvist LP, Borchsenius F, Balslev H (eds) Botánica Económica de los Andes Centrales. Universidad Mayor de San Andrés, La Paz

Harrington HJ (1956) Main morphostructural regions of South America. In: Jenks WF (ed) Handbook of South American geology. Memoir, Geological Society of America, Boulder, p. xii–xviii

Hedin MC, Maddison WP (2001) A combined molecular approach to phylogeny of the jumping spider subfamily Dendryphantinae (Araneae: Salticidae). Mol Phylogenet Evol 18:386–403

Henning T, Rodríguez EF, Weigend M (2011) A revision of the *Nasa ranunculifolia* group (Nasa ser. Grandiflorae pro parte, Loasaceae). Bot J Linn Soc 167:47–93

Illies J (1969) Biogeography and ecology of neotropical freshwater insects, especially those from running waters. In: Fittkau EJ, Illies J, Kling H, Schwabe GH, Sioli H (eds) Biogeography and ecology in South America, IIth edn. Springer, Netherlands, pp 685–708

Jacobsen D (2003) Altitudinal changes in diversity of macroinvertebrates from small streams in the Ecuadorian Andes. Arch Hydrobiol 158: 145–167

Jacobsen D, Marín R (2007) Bolivian Altiplano streams with low richness of macroinvertebrates and large diel fluctuations in temperature and dissolved oxygen. Aquat Ecol 42:643–656

Jacobsen D, Schultz R, Encalada AC (1997) Structure and diversity of stream invertebrate assemblages: the influence of temperature with altitude and latitude. Freshwater Biol 38:247–261

Krosch MN, Baker AM, Cranston PS (2011) Systematics and biogeography of the Gondwanan Orthocladiinae (Diptera: Chironomidae). Mol Phylogenet Evol 59:458–468

Mena P, Hofstede R (2006) Los páramos ecuatorianos, p 91–109. In: Moraes M, Øllgaard B, Kvist L, Borchsenius F, Balslev H (eds) Botánica Económica de los Andes Centrales. Universidad Mayor de San Andrés, La Paz

Nuñez M, Prat N (2010) Efectos de la sequía y las crecidas en los índices biológicos en el río Llobregat. Tecnología del Agua 30:46–55

Prat N, Acosta R, Villamarín C, Rieradevall M (2012) Guía para el reconocimiento de las larvas de Chironomidae (Diptera) de los ríos Altoandinos de Ecuador y Perú. Clave para la determinación de los principales morfotipos larvarios. Grupo de Investigación F.E.M. (Freshwater Ecology and Management), Departament d'Ecologia,

Universitat de Barcelona. http://www.ub.edu/riosandes/index.php/guiachiros.html Accessed 13 May 2013.

Rivera J, Yagui H, Ehrmann R (2011) Mantids in the Mist—taxonomy of the Andean genus *Pseudopogonogaster* Beier, 1942, a cloud forest specialist, with notes on its biogeography and ecology (Mantodea: Thespidae: Miopteryginae). Insect Syst Evol 42:313–335

Roback SS, Coffman WP (1983) Results of the Catherwood Bolivian-Peruvian Altiplano Expedition. Part II. Aquatic Diptera including montane Diamesinae and Orthocaldiinae (Chironomidae) from Venezuela. Proc Acad Nat Sci Philadelphia 135:9–79

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am 87:651–701

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web-servers. Syst Biol 75:758–771

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Villamarín C (2012) Estructura y composición de las comunidades de macroinvertebrados acuáticos en ríos altoandinos del Ecuador y Perú. Diseño de un sistema de medida de la calidad del agua con índices multimétricos. Ph.D. Thesis, Universitat de Barcelona, Barcelona, Spain, p 207.

Villamarín C, Rieradevall M, Paul MJ, Barbour MT, Prat N (2013) A tool to assess the ecological condition of tropical high Andean streams in Ecuador and Peru: the IMEERA index. Ecol Indic 29:79–92

Weigend M, Gottschling M, Hilgeri HH, Nürk N (2010) Five new species of *Lithospermum* L. (Boraginaceae tribe Lithospermeae) in Andean South America: another radiation in the Amotape-Huancabamba Zone. Taxon 59:1161–1179