

## DNA barcode data confirm new species and reveal cryptic diversity in Chilean *Smicridea* (*Smicridea*) (Trichoptera:Hydropsychidae)

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**Abstract.** Mitochondrial deoxyribonucleic acid (mtDNA) sequence data have been both heralded and scrutinized for their ability or lack thereof to discriminate among species for identification (DNA barcoding) or description (DNA taxonomy). Few studies have systematically examined the ability of mtDNA from the DNA barcode region (658 base pair fragment of the 5' terminus of the mitochondrial cytochrome c oxidase I gene) to distinguish species based on range-wide sampling of specimens from closely related species. Here we examined the utility of DNA barcode data for delimiting species, associating life stages, and as a potential genetic marker for phylogeographic studies by analyzing a range-wide sample of closely related Chilean representatives of the caddisfly genus *Smicridea* subgenus *Smicridea*. Our data revealed the existence of 7 deeply diverged, previously unrecognized lineages and confirmed the existence of 2 new species: *Smicridea* (*S.*) *patinae*, new species and *Smicridea* (*S.*) *lourditae*, new species. Based on our current taxonomic evaluation, we considered the other 5 lineages to be cryptic species. The DNA barcode data proved useful in delimiting species within Chilean *Smicridea* (*Smicridea*) and were suitable for life-stage associations. The data also contained sufficient intraspecific variation to make the DNA barcode a candidate locus for widespread application in phylogeographic studies.

**Key words:** mtDNA, integrative taxonomy, cryptic species, DNA taxonomy, larval association.

South America has high levels of biological diversity and associated high levels of endemism (Malcolm et al. 2006). The continent boasts several hyperdiverse regions (*biodiversity hotspots*) that contain >1500 endemic species of vascular plants and have lost at least 70% of their primary vegetation (Myers et al. 2000). The central Chilean Winter Rainfall-Valdivian Forests is one of these hotspots (Myers et al. 2000). The high levels of biodiversity and endemism in these forests are a result of their location at a crossroads between the Neotropical and ancient Gondwanan floristic and faunistic regions (Conservation International 2007). In addition, the central Chilean region (from latitude ~32–48°S) is surrounded by the Pacific Ocean to the west, desert to the north, and very cold and arid regions of Patagonia to the south and east (Cabrera and Willink 1980). Thus, the temperate

terrestrial and, particularly, the freshwater faunas of this area have been very isolated since the southern South American landmass began splitting from Australia in the Eocene, between 52 to 35 million years ago (Sanmartín and Ronquist 2004). The high levels of endemism in the aquatic fauna in this region could be the result of persistence of temperate Gondwanan relicts (de Moor and Ivanov 2008) and sustained isolation of this temperate region since the late Eocene.

In the Neotropics, Chile has one of the best known Trichoptera faunas (e.g., Schmid 1964, Flint 1967, 1969, 1974a, 1989, 2002, Holzenthal 2004), which has a high degree of endemism (Flint 1974a, Rojas 2006). At the species level, the Chilean caddisfly fauna is almost 100% endemic (Holzenthal 2004). Our current knowledge of Neotropical Trichoptera clearly indicates that central Chile is a center of diversification for caddisflies. High levels of diversity and endemism in central Chile also are known for other freshwater organisms, including freshwater crabs (Perez-Losada et al. 2009) and fishes (Dyer 2000, Unmack et al. 2009).

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One reason for the high level of endemism in Chilean caddisflies is that the biogeographic affinity of the Chilean caddisfly fauna lies primarily with Australia, New Zealand, and other biogeographically related regions (e.g., New Caledonia), and not the rest of South America (de Moor and Ivanov 2008). However, this pattern is somewhat different for the Chilean representatives of the genus *Smicridea* McLachlan 1871. The subgenus *Smicridea* (*Smicridea*) currently consists of 113 known species, all occurring in the New World, and most endemic to the Neotropics (Morse 2006). Its closest allies in the family Hydropsychidae are the Neotropical subgenus *Smicridea* (*Rhyacophylax*) Müller 1879 and the Australian genera *Asmicridea* Mosely 1953 (in Mosely and Kimmins 1953) and *Smicrophylax* Neboiss 1977.

In his pioneering work, Flint (1989) revised *Smicridea* from the Chilean subregion of South America. He described 11 new species at the time and increased the number of known species in the region from 3 to 14. Holzenthal (2004) described an additional species and brought the total to 15 (7.0% of the Chilean caddisfly fauna). This total makes *Smicridea* the 3<sup>rd</sup> most species-rich genus of caddisflies in Chile, following *Sortosa* Navás 1918a with 20 and *Verger* Navás 1918b with 19 species (Rojas 2006).

Molecular tools have been used widely for species separation and identification for several decades, and recent technological advances have led to a steep rise in the number of studies (Vogler and Monaghan 2006). The goal of deoxyribonucleic acid (DNA) taxonomy, sensu Vogler and Monaghan (2006), is to use  $\geq 1$  genes to "...identify groups that correspond to entities of reproductively coherent individuals (the species), i.e., to determine a hierarchical level roughly equivalent to the binomials of the traditional system." Recent advances in DNA taxonomy include coalescent-based recognition of species (Monaghan et al. 2009). In contrast, DNA barcoding (Hebert et al. 2003) was proposed as a means for identifying unknown specimens by collecting sequence data from a specific gene region and comparing these data to an established reference database. For animal taxa, a 658 base pair (bp) fragment at the 5' terminus of the mitochondrial cytochrome c oxidase I (COI) gene region has become the standard DNA barcode region.

Intra- and interspecific variation of mitochondrial DNA (mtDNA) sequences from the DNA barcode region can be discriminated in some taxa (e.g., Ball et al. 2005), but not others (e.g., Alexander et al. 2009). Only a few studies have used DNA sequence data to address issues in caddisfly taxonomy. These studies were focused on associating adult and immature life

stages for larval descriptions (e.g., Shan et al. 2004, Graf et al. 2005, Waringer et al. 2007, 2008, Zhou et al. 2007, Zhou 2009) or species delimitation (Bálint et al. 2009, Zhou et al. 2009, 2010). In caddisflies, most studies to date show that intraspecific variation is less than, and generally clearly distinguishable from, interspecific variation (e.g., Graf et al. 2005, Waringer et al. 2008). However, in some taxa, levels of intraspecific and interspecific variation are the same because of incomplete lineage sorting or introgression (Waringer et al. 2007, Pauls et al. 2009). To our knowledge, no study has examined how well DNA sequence data from the DNA barcode region can distinguish species based on a range-wide sampling of closely related species.

We used a range-wide, population-level sample of several *Smicridea* (*Smicridea*) species to test the ability of the DNA barcode to: 1) delimit closely related species, 2) associate juvenile and adult life stages, and 3) examine the utility of DNA barcodes for discerning genetic population structure. We further describe previously unrecognized diversity within Chilean *Smicridea* (*Smicridea*) that was recognized morphologically and supported by our analysis of DNA barcode data or that was recognized initially through the DNA barcode data and subsequently re-examined for diagnostic morphological characters. Thus, our study presents the first use of DNA barcode data as a guide to help confirm and describe new species of caddisflies.

## Materials and Methods

### *Sampling and data collection*

We collected ~5000 *Smicridea* specimens from central Chile during January and February 2000, 2005, and 2008. Specimens were attracted to AA battery-powered closet lights (General Electric Co., Fairfield, Connecticut) outfitted with 8-W fluorescent ultraviolet (UV) lights placed across a shallow white plastic pan (~24 × 34 × 4.5 cm) filled with 96% ethanol. Additional specimens were attracted to a 15-W fluorescent UV light powered by a 12-V car battery and placed in front of a 200 × 140-cm white bed sheet suspended between 2 trees. Specimens attracted to the light were captured and killed in cyanide (KCN) killing jars and pinned or placed in 96% ethanol the following morning. Outgroup taxa included several species of *Smicridea* (*Smicridea*) from other regions in South America, several species of *Smicridea* (*Rhyacophylax*) from Chile and other regions of South America, and representatives of the only other genera within the subfamily Smicrideinae, *Asmicridea* and *Smicrophylax*, from Australia.

TABLE 1. Summary of sampling of Chilean *Smicridea* (S.) specimens showing the number of sites (*N* [sites]) and individuals (*N* [ind]) sampled for each species from each region. Detailed information on sites and individuals is given in Appendix 1.

Species	Coast		Andes		Chiloé		Valle Central		Total
	<i>N</i> [sites]	<i>N</i> [ind]	<i>N</i> [sites]	<i>N</i> [ind]	<i>N</i> [sites]	<i>N</i> [ind]	<i>N</i> [sites]	<i>N</i> [ind]	<i>N</i> [ind]
<i>annulicornis</i>	1	2	6	19	1	2	–	–	23
cf. <i>annulicornis</i>	2	6	16	73	–	–	2	6	85
<i>anticura</i>	–	–	1	5	–	–	–	–	5
cf. <i>anticura</i>	–	–	3	3	–	–	–	–	3
<i>decora</i>	–	–	6	14	–	–	–	–	14
cf. <i>decora</i>	–	–	1	2	–	–	1	4	6
<i>figueroai</i>	–	–	1	1	–	–	–	–	1
<i>frequens</i>	1	4	11	23	1	1	1	1	29
<i>lourditae</i> n. sp.	1	2	–	–	–	–	–	–	2
<i>manzanara</i>	1	8	–	–	–	–	–	–	8
cf. <i>manzanara</i>	1	1	2	23	–	–	–	–	24
<i>mucronata</i>	4	18	11	72	1	8	–	–	98
<i>patinae</i> n. sp.	1	1	–	–	–	–	–	–	1
<i>penai</i>	–	–	4	14	1	7	1	1	22
cf. <i>penai</i>	–	–	3	10	–	–	1	1	11
<i>pucara</i>	5	29	6	27	–	–	–	–	56
<i>redunca</i>	1	1	–	–	–	–	–	–	1
<i>smilodon</i>	–	–	4	5	1	1	–	–	6
<i>turgida</i>	1	10	–	–	–	–	–	–	10
<b>Total</b>	<b>8</b>	<b>82</b>	<b>32</b>	<b>291</b>	<b>1</b>	<b>19</b>	<b>2</b>	<b>13</b>	<b>405</b>

We prepared male and female genitalia for observation using lactic acid as outlined by Blahnik et al. (2007), and identified species using the work of Flint (1989). For each species or putative new species of *Smicridea* (*Smicridea*) in our collections, we selected up to 15 specimens from all sampling sites where the species was collected for final sampling (final sampling scheme is described in Table 1 and Appendix 1; available online from: <http://dx.doi.org/10.1899/09-108.1.s1>).

We extracted whole genomic DNA from individual legs of identified specimens following the procedures of Ivanova et al. (2006) or from unprepared abdomens as follows. We removed abdomens and placed them in tissue lysis buffer (ATL) and Proteinase K for 12 to 24 h (DNeasy extraction protocol; Qiagen, Hilden, Germany). We extracted DNA with DNeasy Tissue Kits or QIAamp Micro Kit (Qiagen) following the manufacturer's protocol. We assigned prepared abdomens and the remaining specimens unique 9-digit accession numbers beginning with the prefix UMSP and entered taxonomic, collection, and locality data in the University of Minnesota Biota (Colwell 2003) database (<http://www.entomology.umn.edu/museum/databases/BIOTAdatabase.html>) and the Barcode of Life Data System (BOLD; <http://www.boldsystems.org/>). We followed methods described by Hebert et al. (2003) for polymerase chain reaction (PCR) amplification and sequencing protocols. We amplified full-length COI DNA barcodes (658 bp) with 2 primer sets:

LepF1/LepR1 (Hebert et al. 2004) and LCO1490/HCO2198 (Folmer et al. 1994). Sequences of the COI barcode region (Hebert et al. 2003) were generated at the Canadian Centre for DNA Barcoding (CCDB), University of Guelph, the University of Minnesota Biomedical and Genomics Center (BMGC), or Functional Bioscience (Madison, Wisconsin). Sequences were edited and aligned in Sequencher 4.9 (Gene Codes, Ann Arbor, Michigan).

#### Data analysis

We included only sequences with <1% ambiguous data in the analyses. All acceptable sequences were collapsed into unique haplotypes using Collapse (version 1.2; <http://darwin.uvigo.es/software/collapse.html>). To examine the utility of DNA barcode data for identifying and delimiting *Smicridea* species, we compared intra- and interspecific variation for each species of Chilean *Smicridea* (*Smicridea*). We calculated absolute pairwise distances between all haplotypes with PAUP\* 4.0b10 (Swofford 2003) and generated a histogram of intraspecific and interspecific differences based on current morphological taxonomic assessment. Our goal in this simple analysis was to identify a potential barcode gap (Hebert et al. 2003).

We did a Bayesian Markov-Chain Monte-Carlo (B/MCMC) phylogenetic analysis with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and the most

appropriate model selected by Modeltest v3.5 (Posada and Crandall 1998) to study the relatedness of haplotypes. We ran 2 parallel analyses with 6 chains run for  $5 \times 10^6$  generations and sampled trees every 1000<sup>th</sup> generation. We discarded the first  $2.5 \times 10^6$  generations as burnin. We plotted the log-likelihood scores of sample points against generation time with TRACER 1.0 software (<http://tree.bio.ed.ac.uk/software/tracer/>) to ensure that likelihood equilibrium was reached after  $2.5 \times 10^6$  generations. From the remaining trees, we calculated a majority-rule consensus tree with average branch lengths with the sumt option of MrBayes. We obtained posterior probabilities (pp) for each clade. Two independent runs were done on the Computational Biology Service Unit of Cornell University (<http://cbsu.tc.cornell.edu/>).

We did a neighbor joining (NJ) analysis using the Kimura-2-Parameter (K2P) model (Kimura 1980) with the unique haplotypes and with all specimens. We evaluated node support with NJ bootstrapping (Felsenstein 1985) with 5000 replicates implemented by PAUP\* 4.0b10 (Swofford 2003). We interpreted bootstrap proportions (bs) between 75 and 90 as indicative of fairly strong support and between 91 to 100 as indicative of strong support of the given relationship (Hillis and Bull 1993). Based on the specimen-level NJ analysis, we considered larvae conspecific with an adult male if they either shared a haplotype with an adult male or were nested within the species boundaries of adult males. If a larva was placed directly basal to the species boundaries based on adult males, we associated the specimen only if it was grouped in a strongly supported and monospecific clade (Zhou et al. 2007).

#### *Species descriptions and specimen deposition*

We used standard methods to examine, illustrate, and describe the species (Holzenthall and Anderson 2004). Morphological terminology followed that of Flint (1989). We deposited holotypes and paratypes in the University of Minnesota Insect Collection (UMSP). Additional paratypes were deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC (NMNH).

### Results

The dataset consisted of 463 individuals from 42 taxa. Of these, 19 taxa (405 individuals) were *Smicridea* (*Smicridea*) from Chile (Table 1, Appendix 1). These taxa included *Smicridea* (*S.*) *annulicornis* Blanchard 1851, *Smicridea* (*S.*) cf. *annulicornis*, *Smicridea* (*S.*) *anticura* Flint 1989, *Smicridea* (*S.*) cf. *anticura*, *Smicridea* (*S.*) *decora* (Navás 1930), *Smicridea* (*S.*) cf. *decora*,

*Smicridea* (*S.*) *figueroai* Holzenthall 2004, *Smicridea* (*S.*) *frequens* (Navás 1930), *Smicridea* (*S.*) *lourditae* new species, *Smicridea* (*S.*) *manzanara* Flint 1989, *Smicridea* (*S.*) cf. *manzanara*, *Smicridea* (*S.*) *mucronata* Flint 1989, *Smicridea* (*S.*) *patinae* new species, *Smicridea* (*S.*) *penai* Flint 1989, *Smicridea* (*S.*) cf. *penai*, *Smicridea* (*S.*) *pucara* Flint 1989, *Smicridea* (*S.*) *redunca* Flint 1989, *Smicridea* (*S.*) *smilodon* Flint 1989, and *Smicridea* (*S.*) *turgida* Flint 1989. The Latin abbreviation cf. (compare) was used to refer to 5 morphologically cryptic (i.e., reliable distinguishing characters not yet discovered) but genetically divergent monophyletic lineages to the morphologically most similar nominal species based on the descriptions and illustrations of Flint (1989), the most authoritative taxonomic reference available (see *The barcode gap*, *Haplotype phylogeny*, and *Taxonomy* below).

In addition, the data set included 18 other species of *Smicridea* (*Smicridea*) (40 individuals) from outside of Chile, 3 species of *Smicridea* (*Rhyacophylax*) (10 individuals), *Smicrophylax parvula* (Mosely 1953) (in Mosely and Kimmins 1953) (1 individual), and *Asmicridea edwardsii* (McLachlan 1866) (7 individuals). The 463 sequences were 658 bp long, and consisted of 203 unique haplotypes based on an analysis using the program Collapse. Haplotype code and GenBank Accession Number for Chilean *Smicridea* (*Smicridea*) individuals are given in Appendix 1. Voucher and sequence information of all material studied are also accessible in BOLD project “DNA barcoding Chilean *Smicridea* (CLSMD)” (<http://www.boldsystems.org>).

#### *The barcode gap*

Samples of several Chilean species extended across a wide geographic range (Fig. 1, Appendices 1, 2; available online from: <http://dx.doi.org/10.1899/09-108.1.s2>). In contrast, our sample of outgroup taxa generally consisted of only a few specimens from few localities. Therefore, we based the histogram of intra- vs interspecific differences between haplotypes on the ingroup only, i.e., the 19 taxa of Chilean *Smicridea* (*Smicridea*). The resulting histogram (Fig. 2) showed a clear gap from 39 to 53 bp differences, where the frequency of divergences in the histogram was always 0. Two isolated groups of data points appeared on the histogram, each comprising multiple peaks. The left group represented almost exclusively intraspecific differences and differences between 2 specimens of *S. annulicornis* that grouped with specimens of *S.* cf. *annulicornis* (see next paragraph). The right group represented interspecific differences or differences between nominal species and the genetically distinct and highly diverged cf. lineages. The number of intraspecific differences was almost exclusively



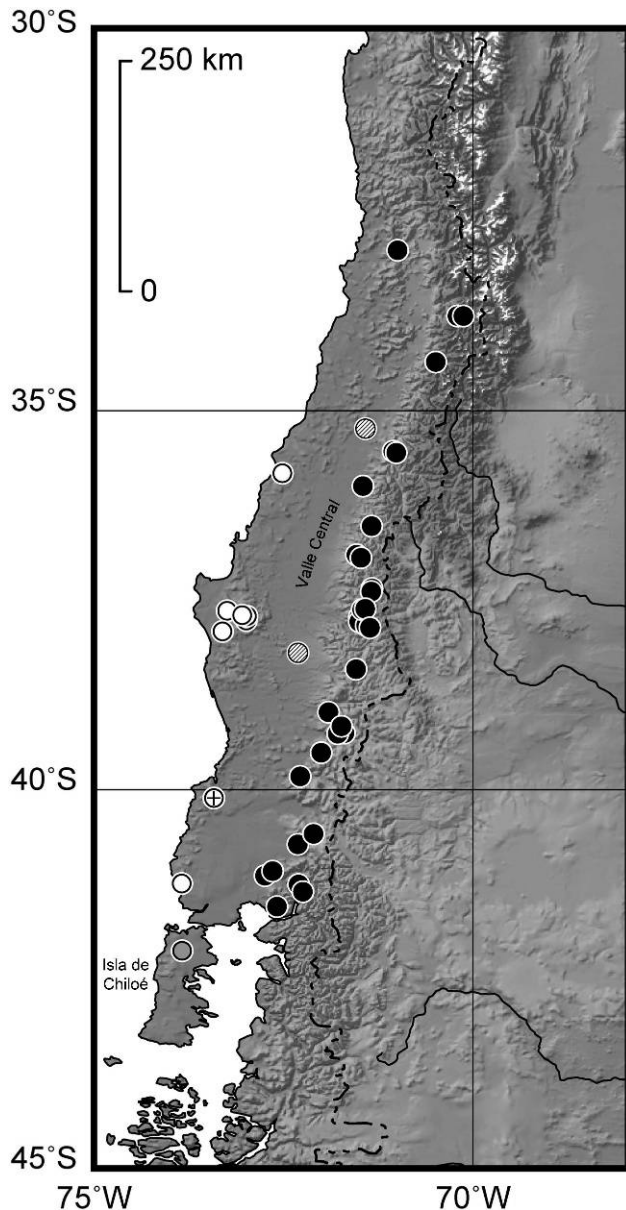


FIG. 1. Map of central Chile indicating relief and sampling localities of *Smicridea* (*S.*) samples used in our study. Collection sites are coded by geographic region: Coastal Range (white), Chiloé (grey), Valle Central (hatched), Andes (black). + marks the holotype locality of *Smicridea* (*S.*) *lourditae*, new species, and *Smicridea* (*S.*) *patinae*, new species.

<39 bp (5.9%), whereas interspecific differences and differences between cf. lineages and nominal species were generally >53 bp (8.05%; for exceptions see below). Several peaks occurred within both groups, a result indicating that differences within and among species varied by taxon and taxon comparisons.

The morphology of 2 specimens (UMSP000113424 and UMSP000133009) was intermediate between *S.*

*annulicornis* and *S.* cf. *annulicornis*, but the specimens were morphologically closer to the nominal species and were identified as such. However, these individuals clustered within *S.* cf. *annulicornis* (Appendix 2). Thus, intraspecific differences between these specimens and other *S. annulicornis* haplotypes ranged from 63 to 75 bp, whereas interspecific differences between these specimens and *S.* cf. *annulicornis* were <14 bp.

In all other cases differences between the nominal and the cf. taxon exceeded the barcode gap: between *S. anticura* and *S.* cf. *anticura* the difference was 68 bp (10.3%); between *S. penai* and *S.* cf. *penai*, and *S. decora* and *S.* cf. *decora* the difference ranged from 73 to 79 bp (11.1–12.0%); and between *S. manzanara* and *S.* cf. *manzanara* the difference ranged from 86 to 94 bp (13.1–14.3%). The minimum distance observed among the species that were clearly distinguishable based on morphology was between *S.* cf. *decora* and *S. annulicornis* and between *S.* cf. *decora* and *S.* cf. *annulicornis* (both 53 bp, 8.05%). The maximum distance was between *S. penai* and *S. patinae*, new species (143 bp, 21.7%).

#### Haplotype phylogeny

The 2 independent B/MCMC analyses yielded the same topology and the same significantly supported clades ( $pp \geq 0.95$ ). The result of the 1<sup>st</sup> analysis for the haplotypes is shown in Fig. 3 and depicts both pp and NJ bs support values. The base of the tree consisted of 3 lineages of *Asmicridea edwardsii*, which were selected as outgroups to root the tree. *Smicrophyllax parvula* was sister to a strongly supported clade ( $pp = 1.0$ ,  $bs = 96$ ) of *Smicridea*. The relationship among the 2 subgenera *Smicridea* (*Smicridea*) and *Smicridea* (*Rhyacophylax*) was unresolved. The 3 species of *Smicridea* (*Rhyacophylax*) formed a strongly supported clade ( $pp = 1.0$ ,  $bs = 97$ ), whereas *Smicridea* (*Smicridea*) species fell into several lineages. Only 2 of these lineages were resolved with fairly strong and strong support, respectively: a clade of Chilean *Smicridea* (*Smicridea*) species ( $pp = 1.0$ ,  $bs = 83$ ), and 1 clade with 2 subclades consisting of *Smicridea* (*S.*) *palifera* Flint 1981 + (*Smicridea* (*S.*) *holzenthali* Flint and Denning 1989 + *Smicridea* (*S.*) *nigripennis* Banks 1920 + *Smicridea* (*S.*) *turrialbana* Flint and Denning 1989 + *Smicridea* (*S.*) *ulva* Flint 1974b) ( $pp = 0.96$ ,  $bs =$  not applicable [n.a.];  $pp = 1.0$ ,  $bs = 100$ , respectively). The 2 subclades collectively conformed to the *nigripennis* group (Flint 1974b, Flint and Denning 1989). Within the Chilean clade, several multispecies clades had fairly strong support ( $pp \geq 0.97$ ,  $bs \geq 84$ ). These clades did not always group with the morphologically most similar taxa. For

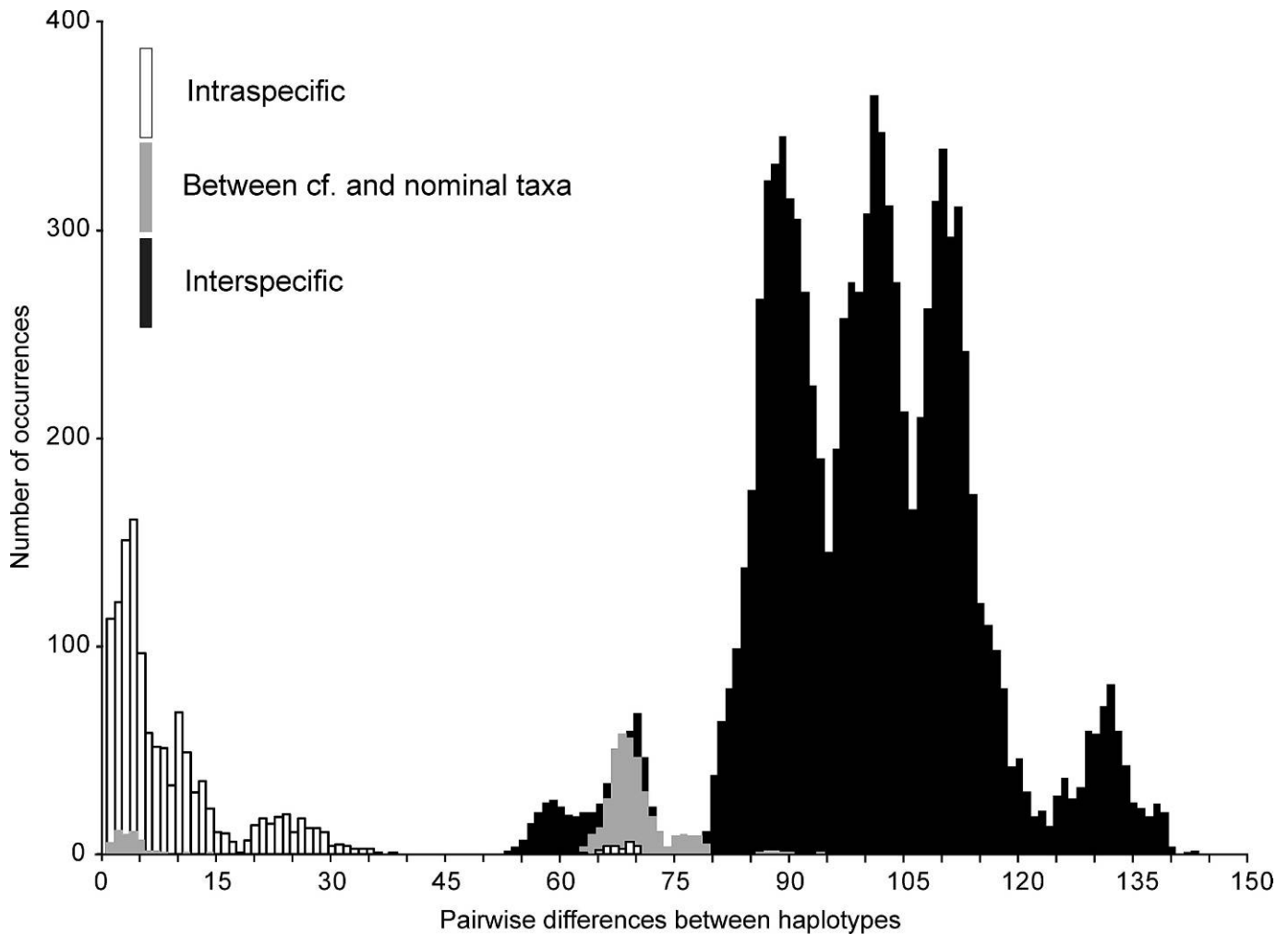


FIG. 2. Histogram of intraspecific and interspecific differences observed in Chilean *Smicridea* (*S.*) species. The black, white, and grey data series indicate interspecific divergence, intraspecific divergence, and divergence between cf. and nominal taxa, respectively.

example, *S. lourditae*, new species is morphologically closest to *S. pucara*, but it grouped with *S. cf. penai* and *S. penai* as its closest relatives (Fig. 3). In other cases, the morphologically most similar species (e.g., *S. turgida* and *S. patinae*) or the nominal and the cf. forms (e.g., *S. anticura* and *S. cf. anticura*) were sister taxa.

At the terminal branches of the tree, most species formed monophyletic groups with strong support (pp = 1.0, bs = 100). Species typically showed deep divergence from congeners. The only exceptions were *Smicridea* (*S.*) *albosignata* Ulmer 1907, which was monophyletic, but with a bootstrap value of 71 (pp = 0.99), and *S. cf. annulicornis*, which contained 2 specimens identified as *S. annulicornis* (UMSP000113424 and UMSP000133009) (see above and Discussion).

We tallied fixed (i.e., observed in all individuals), nonsynonymous mutations among cf. taxa and the respective nominal forms and between the new

species and the morphologically closest taxa (Table 2). In addition, unique fixed amino acid residues were identified for other species of Chilean *Smicridea* (*S.*) (Table 2). Fixed amino acid residues were not observed between *S. anticura* and *S. cf. anticura* or between *S. lourditae*, new species, *S. pucara*, *S. cf. penai* and *S. penai*.

#### Larval association

The specimen-level NJ analysis is presented in Appendix 2. All 31 larvae were unambiguously associated to a species according to the association criteria outlined by Zhou et al. (2007). Many larvae shared identical haplotypes with adult males (criterion 1). All larvae that did not share identical haplotypes were nested within sequences from male specimens and within strongly supported monospe-

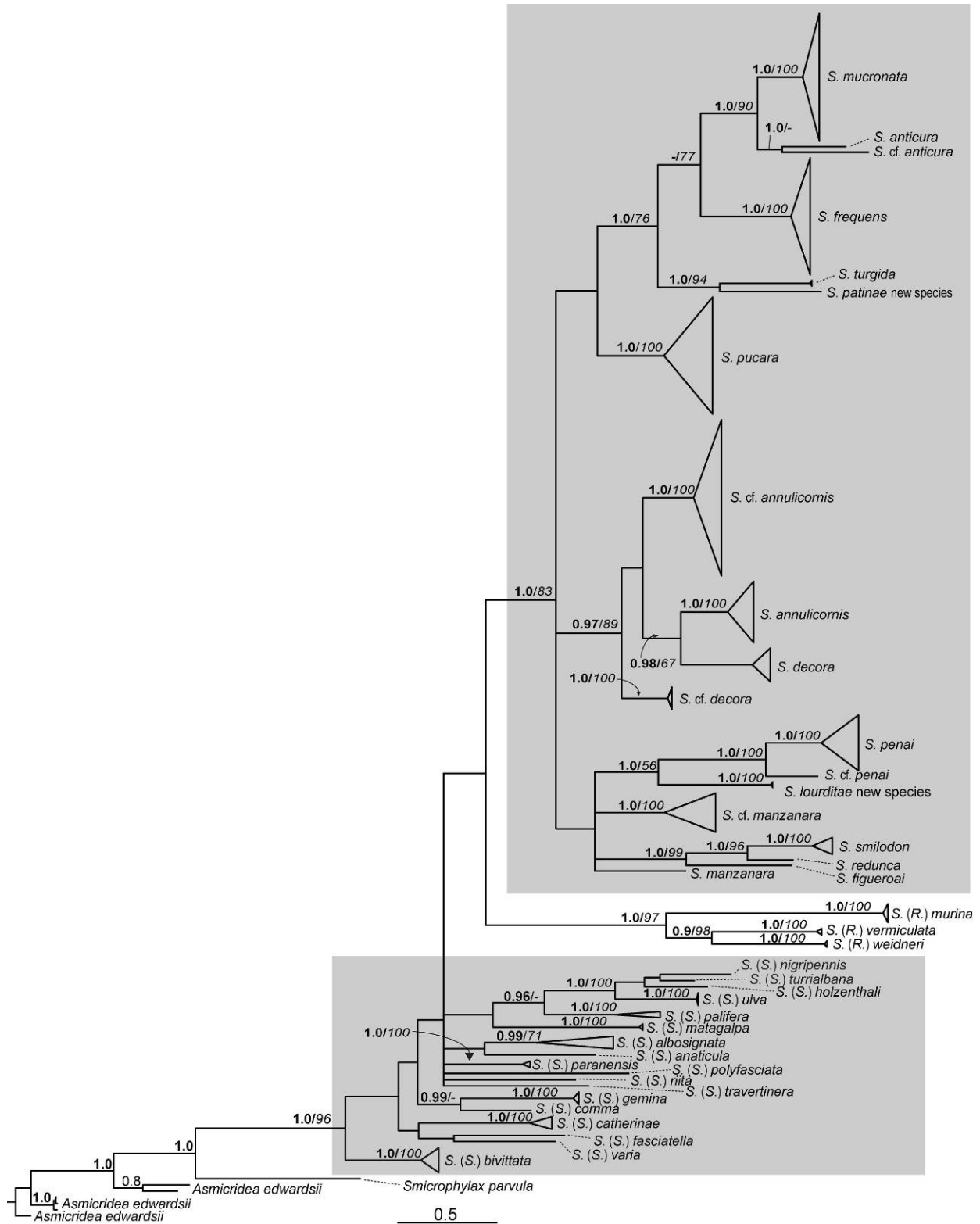


FIG. 3. Bayesian phylogeny of 203 haplotypes recovered in our study. The multihaplotype species-level tip clades were collapsed into triangles (see Appendix 1 for specimen-level details). Height of the triangles is relative to the number of haplotypes within the collapsed clade. Width of triangles indicates degree of divergence among haplotypes within the collapsed clade. Values on branches indicate posterior probabilities  $\geq 0.95$  (bold) and NJ bootstrap support values  $\geq 75$  (italics). *Smicridea* (*Smicridea*) are shaded in grey.

TABLE 2. Fixed differences in amino acid sequences between selected taxon pairs and fixed amino acid residues for individual taxa of *Smicridea* (S.). Amino acid residues are given following International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry and Molecular Biology (IUBMB) recommendations (A=Alanine, F=Phenylalanine, I=Isoleucine, L=Leucine, M=Methionine, S=Serine, T=Threonine, V=Valine).

Comparison	Amino acid residue														
	1	8	20	21	48	95	101	102	139	151	152	164	166	177	195
Taxon pairs															
<i>annulicornis</i> /cf. <i>annulicornis</i>									V/I		I/V			V/I	
<i>decora</i> /cf. <i>decora</i>	S/T		V/I	L/I				V/I			V/I				
<i>manzanara</i> /cf. <i>manzanara</i>							L/M								
<i>patina</i> n. sp./ <i>turgida</i>												F/L			S/A
Species															
<i>decora</i> /others	S/T		V/I	L/I											
<i>nigripennis</i> /others						M/L,F				S/T					
<i>polyfasciata</i> /others		M/I			V/T,I								S/P		

cific clades (criterion 2). Based on these associations we identified 5 previously unknown larvae: *S. decora*, *S. cf. annulicornis*, *S. cf. manzanara*, *S. pucara*, and *S. smilodon*.

### Taxonomy

#### Cryptic lineages

Several individuals and populations formed distinct lineages that were not initially sorted from congeners by morphological characters. After the divergence patterns at COI were revealed, subsequent observation and study did reveal corroborating morphological characters for 3 of these taxa (*S. cf. annulicornis*, *S. cf. decora*, and *S. cf. manzanara*). However, the differences in morphological characters between lineages were small and variable. Thus, we were unable to identify reliable diagnostic characters. A taxonomic resolution was made more difficult by our limited access to or poor condition of type material in some instances. The type of *S. annulicornis*, at the Paris Museum, was unavailable for study. The macerated genitalia stored in a microvial and fixed on the pin holding the body of the type of *S. decora* (at the Museum of Zoology, Barcelona) actually belongs to *S. penai* (Flint 1989, SUP and RWH, personal observation). Moreover, some apparent differences between *S. decora* and *S. cf. decora* in size of the internal sacs proved to be variable and difficult to sort when additional material from the NMNH was examined. The difference in morphology between *S. manzanara* and *S. cf. manzanara* lies in the width of a distinct bulge on the stem of the phallus when observed in ventral view. The bulge is most pronounced in the type of *S. manzanara* and other specimens from or in the vicinity of the type locality. The bulge is almost

entirely lacking in several of our specimens of *S. cf. manzanara*. However, the entire paratype series examined from the NMNH consisted of intermediate forms from a wider distribution and draws into question the reliability of the bulge of the phallus as the only diagnostic character.

The situation was more difficult in the other 2 genetically distinct lineages (*S. cf. anticura* and *S. cf. penai*). The female of *S. cf. anticura* does have a more strongly mesally elongated and narrowed sternum VIII than the female of the nominal species. However, we were unable to identify diagnostic features of the male genitalia, although some minor color differences were present in the forewing. With respect to *S. penai* and *S. cf. penai*, we were unable to identify interspecific morphological differences that were outside of normal intraspecific variation, and the lineages did not exhibit any morphological characteristics that would allow them to be distinguished. The 2 lineages also were not distinctly associated with specific populations or regions (Appendices 1, 2), and specimens from both clades occurred together in sympatry in 3 populations. Thus, we assume that these might be true cryptic species, i.e., deeply divergent, reciprocally monophyletic lineages that evolved independently for a sufficient amount of time to become reproductively isolated, but did not evolve any observable morphological differences. However, we acknowledge that with more sampling of both COI lineages it might be possible to presort enough individuals to identify previously unobserved morphologically diagnostic characters.

#### Description of New Species

For 2 morphologically distinct lineages, the DNA barcode data indicated highly divergent lineages, and thus corroborated our initial morphological assess-



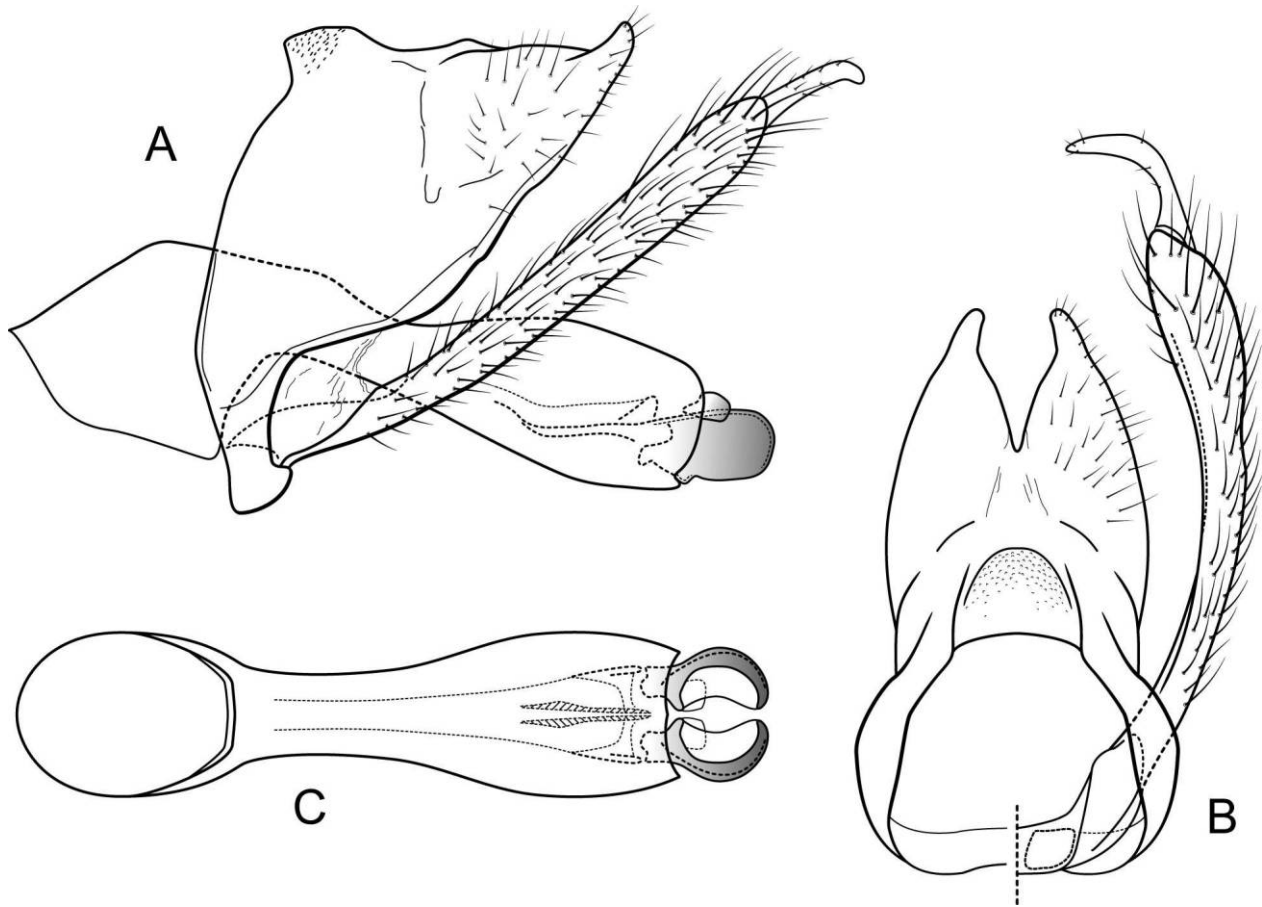


FIG. 4. *Smicridea* (*S.*) *lourditae*, new species, male genitalia, holotype (UMSP000113378). A.—Lateral. B.—Dorsal. C.—Phallus, ventral.

ment of their distinctiveness. Here, we formally describe these new species.

***Smicridea* (*S.*) *lourditae* Pauls, Blahnik,  
Holzenthal, new species  
(Fig. 4A–C)**

*Diagnosis*

*Smicridea lourditae* is most similar to *S. pucara*, with which it was collected in sympatry. The 2 species are genetically clearly distinct (not sister in the phylogeny and diverged by 12.3–14.4%). The differences in morphology between the 2 species are slight but consistent. *Smicridea pucara* has much more heavily mottled forewings and has strikingly darker hair on the 1<sup>st</sup> and 2<sup>nd</sup> tibiae than does *S. lourditae*. There also are consistent differences in the male genitalia, i.e., tergum X, the shape of the stem of the phallus, which is slightly more inflated in ventral view in *S. lourditae*, and the shape of the lateral plates and dorsolateral

lobes at the apex of the phallus. In *S. lourditae*, the inner ventral margin of the lateral plates is more strongly retracted, so that the lateral plates seem to be more open in ventral view than in *S. pucara*. The dorsolateral lobes are shorter and more quadrate in *S. lourditae*. In dorsal view tergum X of *S. lourditae* is more rounded laterally than in *S. pucara*. Apically, tergum X is more abruptly narrowed, with the inner margin of the dorsal cleft angled in *S. lourditae*. In *S. pucara*, tergum X is gradually narrowed, with an almost straight inner margin of the dorsal cleft.

*Description*

Length of forewing 9.5–10 mm. Body color brown; appendages paler; antennae annulate; forewings brown with few markings. Eye of male in frontal aspect with diameter  $\frac{1}{2}$  of interocular distance. Sternum V with anterolateral processes slightly longer than sternum V. Abdominal segments VI and VII with 2 pairs of internal sacs; both pairs as long as their segments.

*Male genitalia* (Fig. 4).—Segment IX with anterior margin nearly vertical. Tergum X elongate; tip with wide dorsomesal cleft,  $\sim 2\times$  as deep as wide at widest part, and with inner margin angled; tergite with apex clearly produced, acutely rounded in dorsal aspect, produced and upturned in lateral aspect. Inferior appendage with basal segment slightly inflated apicad; apical segment narrow, subacute, curved mesad. Phallus tubular, base at right angle to stem; width of basal opening in lateral view  $3\frac{1}{2}\times$  diameter of stem at its narrowest point; stem slightly curved upward, apex distinctly enlarged; lateral plates  $1\frac{1}{2}\times$  longer than broad, with ventral margin more or less confluent with ventral margin of stem, in lateral and ventral aspects with mesobasal shoulder present, cupped, and with inner margins of ventral surface distinctly concave, dorsal margins subparallel; dorso-lateral lobe short, subtruncate, reaching  $\sim \frac{1}{3}$  length of lateral plate.

*Holotype*.—Male. **Chile: Region X – Los Lagos:** Monumento Nacional Alerce Costero, unnamed tributary on trail to Alerce Milenario, 895 m,  $40^{\circ}11.874'S$ ,  $73^{\circ}26.217'W$ , 5-Feb-2008, Holzenthal, Pauls, Mendez: UMSP000113378 (University of Minnesota Insect Collection specimen code and BOLD SampleID), GenBank Accession HM065274, haplotype Hap064.

*Paratypes*.—Male. **Chile: Region X – Los Lagos:** Monumento Nacional Alerce Costero, unnamed tributary on trail to Alerce Milenario, 895 m,  $40^{\circ}11.874'S$ ,  $73^{\circ}26.217'W$ , 5-Feb-2008, Holzenthal, Pauls, Mendez: UMSP000113377 (University of Minnesota Insect Collection specimen code and BOLD SampleID), GenBank Accession HM065273, haplotype Hap063. Male. **Chile: Region “Osorno” [Region X – Los Lagos]:** Bahía, San Pedro West of Purranque, 15 m,  $40^{\circ}56.398'S$ ,  $73^{\circ}51.846'W$ , 5-Mar-1987, Peña: in NMNH, UMSP000127211.

*Distribution and ecology*.—Two specimens of *S. lourditae* were collected at a blacklight placed alongside a very small spring brook in the Valdivian Mountain Ranges of coastal Chile (Fig. 1). The site was densely forested with *Fitzroya cupressoides*, *Lauriopsis philippiana*, *Nothofagus* spp., and other tree species. Other species of *Smicridea* collected at the site were *S. mucronata*, *S. pucara*, and *S. patinae* new species. Other caddisflies collected at the type locality included members of the families Calamoceratidae (*Phylloicus aculeatus* (Blanchard 1851)), Ecnomidae (*Austrotinodes triangularis* Schmid 1958), Helicophidae (*Austrocentrus griseus* Schmid 1964), Hydrobiosidae (*Cailloma pumida* Ross 1956, *Clavichorema chiloeanum* Schmid 1955, *Clavichorema transcasicum* Schmid 1955, *Heterochorema paradoxicum* Flint 1983, *Isochorema flintorum* Schmid 1989, *Neochorema lobiferum* Flint 1969,

*Neopsilochorema tricarinatum* Schmid 1955), Kokiriidae (*Pangullia faziana* Navás 1934), Leptoceridae (*Brachysetodes trifidus* Schmid 1955, *Triplectides nigripennis* Mosely 1936), Limnephilidae (*Austrocosmoecus hirsutus* Schmid 1955), Philopotamidae (*Sortosa elongatoides* (Flint 1967), *Sortosa chilensis* (Navás 1918c)), Philorheithridae (*Mystacopsyche ochracea* Schmid 1955), Polycentropodidae (*Polycentropus quadriappendiculatus* Schmid 1964), and Sericostomatidae (*Myotrichia murina* Schmid 1955, *Notidobiella chacayana* Schmid 1957).

*Etymology*.—This species is named *Smicridea lourditae* in honor of Dr. Maria Lourdes Chamorro for her help in the field and for her help in organizing our trips to Chile, without which this study would not have been possible.

***Smicridea* (S.) *patinae* Pauls, Blahnik,  
Holzenthal, new species  
(Fig. 5A–D)**

*Diagnosis*

This new species is most similar to *S. turgida*. It can be most easily distinguished from that species by the number of internal abdominal sacs; there is only 1 very small pair in *S. patinae*, whereas there are 2 pairs of much larger sacs in *S. turgida*. The 2 species also can be differentiated by the size and position of the lateral plates of the apex of the phallus. In *S. patinae*, the apicodorsal margin of the phallus stem is more projecting and almost completely covers the lateral plates in both lateral and dorsal view; in *S. turgida*, both the lateral plates and the dorsolateral lobes are clearly protruding from the phallus stem in lateral view. Flint (1989) mentioned that *S. turgida* was similar to *S. mucronata* and *S. anticura*. From these species, *S. patinae* differs by the number of internal abdominal sacs and also by the apex of the phallus, particularly the size and position of the lateral plates in ventral and lateral views. In addition, *S. patinae* is uniformly dark brown with light brown legs, and has uniformly dark brown wings. This pattern is in contrast to the other species, which have lighter bodies and lighter wings that are either flecked, spotted, or mottled.

*Description*

Length of forewing 6.5 mm. Body color dark brown; appendages paler; antennae annulate; forewings uniformly dark brown. Eye of male in frontal aspect with diameter  $\frac{1}{2}$  of interocular distance. Sternum V with anterolateral processes slightly longer than sternum V. Abdominal segment VII with 1 pair of small internal sacs, distinctly shorter than length of segment.

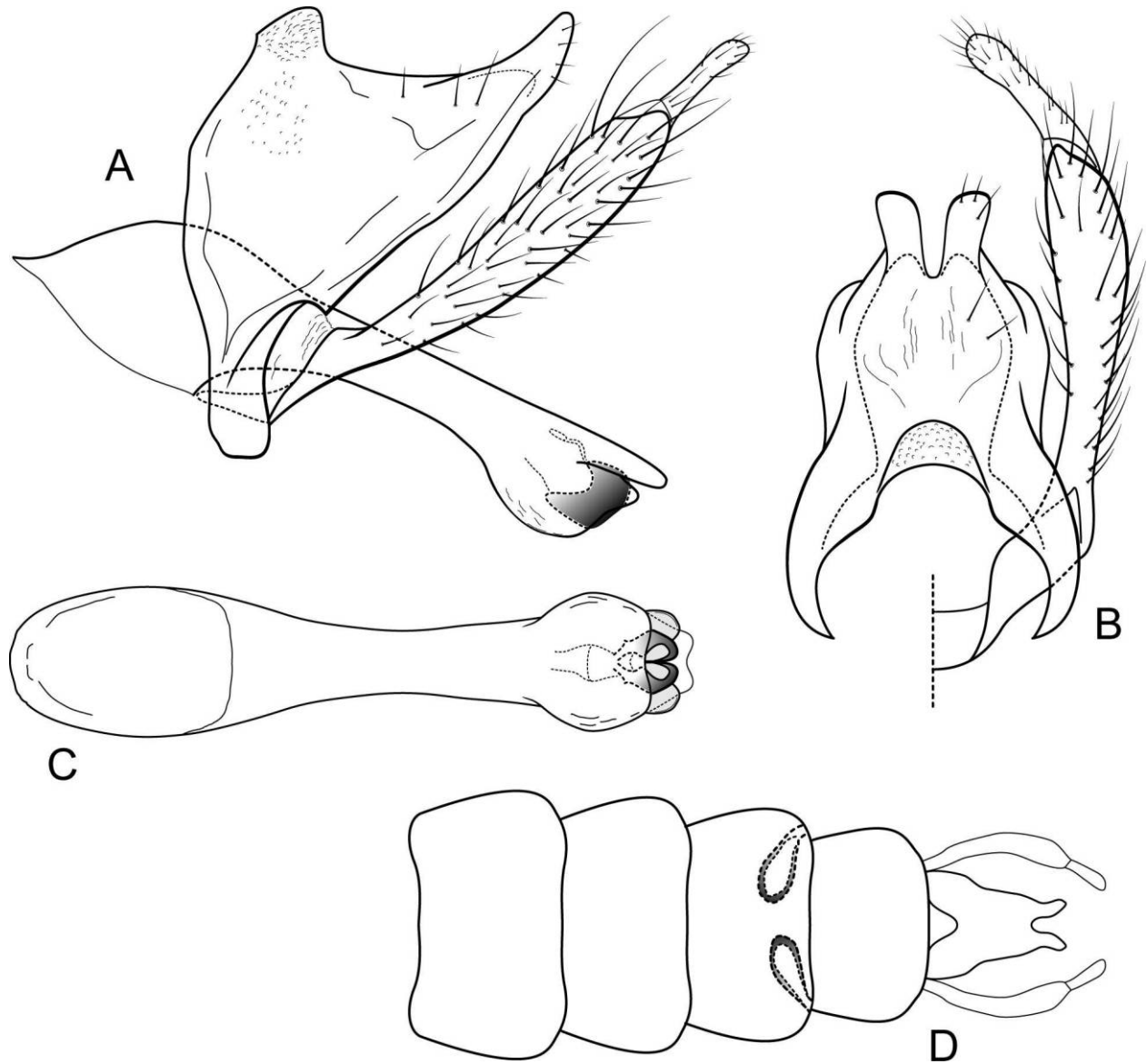


FIG. 5. *Smicridea* (*S.*) *patinae*, new species, male genitalia, holotype (UMSP000113375). A.—Lateral. B.—Dorsal. C.—Phallus, ventral. D.—Abdominal segments V–X, dorsal, showing internal sacs.

*Male genitalia* (Fig. 5).—Segment IX with anterior margin slightly rounded anteriorly. Tergum X elongate; tip with dorsomesal cleft, 3× as deep as wide; tergite with apex clearly produced, bluntly rounded in dorsal aspect, enlarged and upturned in lateral aspect. Inferior appendage with basal segment inflated apicomesally; apical segment elongate, slightly mesally curved, produced apicad, blunt. Phallus tubular, base at right angle to stem; width of basal opening in lateral view 4× diameter of stem at narrowest point; stem more or less straight, apex distinctly enlarged; lateral plates shorter than broad, deflected ventrad by slightly excavated dorsal projection of stem, with ventral margin slightly displaced

from ventral margin of stem, retracted largely into phallus stem, much narrower than stem, cupped, and with inner margins of ventral surface deeply concave; dorsolateral lobe not visible in lateral view, short, but distinct in ventral view.

*Holotype*.—Male. **Chile: Region X – Los Lagos:** Monumento Nacional Alerce Costero, unnamed tributary on trail to Alerce Milenario. 895 m, 40°11.874'S, 73°26.217'W, 5-Feb-2008, Holzenthal, Pauls, Mendez: UMSP000113375 (University of Minnesota Insect Collection specimen code and BOLD SampleID), GenBank Accession HM065396, haplotype Hap095.

*Distribution and ecology*.—A single specimen was collected at a blacklight at the same locality as



*Smicridea lourditae*, new species (see site description under that species) (Fig. 1).

*Etymology*.—This species is named *Smicridea patinae* in honor of Dr. Patina K. Mendez, for her help in the field, without which this study would not have been possible.

## Discussion

### *The DNA barcode gap*

Our data suggests that a barcode gap exists in Chilean *Smicridea* (*Smicridea*) species. A barcode gap has been implicitly proposed for other caddisfly species (e.g., Graf et al. 2009), but Zhou et al. (2007) showed that no distinct gap existed in the species of Hydropsychidae they examined. In their study, a few species exhibited intraspecific divergences close to 12%, whereas minimal interspecific values were near 7%, with most interspecific values ranging between 12% and 18%. However, Zhou et al. (2007) suggested that multiple species might exist in several taxa showing large intraspecific divergence. Based on our results, a distinct barcode gap appears to exist between 5.9% (maximum intraspecific) and 8.05% (minimum interspecific) uncorrected percentage differences in Chilean *Smicridea* (*Smicridea*). These values are relatively high compared with the values observed in other taxa, particularly Lepidoptera, where a 2% lineage divergence between species is often proposed (Hebert et al. 2003, Silva-Brandão et al. 2008). In our study, the gap is obvious among 12 previously described species, 2 new species, and 5 taxa that are morphologically indistinguishable from previously described species, or not reliably diagnosable.

Inconsistencies in the proposed barcode gap are associated with specimens UMSP000113424 and UMSP000133009, which exhibit an intermediate morphology between *S. annulicornis* and *S. cf. annulicornis* but are closer to the nominal species. Morphologically extreme forms of both taxa can be clearly distinguished, and the 2 lineages are genetically deeply diverged (9.6%, Fig. 3), but several specimens in both species exhibited slightly intermediate morphologies. We identified these specimens as belonging to the species they resembled most closely based on morphology as described and illustrated by Flint (1989). With the exception of UMSP000113424 and UMSP000133009, all other species identifications were consistent with the corresponding COI clades. Morphologically intermediate forms exist between *S. annulicornis* and *S. cf. annulicornis*, even though the molecular data suggests 2 very distinct and quite diverged clades. Therefore, the inconsistent results

concerning UMSP000113424 and UMSP000133009 could come from misidentification or possibly from hybridization. The morphological differences among *S. annulicornis* and *S. cf. annulicornis* specimens are slight and grade along a continuum between clearly distinct forms. Thus, distinguishing the 2 species is difficult because variation exists in both forms. Some specimens of intermediate morphology might be impossible to identify correctly. These morphologically very similar, but genetically distinct lineages, also might be subject to rare hybridization events. In our samples, both species occurred in sympatry at 3 sites, which shows that potential exists for interspecific mating. Hybridization in caddisflies might produce offspring that exhibit phenotypes intermediate between the parental phenotypes (Blahnik 1995, Wells 2006), but to our knowledge, this possibility has not been demonstrated experimentally. However, a more thorough analysis using multiple nuclear loci is necessary to examine potential hybridization of the 2 species discussed here.

### *Taxonomy and DNA barcodes*

During our field work in Chile, we collected 10 of the 17 previously described species and subsequently corroborated their status as distinct species using COI sequence divergence data: *S. annulicornis*, *S. anticura*, *S. decora*, *S. figueroai*, *S. manzanara*, *S. mucronata*, *S. pucara*, *S. redunca*, *S. smilodon*, and *S. turgida*. This result confirms that the species were very well circumscribed by Flint (1989) using traditional morphological taxonomy. Subsequent analysis of DNA sequence data helped confirm the existence of 7 additional taxa.

We were readily able to identify and describe 2 new species, but the situation was more difficult for the remaining 5 lineages. For 3 lineages, we found some morphological variation that was distinct in its extreme forms/development. However, the variation of the characters seemed to integrate to some extent, so we currently are unable to distinguish interspecific from intraspecific variation. Additional study, also of more larval material, might reveal reliable diagnostic characters. Difficulty in obtaining types and the poor quality of existing types also prevented us from describing these lineages as new species at this time. We currently think that the 2 other species are true cryptic species that we cannot distinguish morphologically. Resolving this complex of morphologically cryptic lineages will require detailed morphological study of many more specimens from a broader geographic range, including, perhaps, morphometric analysis (e.g., Bálint et al. 2008).



We refrain from describing any of these 5 lineages as new species at this time. Taxonomic description should be based on multiple lines of corroborating evidence and should provide clear diagnostic characters that are useful in identifying and describing diversity. The DNA barcode might provide 1 clearly distinct character, but it is of little use to most biologists assessing diversity based on morphological specimen identification. We think a species description also should be useful in this respect.

With our new species, the total number of formally described Chilean *Smicridea* (*Smicridea*) species has increased to 17. Our barcode data and morphological assessment suggest the potential for 5 additional species in Chile. If verified, they would bring the number of known species to 22, an increase in known species diversity of nearly 47%. These results show that even in recently revised caddisfly taxa from fairly well known regions (i.e., temperate central Chile compared to most of tropical South America), many species are yet to be discovered and described. Our study shows that DNA taxonomy can facilitate and expedite the process of identifying and characterizing this diversity. However, our study also clearly shows the limitations of using only DNA barcode data to identify or circumscribe diversity. We can recognize distinct evolutionary units based on the DNA, but we cannot currently circumscribe or even describe the diversity in a meaningful way that allows biologists without access to DNA facilities to identify species, e.g., for water-quality monitoring or stream assessment.

#### *Phylogenetic relationships within Smicrideinae*

Our study was not designed to resolve phylogenetic relationships among species of *Smicridea* or genera of Smicrideinae, and the sole gene region we used was insufficient to resolve these relationships with phylogenetic rigor or confidence. However, our analyses support monophyly of the subgenus *Smicridea* (*Rhyacophylax*) and monophyly of the Chilean clade of *Smicridea* (*Smicridea*). The subgenus *Smicridea* (*Smicridea*) is paraphyletic in our analysis but this relationship was not supported by high posterior probabilities (Fig. 3). These results should be treated with caution because our sampling of taxa or genetic data was insufficient to resolve deeper relationships fully. Accordingly, only very few basal nodes were highly supported in the B/MCMC analysis. Our analysis did provide interpretable results at the species and population level. In addition to lending support for the monophyly of a Chilean clade of *Smicridea* (*Smicridea*), the B/MCMC analysis of haplotypes

showed that most morphologically distinguished species also were genetically distinct and formed strongly supported monophyletic clades. Exceptions within Chilean *Smicridea* (*Smicridea*) were discussed in detail above. Our outgroup taxa, *S. albosignata* and *A. edwardsii*, exhibited striking intraspecific divergences or were not monophyletic, respectively (Fig. 3). However, these taxa are not the focus of our study, and we have insufficient data to draw conclusions.

#### *Larval associations in Chilean Smicridea*

We were able to associate unambiguously the larvae of *S. annulicornis* and 5 previously undescribed larvae to species (*S. cf. annulicornis*, *S. decora*, *S. cf. manzanara*, *S. pucara*, *S. smilodon*). Our associations fulfilled the rigorous nesting criteria outlined by Zhou et al. (2007). However, Zhou et al. (2007) recommended using more than a single gene for life-stage associations. We only used a single locus, but we are confident in our associations, which were based on deep divergences observed among closely related congeneric species, comprehensive taxon sampling of the focal region, and fulfillment of the association criteria outlined in the methods section. Authors of other studies have used a single marker to associate caddisfly life stages successfully (Graf et al. 2005, 2009, Waringer et al. 2007, 2008). However, when no clear barcode gap or lineage sorting is observed, e.g., for Chinese Hydropsychidae (Zhou et al. 2007), life-stage associations should be based on more than a single locus.

Our associations provided us with another opportunity to examine morphological differences among some of the cryptic species outlined above. Among *S. annulicornis* and *S. cf. annulicornis* larvae, we observed differences in the head coloration (distinctive light and dark markings on the head capsule in *S. annulicornis* and more or less uniformly brown in *S. cf. annulicornis*), and in the scale hairs on the body surface (broader and more truncated in *S. annulicornis* than in *S. cf. annulicornis*). Among *S. manzanara* and *S. cf. manzanara*, we noticed differences in coloration of the head capsule, pronotum, and mesonotum and differences in the scale hairs on the body surface. However, we found considerable variation in these characters between 2 populations of *S. cf. manzanara*. Although these larval differences support the hypothesis that both *S. annulicornis*/*S. cf. annulicornis* and *S. manzanara*/*S. cf. manzanara* represent pairs of distinct species, the number of specimens and geographic variation in our current larval data set was too limited to draw conclusions about diagnostic larval characters.

## Conclusions

Our study shows that combining detailed morphological observation and molecular sequence data, in our case from the DNA barcode region, can be very constructive in discovering and describing new species. To our knowledge, our study represents the first use of COI sequence data from the DNA barcode region to identify and facilitate the description of new caddisfly species. The pace of environmental change, either through habitat destruction or from the detrimental effects of global warming, calls for increasing efforts to discover, describe, and protect the world's biota, especially in biodiversity hotspots. DNA taxonomy or DNA barcoding alone can reveal previously unknown evolutionary units, i.e., species. With formal taxonomic description of the newly recognized diversity, results of such studies will become more valuable to biodiversity surveys or conservation efforts that rely on noninvasive sampling. Thus, we advocate the use of *integrative taxonomy* (sensu Will et al. 2005, Meier 2008), the collaborative and mutually beneficial integrative application of molecular biology, comparative morphology, and descriptive taxonomy to circumscribe and describe species.

Our study shows that DNA barcode data were useful in delimiting species within Chilean *Smicridea* (*Smicridea*) and suitable for life-stage associations. These species are closely related and presumably evolved as a monophyletic radiation in the region. These data are promising for the utility of DNA barcoding for delimiting and associating other *Smicridea* species. However, many of the other species of *Smicridea* (*Smicridea*) have vastly larger ranges across the Neotropical lowlands, over which intraspecific divergences could potentially exceed the ones we observed within Chile. The DNA barcode region of COI also was sufficient for discovering and analyzing intraspecific variation. Thus, it is a candidate locus for widespread application in phylogeographic studies in insects. Furthermore, it is easy to amplify and align. This finding is not novel (Graf et al. 2008, Stradner 2008, Ståhls and Savolainen 2008, Craft et al. 2010), but we think it important to stress that DNA barcodes can be used in insect phylogeography in many cases, perhaps universally. We agree with the growing body of literature that promotes the use of multiple independent, preferably nuclear and mitochondrial, loci in phylogeographic studies (e.g., Hickerson et al. 2010). However, if used widely for intraspecific studies, the barcode region of the COI gene will provide researchers with great opportunities for comparative phylogeographic analysis across taxa

and regions with homologous data because a huge amount of barcode data is being accumulated rapidly across animal groups (Hebert et al. 2010). Our results are inconclusive on the utility of the DNA barcoding region for resolving phylogenetic relationships among species within *Smicridea* or among higher taxa (e.g., genera). At this level, it probably will be necessary to use more genes that are less prone to homoplasy (Rubinoff 2006). In caddisflies, ribosomal 28S D2, elongation-factor 1 alpha (Kjer et al. 2001, 2002, Espeland et al. 2008), and wingless (Pauls et al. 2008) have been useful as additional genes for phylogenetic studies.

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