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Associating immatures and adults of aquatic insects using DNA barcoding in high Andean streams

Asociando inmaduros y adultos de insectos acuáticos utilizando códigos de barras de ADN en ríos altoandinos

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Abstract

We analyzed the feasibility of using DNA-barcoding as a tool to achieve a correct and rapid association between different life stages of Ephemeroptera, Plecoptera, and Trichoptera insects in high Andean streams from La Paz, Bolivia. We focused on this particular environment because the water of streams from the glaciers is becoming scarce, and this could create a risk of local disappearance of these aquatic species. Using cytochrome c oxidase subunit 1 (COI) gene sequences, we found high genetic interspecific divergence between specimens from different families and genera (maximum 20%), whereas the intraspecific genetic divergences were lower between specimens of the same species (ranged from 0.1-2%). In this manner, we associate the larval or nymphal instar with their respective adults (female and male), for one species of mayfly (*Meridialaris tintinnabula* Pescador & Peters, 1987), three species of stoneflies (*Anacroneuria vagante* Stark & Baumann, 2011, *Claudioperla tigrina* Klapálek, 1904 and C. *ruhieri* Gibon & Molina, 2013) and one species of caddisfly (*Anomalocosmoecus* cf. *illiesi* Marlier, 1962). We concluded that COI Barcoding can be an effective tool for associating life stages and this tool could speed up the exploring the biodiversity patterns of aquatic insects in the High Andean region.

Key words: Biodiversity pattern, DNA-barcode, High Andean region, Larval/nymphal-adult association, Taxonomy.

Resumen

Analizamos la viabilidad del uso de códigos de barras de ADN como una herramienta para lograr una asociación rápida y correcta entre las diferentes etapas de vida en algunos insectos de los órdenes Ephemeroptera, Plecoptera y Trichoptera, correspondiente a cursos de aguas corrientes de la región Andina de La Paz, Bolivia. Nos enfocamos en estos ambientes, debido a que el agua de los arroyos de los glaciares empieza a escasear, y esto podría crear un riesgo en

la desaparición local de estas especies acuáticas. Por medio del uso de las secuencias del gen citocromo c-oxidasa subunidad 1 (COI), encontramos alta divergencia interespecífica genética entre muestras de diferentes familias y géneros (máximo 20%), mientras que las divergencias genéticas intraespecíficas fueron menores entre ejemplares de la misma especie (osciló entre 0.1-2%). De esta manera asociamos el estadio larval o de ninfa con sus respectivos adultos (hembras y machos), para una especie de efemeróptero (*Meridialaris tintinnabula* Pescador & Peters 1987), tres especies de plecópteros (*Anacroneuria vagante* Stark & Baumann 2011, *Claudioperla tigrina* Klapálek 1904 y *C. ruhieri* Gibon & Molina 2013) y una especie de tricóptero (cf. *Anomalocosmoecus illiesi* Marlier, 1962). Concluimos que el código de barras de ADN podría ser una herramienta eficaz para asociar etapas de la vida y esto podría acelerar el estudio de los patrones de biodiversidad de insectos acuáticos de la región altoandina.

Palabras claves: Asociación de larvas / ninfa-adulto, Código de barras de ADN, Patrón de la biodiversidad, Región altoandina, Taxonomía.

Introduction

Traditionally, the composition of macroinvertebrate communities has been used for assessing the health status of freshwater environments. Within aquatic macroinvertebrate communities, insects belonging to the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT) are the most used bioindicators due to high sensitivity to changing water conditions (Armitage *et al.* 1983, Doledec & Statzner 2008, Moya *et al.* 2011), which also means that climate change and habitat fragmentation poses a particularly serious threat to EPT taxa (Sandin *et al.* 2014). Unfortunately, in spite of their abundance in running waters of the Andean region, their diversity and fantastic knowledge remain poorly documented at the species level in the biodiversity era.

EPT insects spend most of their life cycle as larvae or nymphs in the aquatic environment (Molina & Puliafico 2016). Since the formal description of these species usually is based on the morphology of adult males, it is often complex or even impossible to achieve good taxonomic resolution at the species level for samples of larvae or nymphs without rearing these insects to adulthood. However, with development and accessibility of molecular phylogenetic technology, it is possible to also determine the species of immature stages (Zhou *et al.* 2009, Ruiter *et al.* 2013). Through concerted efforts of systematists and ecologists, the taxonomic knowledge of South American aquatic insects has improved recently (Fernández & Dominguez 2001, Dominguez & Fernández 2009), but many species still remain known only from their adult life stage, or they are not known at all.

Obtaining a good taxonomic resolution for immature stages of aquatic insects has never been an easy task. The conventional techniques of the immature-adult associations require many samples and include field or laboratory rearing. However, these procedures are complicated and time consuming and do not always provide reliable results due to the manipulation of the live specimens (Molina *et al.* 2008, Silva *et al.* 2012). Obtaining a good taxonomic resolution for immature stages will allow for more detailed ecological studies and permit a closer monitoring of species turnover caused by the subtle changes associated with climate changes.

Today, DNA barcoding is mostly providing a framework for clarifying the taxonomy of poorly known groups and used as an identification tool. For insects, good species delimitation can often be achieved by DNA barcoding using a short fragment of the mitochondrial cytochrome c oxidase subunit I (COI) (Hebert & Gregory 2005, Pons *et al.* 2006). Given an adequate database of reference sequences or DNA library, sequences can be used to group unidentified individuals (or even parts of these) with *a priori*-defined taxonomic entities based on similarity of the sequences.

The applicability of COI for species identification has been examined in several aquatic insect groups (Hogg *et al.* 2009, Zhou *et al.* 2009, Gattolliat & Monaghan 2010, Ruiter *et al.* 2013), but the shortage of sufficiently extensive databases of reference sequences hinders the possibility of associating sequences at lower taxonomic levels (Gattolliat & Monaghan 2010). In the Andean tropical region, DNA barcoding of aquatic insects has been scarcely attempted to date (Rozo-Lopez & Mengual 2015), but could potentially accelerate the knowledge of both adults and immatures, as vividly exemplified by Strutzenberger *et al.* (2011), who increased local species richness of geometrid moths in a well-known Andean mountain forest by 50%.

In this paper, we evaluate the feasibility of using DNA barcodes for the larval/nymphal-adult association for some EPT species of the high Andes. With the shrinking of the tropical glaciers leading to water shortage in this region (Soruco *et al.* 2009, Francou & Vincent 2010), we believe it is imperative and highly timely to develop the use of DNA barcoding of indicator species to more efficiently monitor this particular environment (Molina *et al.* 2008, Gibon & Molina 2013).

Methods

An aquatic insect survey was conducted over several days in 2014, sampling in different streams between the Apolobamba and Quimsa Cruz mountains (La Paz Department, Bolivia). Collected specimens were preserved in 95% ethanol. Larvae/nymphs and adults were preliminarily sorted to family and genus using a stereo microscope and the morphological keys provided by Dominguez & Fernández (2009). We focused on obtaining reliable species identifications of the adult specimens. For this, one pair of legs, head, thorax, abdomen, wings and genitalia were dissected and mounted in Euparal on a slide. Another pair of legs was preserved in 95% of ethanol and kept at -18°C until DNA extraction. The identified, slide-mounted specimens are deposited in the Entomological collection of the Natural History Museum of Denmark, University of Copenhagen (ZMUC).

Molecular analysis

DNA was extracted using the DNeasy® Blood &Tissue kit (Qiagen, CA, USA) according the manufacturer's instructions and a 658 base pair (bp) long region of the cytochrome c oxidase subunit 1 (COI) gene was amplified and sequenced using the forward primer LCO1490 - GGTCAACAAATCATAAAGATATTGG; and the reverse primer: HCO2198 – TAAACTTCAGGGTGACCAAAAAATCA (Folmer *et al.* 1994). PCR amplification was performed as 25µl reactions with 2 µl of DNA template, 2.5µl TQ, 10 µl dNTP mix, 2.5 µl each of primers LCO1490, and HCO2198, 0.1 µl DNA polymerase and 6 µl of ultrapure water. The PCR program consisted of an initial premelt of 94°C 1 min and 35 cycles following the profile: denaturing step at 95˚C for 30 s, annealing step at 50˚C for 30 s, extending step at 72˚C for 54 s and finally the sample was stored at 4°C. PCR products were checked by Agarose gel electrophoresis (Invitrogen). DNA purification was performed with the Qiagen kit QIAquick® (Qiagen, CA, USA) following the manufacturer's protocol. Direct sequencing of purified PCR products was performed using BIGDYE v 1.1 (Applied Biosystems, Wellesley, Massachusetts, U.S.A.) and purified sequencing products were run on an AB3130x1 automated sequencer (Applied Biosystems).

Data analysis

The forward and reverse sequences were edited and assembled using Sequencher (2011). All the sequences were aligned in MEGA v 6.0.6 (Tamura *et al.* 2013), using the ClustalW algorithm with default parameters and according to the *Drosophila yukaba* sequence (Folmer *et al.* 1994, GenBank accession No. X03240). In order to evaluate the interspecific and intraspecific genetic divergences among our morphological species identified, we obtained other genetic sequences for a close related genus or same genus according to the pairwise alignment similarity (GenBank® database: www.ncbi.nlm.nih.gov/genbank/). All genetic

sequences were subjected to pairwise nucleotide sequence divergence calculations using the Kimura 2-parameter (K2P) model. The species boundary associating were defined using the criteria that less than 2% genetic divergence indicates conspecificity (Hebert *et al.* 2003, Hogg *et al.* 2009). Finally, we built a gene tree using a Neighbour-joining (NJ) method with pairwise deletion of missing sites and K2P distance options (Kimura 1980).

All sequences were deposited under the project "Barcode Andean Aquatic Insects" (BAAI) in the Barcode of Life Data Systems (BOLD) (Ratnasinghan & Hebert (2007); www.barcodeoflife.org; sequence pages BAAI0002-15–BAAI0016-15) and cross-referenced to GenBank®.

Results

The geographic references of the specimens identified are shown in Table 1. DNA sequences from adult males identified to species based on morphology were used to explore their possible female and larval/nymphal complement specimens in order to verify the associations.

An average of 556 base pairs of the COI gene was recovered from 15 specimens. Compared with *Drosophila yukaba* sequences, our alignment covered a region between bp positions 1571 and 2127.

The Ephemeroptera specimens, the genetic sequence alignments we compared with a genetic sequence of the closely related genus: *Delatidium cerinum* (GenBank accession No.KX038186), which one showed 84% of pairwise identical with our alignments. The interspecific genetic divergences showed an average of 17% and the interspecific divergence was 0.2% (Table 2).

The Plecoptera species, on one side *Anacroneuria vagante* were compered with the same genus (*Anacroneuria* sp. GenBank accession No.KR134821), and showing 86% of pairwise alignment similarity. The interspecific genetic divergences were on average 16% and the interspecific divergences were also 0.2%. On the other side, the *Claudioperla* species were compared with close related genus (*Dinotoperla uniformis*, GenBank accession No.KX078023), with 83% of pairwise alignment similarity. The interspecific and intraspecific genetic divergences were 19% and 2%, respectively (Table 3).

The Trichoptera specimens were compared with a specimen the same genus (*Anomalocosmoecus* sp., GenBank accession No.KM507945), showing 97% of pairwise alignment similarity and likely belong to close related species. The interspecific and intraspecific genetic divergences were 3% and 1%, respectively (Table 4).

In general, small intraspecific genetic divergences we observed among specimens of different developmental stages and among specimens of different sexes in the same species (genetic divergence range from 0.1% to 2%).

Based on the NJ analysis we were able to associate the larval/nymphal instars with their conspecific adults for one species of Ephemeroptera (Fig 1), three species of Plecoptera (Fig 2), and one species of Trichoptera (Fig 3). Three specimens (one larva/nymph, one adult male, one adult female) were always associated in a clade with high bootstrap support (100%). As these specimens show a <2% divergence, they are here considered as conspecific.

Discussion

To reliably identify specimens for precise species level delimitation, an extension of existing databases with genetic sequences (Gattolliat & Monaghan 2010) with data for EPT insects from the Andean region is needed. The two species of the genus *Claudioperla* Illies, 1963 (Plecoptera) showed an interspecific divergence <20%, and each had maximum intraspecific divergences <2% (Table 3). *Claudioperla tigrina* (Klapálek 1904) is a well-known species throughout the Andes, whereas the recently described *C. ruhieri* Gibon & Molina is known only from Bolivia (Gibon & Molina 2013).

Table 1. List of specimens, voucher information and accession numbers included in this study

T a x a	Stage	Museum ID	Stream	Latitude	Longitude	Altitude (m s.a.l.)	DNAbarcode
Ephemeroptera							
Leptophlebiidae							
Massartellopsis tintinabula	Imago ♂	zmuc00033538	Choquetanga	-16.871735	-67.30719	3656	BAAI0002-15
Massartellopsis cf. *tintinabula*	Nymph	zmuc00033539	Charazani	-15.23685	-69.057936	4200	BAAI0003-15
Massartellopsis cf. *tintinabula*	Subimago ♀	zmuc00033540	Charazani	-15.23685	-69.057936	4200	BAAI0004-15
Plecoptera							
Gripopterygidae							
Claudioperla tigrina	Adult ♂	zmuc00033532	Choquetanga	-16.871735	-67.30719	3656	BAAI0005-15
Claudioperla cf. *tigrina*	Nymph	zmuc00033533	Suches affluent	-15.304704	-69.044995	4340	BAAI0006-15
Claudioperla cf. *tigrina*	Adult ♀	zmuc00033534	Suches affluent	-15.304704	-69.044995	4340	BAAI0007-15
Claudioperla ruhieri	Adult ♂	zmuc00033535	Quime affluent	-16.948605	-67.3179	4719	BAAI0008-15
Claudioperla cf. *ruhieri*	Nymph	zmuc00033536	Quime affluent	-16.96504105	-67.31133505	4457	BAAI0009-15
Claudioperla cf. *ruhieri*	Adult ♀	zmuc00033537	Quime affluent	-16.96504105	-67.31133505	4457	BAAI0010-15
Perlidae							
Anacroneuria vagante	Adult ♂	zmuc00033528	Choquetanga	-16.871735	-67.30719	3656	BAAI0011-15
Anacroneuria cf. *vagante*	Nymph	zmuc00033526	Choquetanga	-16.871735	-67.30719	3656	BAAI0012-15
Anacroneuria cf. *vagante*	Adult ♀	zmuc00033527	Choquetanga	-16.871735	-67.30719	3656	BAAI0013-15
Trichoptera							
Limnephilidae							
Anomalocosmuecus cf. *illiesi*	Adult ♂	zmuc00033529	Charazani	-15.23685	-69.057936	4200	BAAI0014-15
Anomalocosmuecus cf. *illiesi*	Larva	zmuc00033530	Charazani	-15.23685	-69.057936	4200	BAAI0015-15
Anomalocosmuecus cf. *illiesi*	Adult ♀	zmuc00033531	Charazani	-15.23685	-69.057936	4200	BAAI0016-15

The **cf.** abbreviation came from Latin word, which means “compare” or “confer.”. We are using the **cf.** abbreviation in order to explain that morphology identification is not certain because in such cases the taxonomic species descriptions are unknown at the immature stage and the adult stage we are not pretty sure especially to the female sex.

Table 2. Pairwise nucleotide sequence divergence calculations (K2P) among COI mitochondrial DNA sequences for Ephemeroptera specimens. The details about code references are showed in Table 1.

Specie name and identifier code	*M.* cf. *tintinnabula* ♀ (zumc00033540)	*M. tintinnabula* ♂ (zumc00033538)	*M.* cf. *tintinnabula* (zumc00033539)
M. tintinnabula ♂ (zumc00033538)	0		
M. cf. *tintinnabula* (zumc00033539)	0.003	0.003	
D. cerinum (KX038186)	0.174	0.174	0.174

Table 3. Pairwise nucleotide sequence divergence calculations (K2P) among COI mitochondrial DNA sequences for Plecoptera species. The details about code references are showed in Table 1.

Species name and identifier code	*Anacroneuria* sp. (KR134821)	*A.* cf. *vagante* ♀ (zumc00033527)	*A. vagante* ♂ (zumc00033528)	*A.* cf. *vagante* (zumc00033526)	*D. uniformis* (KY078023)
A. cf. *vagante* ♀ (zumc00033527)	0.161				
A. vagante ♂ (zumc00033528)	0.163	0.001			
A. cf. *vagante* (zumc00033526)	0.159	0.001	0.003		
D. uniformis (KY078023)	0.261	0.245	0.247	0.243	
C. ruiheri ♀ (zumc00033535)	0.256	0.258	0.263	0.256	0.19
C. cf. *ruiheri* ♂ (zumc00033537)	0.249	0.245	0.249	0.243	0.192
C. cf. *ruiheri* (zumc00033536)	0.249	0.248	0.252	0.246	0.192
C. tigrina ♂ (zumc00033532)	0.283	0.271	0.273	0.269	0.195
C. cf. *tigrina* ♀ (zumc00033534)	0.284	0.281	0.283	0.278	0.201
C. cf. *tigrina* (zumc00033533)	0.28	0.266	0.27	0.264	0.193

Species name and identifier code	*C. ruiheri* ♀ (zumc00033535)	*C.* cf. *ruiheri* ♂ (zumc00033537)	*C.* cf. *ruiheri* (zumc00033536)	*C. tigrina* ♂ (zumc00033532)	*C.* cf. *tigrina* ♀ (zumc00033534)
A. cf. *vagante* ♀ (zumc00033527)					
A. vagante ♂ (zumc00033528)					
A. cf. *vagante* (zumc00033526)					
D. uniformis (KY078023)					
C. ruiheri ♀ (zumc00033535)					
C. cf. *ruiheri* ♂ (zumc00033537)	0.034				
C. cf. *ruiheri* (zumc00033536)	0.032	0			
C. tigrina ♂ (zumc00033532)	0.117	0.11	0.111		
C. cf. *tigrina* ♀ (zumc00033534)	0.121	0.117	0.117	0	
C. cf. *tigrina* (zumc00033533)	0.118	0.11	0.112	0.001	0.002

Table 4. Pairwise nucleotide sequence divergence calculations (K2P) among COI mitochondrial DNA sequences for Trichoptera specimens. The details about code references are showed in Table 1.

Species name and identifier code	*A.* cf. *illiesi* ♂ (zumc00033529)	*A.* cf. *illiesi* ♀ (zumc00033531)	*A.* cf. *illiesi* (zumc00033530)
A. cf. *illiesi* ♀ (zumc00033531)	0.015		
A. cf. *illiesi* (zumc00033530)	0.012	0.003	
Anomalocosmoecus sp. (KM507945)	0.024	0.028	0.028

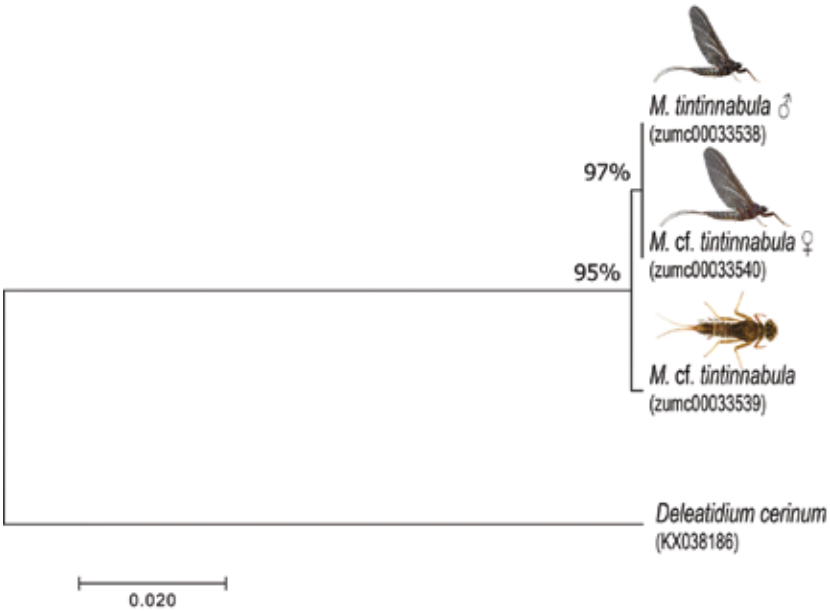

Figure. 1. Neighbor joining tree of COI mitochondrial DNA sequences for Ephemeroptera specimens. Numbers below branches indicate percentage nonparametric bootstrap support (>50%) from 1000 pseudoreplicates. The details of code references are given in Table 1 and Table 2.

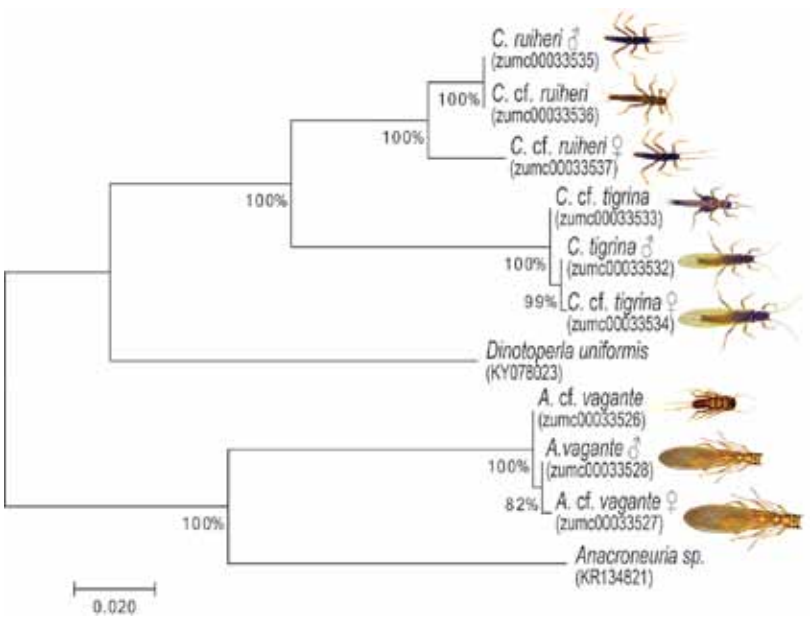

Figure. 2. Neighbor joining tree of COI mitochondrial DNA sequences for Plecoptera species. Numbers below branches indicate percentage nonparametric bootstrap support (>50%) from 1000 pseudoreplicates. The details of code references are given in Table 1 and Table 3.

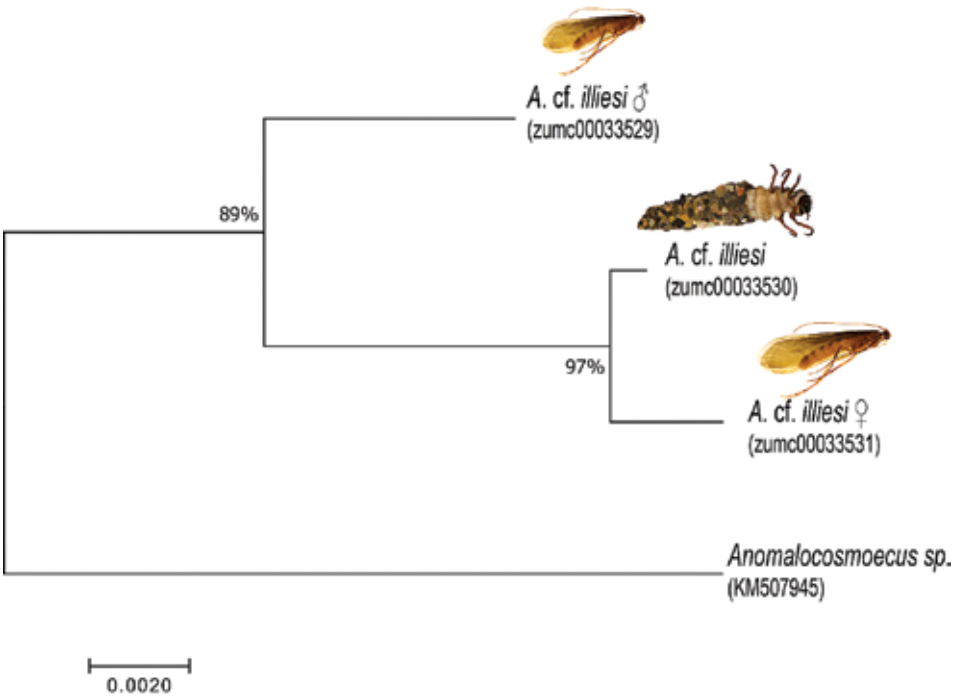

Figure. 3. Neighbor joining tree of COI mitochondrial DNA sequences for Trichoptera specimens. Numbers below branches indicate percentage nonparametric bootstrap support (>50%) from 1000 pseudoreplicates. The details of code references are given in Tables 1 and Table 4.

The adult and immature *Anacroneuria vagante* Stark & Baumann, 2011 (Plecoptera) had a low interspecific divergence of 0.2%. This genus contains a large number of species, many of which were described from a single location or based on a single male specimen and without nymphal association. In Bolivia, 21 species of *Anacroneuria* are known (Stark & Baumann 2011), all without clear nymphal associations to the adult stoneflies.

Ephemeroptera and Trichoptera specimens showed low intraspecific divergence (1%). The Ephemeroptera species was difficult to identify even at genus level based on nymph and female subimago specimens. The single male imago that was obtained in the Choquetanga stream was identified as *Meridialaris tintinnabula* Pescador & Peters. The classical taxonomic key for nymphs of this group indicate a strong similarity between this species and the single species of *Massartellopis* Demoulin, 1955 (Dominguez *et al.* 2006), and for this reason, identifications based only on nymphal and subimaginal instars are highly uncertain. The utility of morphology traits is clearly restricted with this example where nymphal characteristics are not diagnostic.

The Trichoptera specimens were readily identified to *Anomalocosmoecus* cf. *illiesi* Marlier based on the distinctive features given by Flint (1982). However, both male and female genitalia of this species have no known differences from the congeneric species *A. blancasi* Schmid, and these species could potentially be sympatric beyond their currently known co-occurrence in Lake

Titicaca (Flint 1982). These difficulties suggest that more morphological and molecular studies are needed in order to clarify the systematics and distribution of these species.

PCR amplification success largely depends on choosing a suitable primer or set of primers. Previous studies of COI barcoding of EPT insects usually combined two primer sets (LCO1490/HCO2198 and LepF1/LepR1, Gill *et al.* 2014) or even included a third primer set for some caddisfly species (COI 2191/COI 1709, Zhou *et al.* 2009). In our study we found that the primer set of Folmer *et al.* (1994) (LCO1490/HCO2198) worked very well for all specimens analysed and no differences were observed in sequence success for different life stages and sexes.

During our fieldwork, we obtained several immature aquatic insect but their adults were a weak collection due the hard climate condition of the high Andean mountain (strong wind and high humidity), because these adults are terrestrial flyers. The main reason for this is because we associated few specimens of the some exemplar EPT insects, but through of COI barcoding we demonstrate the reliability of associations with a specimen among larval/nymphal and their respective adults at species level. We advocate establishing barcode sequence libraries for aquatic Andean insects to allow associations of specimens of different instars with their adult males and females. With eDNA surveillance (Thomsen *et al.* 2012, Bohmann *et al.* 2014) and next generation metabarcoding (Zhou *et al.* 2012) now being developed and refined, and with material costs for DNA barcodes rapidly decreasing (Meier *et al.* 2015), the major obstacle would seem to be funding and implementing the necessary large-scale inventories needed for securing a proper voucher material. Improved taxonomic knowledge of immature stages will lead to insights in their biology and allow for elaborating and testing of ecological hypotheses. Similarly, improving our knowledge and understanding of biodiversity patterns will enable us to detect and meet potential risks, e.g., as a result of climate change, in the vulnerable lotic ecosystems of the high Andean region.

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