Non-biting midges (Diptera: Chironomidae) research in South America: subsidizing biogeographic hypotheses

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Abstract – We present data on Chironomidae (Insecta: Diptera) collected in South America together with results on the mitochondrial DNA diversity within selected megadiverse genera. This work is part of an on-going project on the ancient origin of South American biodiversity using non-biting midges. Collections were made at 42 localities, in March 2014 and February 2015, in a diverse array of habitats, including small streams, rivers, ponds, lakes and bays. In total, 3196 representatives of six subfamilies were collected. Sixty-one genera were identified, containing at least 211 species. The subfamilies Chironominae and Orthocladiinae predominated in all samples. Tanypodinae were often present, but rarely in large numbers. Except for *Podonomus pepinellii*, reported from Brazil, Podonominae were collected in a few localities in Argentina (Arroyo Lopez, and Arroyo Gutierrez and Gutierrez Lake) and Chile (Llanquihue Lake). Prodiamesinae were only recorded in Chile. Analysis of DNA barcode sequences using neighbor-joining estimation supported 66 species within the selected genera. The chironomid fauna of South America includes multiple genera with worldwide distributions, with Australian, Nearctic and Neotropical components.

Key words: Biodiversity / taxonomy / biogeography / Southern Hemisphere / cytochrome oxidase sub-unit I / DNA barcoding

Introduction

Non-biting midges, belonging to the family Chironomidae, are true flies (order Diptera), and the most widely distributed free-living holometabolous insects (Ferrington, 2008). This cosmopolitan group occurs in all zoogeographical regions of the world, including Antarctica. The immature stages of most species occur in freshwater, but numerous terrestrial or marine species are known (Sæther and Ekrem, 2003). The adult life stage of chironomids is short, and they have limited ability for aerial dispersal compared with many other freshwater insects. Presumably, the great species and habitat diversity in this family is a product of its antiquity and evolutionary plasticity (Ferrington *et al.*, 2008), which not only makes the family a valuable source of indicator species for lentic and lotic aquatic ecosystems, but also an interesting group for phylogenetic and biogeographical analyses (Silva and Ekrem, 2016).

Chironomidae probably originated in the middle Triassic approximately 248–210 million years ago (Cranston *et al.*, 2010). This family comprises at least 10 000 species in more than 400 genera (Armitage *et al.*, 1995; Sæther, 2000) and roughly 6200 of these are known to science (P. Ashe, 2014, personal communication). Although the distribution of the species in many genera is relatively well known, detailed analyses of distribution patterns and historical biogeography are rare in chironomids, especially from the austral regions. Historical biogeography is intimately associated with the Chironomidae (Silva and Ekrem, 2016), particularly the austral taxa, which were monographed by Lars Brundin in 1966. Brundin's work had a great impact on biogeography because of the great clarity of his method and the fact that phylogeny played an important role in generating biogeographical hypotheses (Funk, 2004). More recently, chironomids have been used to better understand faunal relationships in southern South America and other southern temperate regions (Cranston *et al.*, 2010; Cranston and Krosch, 2011; Krosch *et al.*, 2011; Krosch and Cranston, 2013). All these studies have in common the idea of a close

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connection among Chironomidae taxa from Australia, New Zealand and southern South America, and reveal the presence of numerous endemic chironomid genera in temperate South America.

The biota of South America always captivated biogeographers because of the interesting distributional patterns exhibited by its flora and fauna (Hooker, 1843; Darwin, 1859; Wallace, 1876). For more than a century, biogeographers have proposed theories to explain the origin and relationships of biotas found in southern South America and other southern temperate regions such as Australia, New Zealand and South Africa. Moreover, numerous theories have attempted to explain the origin and relationships of biotas found in tropical northern and temperate southern South America (Brundin, 1966; Fittkau *et al.*, 1969; Amorim and Tozoni, 1994). Currently, such explanations are challenged by phylogenetic studies, which use molecular data to test whether speciation is driven by dispersal or vicariance (*e.g.*, Brumfield and Capparella, 1996; Klicka and Zink, 1997; Conti *et al.*, 2002; Xiang *et al.*, 2005; Stelbrink *et al.*, 2012; Silva *et al.*, 2015).

Although new species of Chironomidae have recently been described from South America (Silva *et al.*, 2010, 2012, 2014a, 2014b; Oliveira and Silva, 2011; Trivinho-Strixino and Silva, 2011; Dantas and Hamada, 2013; Trivinho-Strixino *et al.*, 2013, 2015; Andersen and Pinho, 2014; Siri and Donato, 2015), none of these studies focused on biological processes such as vicariance, dispersal and extinction in a biogeographical context. To address these questions, a 2-year study, entitled “Understanding the ancient origin of South American biodiversity: a molecular perspective on the evolution and biogeography of non-biting midges (Diptera: Chironomidae)” and supported by the CAPES Foundation, Ministry of Education of Brazil, and the Museum of Comparative Zoology, Department of Organismic and Evolutionary Biology, Harvard University, began in 2014. Here we present a contribution to data on Chironomidae collected during the project, as well as results on the mitochondrial DNA diversity within selected megadiverse genera.

Material and methods

Study area

South America is the fourth largest continent of the world (17 840 000 km²). It is located mostly in the Southern Hemisphere, with a relatively small portion in the Northern Hemisphere. It is bordered on the west by the Pacific Ocean and on the north and east by the Atlantic Ocean; North America and the Caribbean Sea lie to the northwest. This continent is a natural geographic unit and has a varied terrain comprising grasslands, tropical and subtropical rain forests, tropical deciduous forests, caatinga, subantarctic rain forests and mountain vegetation. Climate in South America can be divided into four

major climatic regions: tropical, temperate, arid and cold. The mean annual precipitation ranges from 0 to 500 cm. The Neotropical location's complex topography and variety of local weather patterns generate numerous microhabitats, which support a rich flora and fauna with significant numbers of endemic species.

Taxon sampling and identification

Collections were made at 42 localities, in March 2014 and February 2015 (Table 1, Fig. 1), in a diverse array of habitats, including small streams and ponds to lakes, rivers and bays. The main emphasis was on adult sampling, collected with a sweep net near aquatic systems. A 20 cm diameter dip-net (mesh size 250 µm) was also used to collect immature stages at some localities. All samples were preserved in the field using 96% ethanol. In the laboratory, after initial sorting and identification, representatives of every taxon in each sample were slide mounted in Euparal for species identification following the procedure outlined by Pinder (1986, 1989) and Andersen *et al.* (2013a). Morphological identification was made based on keys of Wiederholm (1986, 1989), Epler (2001), Spies *et al.* (2009), Andersen *et al.* (2013b), Trivinho-Strixino (2014), original descriptions and eventual examination of type material. Morphological terminology and abbreviations essentially follow Sæther (1980). Voucher specimens are deposited in the Museum of Comparative Zoology (MCZ) at Harvard University, the National Institute of Amazonian Research (INPA) and University Museum, at the Norwegian University of Science and Technology (NTNU-VM).

DNA extraction, amplification, sequencing and alignment

The more taxonomically and ecologically diverse the species compared in biogeographic studies, the more generally applicable the results will be (Zink, 1996). Therefore, to extend the relevance of the results and verify their suitability as subjects for biogeographical analysis, four megadiverse genera were selected for molecular analyses: *Ablabesmyia*, *Chironomus*, *Labrundinia* and *Polypedilum*. These are both species rich and widespread in South America. Specimens for DNA were mostly collected as adults and preserved in 80–96% ethanol and stored dark at 4 °C before molecular analyses. DNA was extracted from single individuals in a buffered solution with the enzyme proteinase-K at 56 °C overnight, following the standard DNeasy Blood & Tissue Kit (Qiagen, USA), according to the manufacturer's protocol. The final elution volume was 100 µL. After DNA extraction, the exoskeleton was washed with 96% ethanol and mounted in Euparal on the same microscope slide as its corresponding head, antennae, wings, legs and abdomen following the procedure outlined by Sæther (1969). Voucher specimens are deposited in the Museum of

Table 1. Localities and geographic coordinates of chironomid collections in South America in March 2014 and February 2015.

Code	Country	City or Province	Locality	Latitude	Longitude
1	Argentina	San Carlos de Bariloche	Arroyo Gutierrez	41°09'36.16"S	71°24'42.40"W
2	Argentina	San Carlos de Bariloche	Arroyo Lopez	41°05'02.29"S	71°32'50.92"W
3	Argentina	San Carlos de Bariloche	Arroyo Ñireco	41°08'00.92"S	71°17'06.27"W
4	Argentina	San Carlos de Bariloche	Escondido Lake	41°03'38.00"S	71°34'01.67"W
5	Argentina	San Carlos de Bariloche	Gutierrez Lake	41°09'59.07"S	71°24'54.46"W
6	Argentina	San Carlos de Bariloche	Nahuel Huapi Lake	41°07'57.82"S	71°17'37.00"W
7	Argentina	San Carlos de Bariloche	Virgen de las Nieves	41°07'58.95"S	71°24'52.71"W
8	Argentina	Viedma	Río Negro at outfall	40°48'00.34"S	62°59'49.28"W
9	Argentina	Viedma	Río Negro	40°47'56.88"S	63°00'13.68"W
10	Argentina	Viedma	Río Negro near bridge	40°49'03.41"S	62°58'31.50"W
11	Argentina	Ushuaia	Bahia Lapataia	54°50'53.38"S	68°29'14.46"W
12	Argentina	Ushuaia	Roca Lake	54°49'01.43"S	68°36'34.91"W
13	Brazil	Bossoroca	Rio Piratini	28°31'46.70"S	54°55'10.80"W
14	Brazil	São Luiz Gonzaga	Serra do Caparaó	20°25'04.00"S	41°48'59.60"W
15	Brazil	Goiás	Córrego dos Buritis	14°10'05.77"S	47°47'28.49"W
16	Brazil	Goiás	Córrego da Siriema	14°10'26.48"S	47°49'26.23"W
17	Brazil	Igarassu	Refúgio Charles Darwin	07°48'56.09"S	34°57'16.15"W
18	Brazil	Manaus	Igarapé Acará	02°56'29.30"S	59°56'07.40"W
19	Brazil	Manaus	Igarapé Barro Branco	02°54'32.90"S	59°57'16.11"W
20	Brazil	Mato Grosso do Sul	Rio Miranda	19°34'30.06"S	57°00'52.40"W
21	Brazil	Presidente Figueiredo	Cachoeira da Suframa	02°02'17.01"S	60°01'41.46"W
22	Brazil	Presidente Figueiredo	Recanto da Pantera	02°02'31.38"S	59°50'56.40"W
23	Brazil	Recife	Córrego Oficina Brennand	08°03'60.42"S	34°58'28.97"W
24	Brazil	Recife	Estação de Aquicultura	08°01'10.02"S	34°56'42.12"W
25	Brazil	Recife	Lago Apipucos	08°01'10.00"S	34°56'02.00"W
26	Brazil	Roraima	Igarapé da Jesine	00°54'47.40"N	59°34'19.70"W
27	Brazil	Roraima	Lagoa Sítio da Ivone	00°52'57.60"N	59°42'17.40"W
28	Brazil	Roraima	Rio Água Boa	02°43'32.60"N	60°48'39.80"W
29	Brazil	São Carlos	Córrego do Fazzari	21°58'08.84"S	47°53'10.21"W
30	Brazil	São Carlos	Córrego do Espraído	21°58'12.35"S	47°52'16.42"W
31	Brazil	São Carlos	Córrego do Monjolinho	21°59'09.51"S	47°52'40.46"W
32	Brazil	São Carlos	Parque Ecológico	21°59'09.84"S	47°52'32.34"W
33	Brazil	São Carlos	Represa do Fazzari	21°58'17.87"S	47°53'14.92"W
34	Brazil	São Carlos	UFSCar	21°59'00.51"S	47°52'42.39"W
35	Brazil	Tamandaré	REBio Saltinho	08°43'24.88"S	35°11'11.23"W
36	Chile	Puerto Montt	Lago Chapo	41°25'51.17"S	72°34'11.48"W
37	Chile	Puerto Montt	Rio Chamiza	41°26'04.85"S	72°35'09.43"W
38	Chile	Puerto Varas	Lago Todos los Santos	41°07'55.66"S	72°23'59.30"W
39	Chile	Puerto Varas	Laguna la Poza	41°16'40.22"S	72°51'17.81"W
40	Chile	Puerto Varas	Llanquihue Lake 1	41°16'37.51"S	72°51'11.71"W
41	Chile	Puerto Varas	Llanquihue Lake 2	41°18'53.55"S	72°58'56.40"W
42	Chile	Puerto Varas	Rio Petrohué	41°08'27.62"S	72°24'19.58"W

Comparative Zoology (MCZ) at Harvard University and University Museum, National Institute of Amazonian Research (INPA) and University Museum, at the Norwegian University of Science and Technology (NTNU-VM).

PCR amplification was conducted in 25 µL reactions and contained 2 µL DNA template (concentration not measured), 2.5 µL 5 × buffer, 2 µL MgCl₂ in 25 µM concentration, 0.2 µL of dNTPs in 10 mM concentration, 1 µL of each of the universal standard barcode primers (Folmer *et al.*, 1994) LCO1490 (50-GGTCAACAAA TCATAAAGATATTG-30) and HCO2198 (50-TAA ACTTCAGGGTGACCAAAAAATC-30), in 10 µM concentration, 0.2 µL of HotStarTaq (all from Qiagen, Germany) and 16.1 µL of ddH₂O. Amplifications for

the COI region were performed in a thermocycler with an initial denaturation step at 95 °C for 15 min, followed by five cycles at 94 °C for 30 s, 45 °C for 30 s, 72 °C for 1 min, followed by 35 cycles at 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min and one cycle at 72 °C for 5 min, and then held at 4 °C.

The PCR products were purified using shrimp alkaline phosphatase and exonuclease I (USB Corp., USA). For bidirectional sequencing, we used the ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies, USA), and cycle sequencing reactions were performed on ABI PRISM 3130xl or 3730xl automated sequencers (Life Technologies, USA) at Harvard University. All sequences generated in this study are available through the dataset “Megadiverse Chironomidae

Fig. 1. Map of South America showing sampling sites in Argentina, Brazil and Chile (red dots). Some sites overlap due to their proximity and the map scale. Blue numbers indicate sampling localities. See Table 1 for more details.

DNA barcoding 2016 (DS-MDCBAR)” on BOLD, DOI: dx.doi.org/10.5883/DS-MDCBAR. Sequences were assembled and edited using Geneious 8.1.5 (Drummond *et al.*, 2010), checked for stop codons and aligned as translated amino acids using the Muscle algorithm (Edgar, 2004) on amino acids as implemented in MEGA7 (Kumar *et al.*, 2016). The alignment was trivial as no indels or introns were observed in the sequences.

Data analysis

A neighbor-joining (NJ) tree was produced in MEGA7 using the Kimura-2-parameter (K2P) model (Kimura, 1980), to make our results comparable with most other DNA barcode studies on insects. Bootstrap analysis used

1000 pseudoreplicates (Felsenstein, 1985) and the “pair-wise deletion” option of missing data. The nucleotide statistics and pairwise distances were also calculated based on the K2P model in MEGA7. Tree graphics were produced using TreeView (Page, 1996).

Results

In total, 3196 representatives of six subfamilies were collected (Table 2). Sixty-one genera have been identified, containing at least 211 species. At least 11 additional species were collected that could not be assigned to described genera and were identified as Chironominae sp. 1–3 and Orthocladiinae sp. 1–8. Chironomidae was represented in the tropical northern South America by

Table 2. Chironomid taxa collected in South America, March 2014 and February 2015.

Voucher number	Chironomidae taxa	Life stage	Locality
	Chironomidae		
	Chironomini		
MCZ Cat # 596143, 596242	<i>Apedilum beckae</i>	M, L	13, 18, 33, 41
MCZ Cat # 596293, 596296	<i>Beardius</i> sp. 1	L	15, 17, 30
MCZ Cat # 596299	Chironominae sp. 1	M	27
MCZ Cat # 596199	Chironominae sp. 2	M	38
MCZ Cat # 596283	Chironominae sp. 3	M	32
MCZ Cat # 595946-50	<i>Chironomus</i> sp. 1	M, L	3, 6, 8, 9, 37
MCZ Cat # 596261-63	<i>Chironomus</i> sp. 2	L	31
MCZ Cat # 595932	<i>Chironomus</i> sp. 3	L	23
MCZ Cat # 595849-51	<i>Chironomus</i> sp. 4	M	21, 23
MCZ Cat # 596373-74	<i>Chironomus</i> sp. 5	M	35
MCZ Cat # 596377	<i>Chironomus</i> sp. 6	M	35
MCZ Cat # 596266-68	<i>Chironomus</i> sp. 7	L	34
MCZ Cat # 595793	<i>Chironomus</i> sp. 8	L	19
MCZ Cat # 595918-20	<i>Chironomus</i> sp. 9	L	17
MCZ Cat # 595829	<i>Chironomus</i> sp. 10	L	18
MCZ Cat # 595832	<i>Chironomus</i> sp. 11	L	18
MCZ Cat # 595780-84	<i>Cladopelma</i> sp. 1	L	18, 19
MCZ Cat # 596298	<i>Cladopelma</i> sp. 2	L	30
MCZ Cat # 596329	<i>Cladopelma</i> sp. 3	L	15
MCZ Cat # 596240	<i>Cryptochironomus</i> sp. 1	L	33
MCZ Cat # 596342, 596349	<i>Cryptochironomus</i> sp. 2	M	8, 9
MCZ Cat # 595990, 596007	<i>Cryptochironomus</i> sp. 3	M	1, 5, 7, 36, 39
MCZ Cat # 595878	<i>Cryptochironomus</i> sp. 4	M	13, 26, 27
MCZ Cat # 595859, 595877	<i>Dicrotendipes</i> sp. 1	M	26, 27
MCZ Cat # 596017, 596144	<i>Dicrotendipes</i> sp. 2	M	1, 38, 39, 40-2
MCZ Cat # 596273	<i>Endotribelos</i> sp. 1	L	32, 33
MCZ Cat # 595803	<i>Endotribelos</i> sp. 2	L	19
MCZ Cat # 595907-08	<i>Endotribelos</i> sp. 3	L	17
MCZ Cat # 596330	<i>Endotribelos</i> sp. 4	L	15
MCZ Cat # 595923-26	<i>Goeldichironomus neopictus</i> Trivinho-Strixino and Strixino	L	17, 24, 25
MCZ Cat # 595906, 595921	<i>Harnischia</i> sp. 1	L	17
MCZ Cat # 596355	<i>Kiefferulus</i> sp. 1	M	9
MCZ Cat # 596276	<i>Kiefferulus</i> sp. 2	L	32
MCZ Cat # 596135-36	<i>Nilothauma aripuanense</i> Mendes and Andersen	M	13
MCZ Cat # 595768, 595887	<i>Nilothauma</i> sp. 1	M	27
INPA # FA01/08	<i>Nilothauma</i> sp. 2	L	19
MCZ Cat # 596370-71	<i>Oukuriella intermedia</i> Messias, Fittkau and Oliveira	M	35
MCZ Cat # 595879-80, 595882-84	<i>Parachironomus lupus</i> Trivinho-Strixino, Silva and Roque	M	28
MCZ Cat # 595807, 596137	<i>Parachironomus manaos</i> Spies	M	13, 19
MCZ Cat # 596187	<i>Parachironomus puberulus</i> Edwards	M	42
MCZ Cat # 596229-30	<i>Parachironomus supparilis</i> Edwards	M	12, 36, 37
MCZ Cat # 596204, 596345	<i>Parachironomus valdiviensis</i> Spies	M	8, 38, 40, 41
MCZ Cat # 596077, 596087	<i>Parachironomus</i> sp. 1	M, L	4, 7
MCZ Cat # 595857, 596127	<i>Parachironomus</i> sp. 2	L	29
MCZ Cat # 596358	<i>Paralauterborniella nigrohalterale</i> Malloch	M	9
MCZ Cat # 595816, 595852	<i>Paratendipes</i> sp. 1	M, L	21, 22
MCZ Cat # 596331	<i>Paratendipes</i> sp. 2	L	15
MCZ Cat # 595861-62	<i>Pelomus</i> sp. 1	M, L	22, 26
MCZ Cat # 595914	<i>Pelomus</i> sp. 2	L	17
MCZ Cat # 596294-95	<i>Pelomus</i> sp. 3	L	30
MCZ Cat # 596333	<i>Pelomus</i> sp. 4	L	15
MCZ Cat # 596258, 596260	<i>Polypedilum (Asheum) beckae</i> Sublette & Sublette	M, L	33
MCZ Cat # 596178, 596181	<i>Polypedilum</i> sp. 1	M	1, 2, 5, 7, 39, 41
INPA # FA40/14	<i>Polypedilum</i> sp. 2	M	15
MCZ Cat # 596149	<i>Polypedilum</i> sp. 3	M	41
MCZ Cat # 596084-85	<i>Polypedilum</i> sp. 4	L	4
MCZ Cat # 596125-26	<i>Polypedilum</i> sp. 5	L	12
INPA # FA34/21	<i>Polypedilum</i> sp. 6	L	32
INPA # FA31/14	<i>Polypedilum</i> sp. 7	L	33
INPA # FA32/04	<i>Polypedilum</i> sp. 8	L	31, 32
INPA # FA02/14	<i>Polypedilum</i> sp. 9	M	22

Table 2. (Contd.)

Voucher number	Chironomidae taxa	Life stage	Locality
INPA # FA32/05	<i>Polypedilum</i> sp. 10	L	31
INPA # FA01/37	<i>Polypedilum</i> sp. 11	L	19
INPA # FA03/04	<i>Polypedilum</i> sp. 12	L	18
INPA # FA01/42	<i>Polypedilum</i> sp. 13	L	19
INPA # FA23/10	<i>Polypedilum</i> sp. 14	M	13
INPA # FA44/04	<i>Polypedilum</i> sp. 15	L	10
MCZ Cat # 595895	<i>Polypedilum</i> sp. 16	M	27
INPA # FA06/15	<i>Polypedilum</i> sp. 17	M	26
INPA # FA40/18	<i>Polypedilum</i> sp. 18	L	15
INPA # FA02/16	<i>Polypedilum</i> sp. 19	L	22
INPA # FA31/24	<i>Polypedilum</i> sp. 20	M	33
INPA # FA02/05	<i>Polypedilum</i> sp. 21	L	22
MCZ Cat # 595853-54	<i>Polypedilum</i> sp. 22	M	21
MCZ Cat # 596189-92	<i>Polypedilum</i> sp. 23	M	38, 42
INPA # FA04/24	<i>Polypedilum</i> sp. 24	M	38
INPA # FA46/09	<i>Polypedilum</i> sp. 25	M	35
INPA # FA40/16	<i>Polypedilum</i> sp. 26	L	15, 16
MCZ Cat # 595894	<i>Polypedilum</i> sp. 27	M, L	28
MCZ Cat # 596354	<i>Saetheria</i> sp. 1	M	8
MCZ Cat # 595763	<i>Stenochironomus</i> sp. 1	L	19
MCZ Cat # 595909	<i>Stenochironomus</i> sp. 2	L	17
MCZ Cat # 96128	<i>Xestochironomus</i> sp. 1	M	13
Pseudochironomini			
MCZ Cat # 595888, 596346	<i>Pseudochironomus</i> sp. 1	M	8, 28
MCZ Cat # 595826-27	<i>Riethia manauara</i> Neubern, Trivinho-Strixino and Silva	L	18
MCZ Cat # 595994-96	<i>Riethia truncatocaudata</i> Edwards	L	1, 5
MCZ Cat # 596030	<i>Riethia</i> sp. 1	M	28
Tanytarsini			
NTNU-VM # 150005, 150006	<i>Caladomyia hoeferi</i> Reiff	M	28
NTNU-VM # 150010	<i>Caladomyia kapilei</i> Trivinho-Strixino	M	28
NTNU-VM # 150009, 150049	<i>Caladomyia ortonii</i> Säwedäl	M	28, 32
NTNU-VM # 149999, 150000	<i>Caladomyia</i> sp. 1	M	26
NTNU-VM # 150007	<i>Caladomyia</i> sp. 2	M	28
NTNU-VM # 150008	<i>Caladomyia</i> sp. 3	M	28
MCZ Cat # 595809, 596031-2	<i>Cladotanytarsus</i> sp. 1	L	1, 7, 22
MCZ Cat # 595889-90	<i>Nandeva</i> sp. 1	M	22
INPA # FA36/05	<i>Nandeva</i> sp. 2	M	29
MCZ Cat # 595801-02	<i>Rheotanytarsus</i> sp. 1	L	19
MCZ Cat # 595885-86	<i>Rheotanytarsus</i> sp. 2	M	28
MCZ Cat # 596305	<i>Rheotanytarsus</i> sp. 3	L	29
MCZ Cat # 595765	<i>Stempellinella</i> sp. 1	L	19
NTNU-VM # 150026	<i>Tanytarsus clivus</i> Reiss	M	5
MCZ Cat # 596088, 596324	<i>Tanytarsus fastigatus</i> Reiss	M	4, 5, 7, 40
NTNU-VM # 150038-41	<i>Tanytarsus hamatus</i> Reiss	M	37
NTNU-VM # 150033-34	<i>Tanytarsus jacaretingensis</i> Sanseverino	M	13
NTNU-VM # 150001	<i>Tanytarsus kiche</i> Vinogradova, Riss and Spies	M	26
NTNU-VM # 150045-46	<i>Tanytarsus longitubuli</i> Trivinho-Strixino, Wiedenbrug and Silva	M, L	32, 33
NTNU-VM # 150044	<i>Tanytarsus obiriciae</i> Trivinho-Strixino and Sonoda	M, L	33
NTNU-VM # 149978	<i>Tanytarsus rhabdomantis</i> Trivinho-Strixino and Strixino	M, L	21, 22
NTNU-VM # 150011	<i>Tanytarsus riopreto</i> Fittkau and Reiss	M	28
MCZ Cat # 596206, 596256	<i>Tanytarsus</i> sp. 1	L	15
MCZ Cat # 596337, 596338	<i>Tanytarsus</i> sp. 2	L	16
MCZ Cat # 595785, 595828	<i>Tanytarsus</i> sp. 3	L	18, 19
NTNU-VM # 149976, 149979	<i>Tanytarsus</i> sp. 4	M	22
NTNU-VM # 149980	<i>Tanytarsus</i> sp. 5	M	22
Orthocladiinae			
MCZ Cat # 596379	<i>Allocladius quadrus</i> Andersen, Sæther et Mendes	M	11
MCZ Cat # 596108-09	<i>Allocladius</i> sp. 1	M	2
MCZ Cat # 596076	<i>Botryocladius</i> sp. 1	L	7
MCZ Cat # 595817, 595917	<i>Corynoneura</i> sp. 1	L	17, 22
MCZ Cat # 596035, 596057	<i>Corynoneura</i> sp. 2	M, L	1, 7, 9, 41
INPA # FA23/G	<i>Corynoneura</i> sp. 3	M	13
MCZ Cat # 596335, 596340	<i>Corynoneura</i> sp. 4	L, M	15, 16

Table 2. (Contd.)

Voucher number	Chironomidae taxa	Life stage	Locality
MCZ Cat # 595779, 595810	<i>Cricotopus</i> sp. 1	L	19, 22, 21
MCZ Cat # 595954-55	<i>Cricotopus</i> sp. 2	M, P	1, 3, 5, 6, 7, 9
MCZ Cat # 596139, 596142	<i>Cricotopus</i> sp. 3	M	13
MCZ Cat # 596166-67	<i>Cricotopus</i> sp. 4	M	37, 39, 40, 41
MCZ Cat # 596339	<i>Cricotopus</i> sp. 5	L	16
MCZ Cat # 595956, 596066	<i>Eukiefferiella</i> sp. 1	M, L	2, 3, 7, 42
MCZ Cat # 596195, 596208	<i>Ferringtonia patagonica</i> Edwards	M	38, 42
MCZ Cat # 596208	<i>Ichthyocladus</i> sp. 1	L	15
MCZ Cat # 596380	<i>Limnophyes</i> sp. 1	M, L	6, 7, 11, 41
INPA # FA25/L	<i>Lipurometriocnemus</i> sp. 1	M	36, 38, 39, 41
MCZ Cat # 595863-64	<i>Lopescladius</i> (<i>Cordiella</i>) sp. 1	M	26
MCZ Cat # 595867-68	<i>Lopescladius</i> (<i>Lopescladius</i>) sp. 1	M	26
MCZ Cat # 596232	<i>Mesosmittia</i> sp. 1	M	36
MCZ Cat # 595814, 595858	Orthoclaadiinae sp. 1	L	22
MCZ Cat # 595969, 595974	Orthoclaadiinae sp. 2	L	6
MCZ Cat # 595997, 595999	Orthoclaadiinae sp. 3	L	5, 7
MCZ Cat # 596110, 596111	Orthoclaadiinae sp. 4	M	2
MCZ Cat # 596138	Orthoclaadiinae sp. 5	M	13
MCZ Cat # 596186	Orthoclaadiinae sp. 6	M	39
MCZ Cat # 596213, 596218	Orthoclaadiinae sp. 7	M	36, 37
MCZ Cat # 596034, 596036	Orthoclaadiinae (<i>Paracladius</i> ?) sp. 8	L	1
MCZ Cat # 596104-05	<i>Orthocladus</i> sp. 1	M, P	2, 6, 7, 41
MCZ Cat # 596194	<i>Parakiefferiella</i> sp. 1	M, L	2, 39, 42
MCZ Cat # 595981	<i>Parametriocnemus</i> sp. 1	L	3, 6
MCZ Cat # 596378	<i>Parapsectrocladius acuminatus</i> Edwards	M	11
MCZ Cat # 596011, 596174	<i>Parapsectrocladius longistylus</i> Cranston	M	5, 41
MCZ Cat # 595951-53	<i>Parapsectrocladius</i> sp. 1	L, P	3
MCZ Cat # 595804-06	<i>Pseudosmittia</i> sp. 1	L	19
MCZ Cat # 596251	<i>Thienemanniella sancticaroli</i>	M	7
MCZ Cat # 596058	<i>Thienemanniella</i> sp. 1	L	33
MCZ Cat # 596325	<i>Thienemanniella</i> sp. 2	L	15
INPA # FA02/10	<i>Ubatubaneura</i> sp. 1	L	22
Podonominae			
Podonomini			
MCZ Cat # 596095-99	<i>Parochlus araucanus</i> Brundin	M	2
MCZ Cat # 595941-45	<i>Podonomus pepinellii</i> Roque and Trivinho-Strixino	L	14
MCZ Cat # 596100-02	<i>Podonomus</i> sp. 1	L	2
MCZ Cat # 596000-03	<i>Podonomus</i> sp. 2	L	5
MCZ Cat # 596025	<i>Podonomus</i> sp. 3	L	1
MCZ Cat # 596152-53	<i>Podonomus</i> sp. 4	L	41
Prodiamesinae			
MCZ Cat # 596170	<i>Monodiamesa mariae</i> Andersen	M	41
Tanypodinae			
Clinotanypodini			
MCZ Cat # 596272	<i>Clinotanypus</i> sp. 1	L	32
MCZ Cat # 595818-19	<i>Coelotanypus</i> sp. 1	L	18
Fittkauimyini			
MCZ Cat # 595771-75	<i>Fittkauimyia</i> sp. 1	L	19
MCZ Cat # 596290-92	<i>Fittkauimyia</i> sp. 2	L	30
Macropelopiini			
MCZ Cat # 596210	<i>Alotanypus vittiger</i> Edwards	M	37
MCZ Cat # 596211	<i>Alotanypus</i> sp. 1	M	37
INPA # FA18/11	<i>Alotanypus</i> sp. 2	M	4, 5
INPA # FA22/8	<i>Alotanypus</i> sp. 3	M	2
MCZ Cat # 596051- 596052	<i>Apsectrotanypus</i> sp. 1	L	7
Pentaneurini			
MCZ Cat # 596318	<i>Ablabesmyia</i> sp. 1	L	15
MCZ Cat # 596300-02	<i>Ablabesmyia</i> sp. 2	L	15, 17, 29
MCZ Cat # 595761	<i>Ablabesmyia</i> sp. 3	L	18
MCZ Cat # 596317	<i>Ablabesmyia</i> sp. 4	L	15
MCZ Cat # 596200-03	<i>Ablabesmyia</i> sp. 5	M, L	1, 2, 4, 5, 7, 36, 38
MCZ Cat # 596362, 596363	<i>Ablabesmyia</i> sp. 6	M	12
MCZ Cat # 595764, 595767	<i>Ablabesmyia</i> sp. 7	L	18

Table 2. (Contd.)

Voucher number	Chironomidae taxa	Life stage	Locality
MCZ Cat # 596113	<i>Ablabesmyia</i> sp. 8	M	13
INPA # FA06/02	<i>Ablabesmyia</i> sp. 9	M	26
MCZ Cat # 596234-37	<i>Ablabesmyia</i> sp. 10	M, L	13, 30, 33
MCZ Cat # 595870	<i>Ablabesmyia</i> sp. 11	M	28
MCZ Cat # 595898, 595929	<i>Ablabesmyia</i> sp. 12	L	17, 24, 25
MCZ Cat # 595869, 595874	<i>Ablabesmyia</i> sp. 13	M	28
INPA # FA07/07	<i>Labrundinia amazonica</i> Silva	M	28
INPA # FA23/05	<i>Labrundinia gregi</i> Silva	M	13
INPA # FA34/11	<i>Labrundinia multidentata</i> Silva	L	32
MCZ Cat # 595762, 595769	<i>Labrundinia paulae</i> Silva	L	19, 33
INPA # FA40/07	<i>Labrundinia pilosella</i> Loew	M	15
INPA # FA37/01	<i>Labrundinia semicurvata</i> Silva	M, P	20
INPA # FA40/05	<i>Labrundinia separata</i> Edwards	M	15
INPA # FA31/09	<i>Labrundinia tenata</i> Roback	L	33
INPA # FA1/17	<i>Labrundinia</i> sp. 1	L	19
INPA # FA36/01	<i>Labrundinia</i> sp. 2	L	29
INPA # FA7/01	<i>Labrundinia</i> sp. 3	M	28
INPA # FA40/01	<i>Labrundinia</i> sp. 4	L	15
MCZ Cat # 595777, 595778	<i>Labrundinia</i> sp. 5	L	19
INPA # FA09/09	<i>Labrundinia</i> sp. 6	M, P	17, 20
INPA # FA40/08	<i>Labrundinia</i> sp. 7	L	15
MCZ Cat # 595844-45	<i>Labrundinia</i> sp. 8	L	21
MCZ Cat # 595846-47	<i>Larsia</i> sp. 1	M, L	17, 19, 21, 24
MCZ Cat # 596248, 596259	<i>Larsia</i> sp. 2	L	31, 32, 33
MCZ Cat # 595871	<i>Monopelopia</i> sp. 1	M	28
MCZ Cat # 596246, 596277	<i>Monopelopia</i> sp. 2	M, L	32, 33
MCZ Cat # 595773, 595836	<i>Pentaneura</i> sp. 1	L	19, 21
MCZ Cat # 595902, 595928	<i>Pentaneura</i> sp. 2	L	17, 24
MCZ Cat # 596271, 596278	<i>Pentaneura</i> sp. 3	L	32, 30
MCZ Cat # 596320, 596322	<i>Pentaneura</i> sp. 4	L	15
INPA # FA31/06	<i>Thienemannimyia</i> sp. 1	L	33
Procladiini			
MCZ Cat # 596117-18	<i>Djalmabatista scopulata</i> Hagenlund, Andersen and Mendes	M	13
MCZ Cat # 596323	<i>Djalmabatista</i> sp. 1	L	15
MCZ Cat # 596319	<i>Djalmabatista</i> sp. 2	L	15
MCZ Cat # 596008-10	<i>Djalmabatista</i> sp. 3	L	4, 5

Data in the “Voucher number” column refer partially to what is housed at INPA, MCZ and NTNU-VM only, and for some species this is less than the full material examined in the present study.

M, male; L, larva; P, pupal exuviae. Sampling localities according to Table 1.

151 species, while the temperate southern comprised 57; only three species were common to both regions. The subfamilies Chironominae and Orthocladiinae predominated in all samples. Tanypodinae were present, but rarely in large numbers. Podonomininae were collected in a few localities. Prodiamesininae were recorded only in Chile.

Chironominae

Chironominae is the largest subfamily in terms of described species. This group dominates samples from tropical and subtropical lowlands, with immature stages occurring in a wide range of freshwater habitats, including brackish and marine waters (Ashe *et al.*, 1987). Chironominae is subdivided into three tribes: Chironomini, Pseudochironomini and Tanytarsini.

Chironomini was represented by at least 20 genera, comprising at least 83 species. *Polypedilum* was the most

widespread and rich in species. At least 27 species were collected, about half of which have previously been recorded from the Neotropical Region (Spies and Reiss, 1996). *Polypedilum* is one of the largest chironomid genera containing about 440 described species (Sæther *et al.*, 2010). Larvae of *Polypedilum* occur in nearly all types of still and flowing waters. A few species are also found in bromeliad tanks (Epler *et al.*, 2013). Other commonly sampled Chironominae were *Chironomus*; with 11 species, it was the second most speciose Chironomini genus. *Chironomus* is a cosmopolitan genus with several hundred species. Numerous species of this group are very abundant in heavily polluted standing or running waters, whereas some species commonly colonize small water bodies such as fish breeding tanks, where they complete their life cycle in a few days (Correia *et al.*, 2013). Spies and Reiss (1996) catalogued 19 species from the Neotropical region. However, this number clearly represents outdated knowledge and significantly underestimates real diversity of

Chironomus in the Neotropics, considering that several new species have been described more recently (Correia and Trivinho-Strixino, 2005, 2007; Correia *et al.*, 2005, 2006, 2013).

The tribe Pseudochironomini was represented by *Pseudochironomus* sp. 1, *Riethia manauara* Neubern, Trivinho-Strixino and Silva, *R. truncatocaudata* (Edwards) and an undescribed species of the latter genus. Larvae of *Riethia* inhabit the bottom of lotic and lentic systems associated with sandy and fine particulate organic matter (Neubern *et al.*, 2011). In the Neotropics, pupal exuviae of *Riethia* have been recognized by Ospina-Torres (1992), Wiedenbrug (2000) and Roque *et al.* (2004) from disparate sites in Brazil. The group has close relatives recorded from Australasia, such expanded distribution seems to support Freeman's (1959, 1961) hypothesis that *Riethia* is an ancient Southern Hemisphere group, including the related *Aedokritus*, *Megacentron* and *Manoa*.

Tanytarsini was represented by six genera: *Caladomyia*, *Cladotanytarsus*, *Nandeva*, *Rheotanytarsus*, *Stempellinella* and *Tanytarsus*. The latter was represented by nine species and five related undescribed species. This genus is one of the most species rich genera of Chironomidae in the Neotropical region (Trivinho-Strixino *et al.*, 2015). The identification of adults of *Tanytarsus* is problematical due to the large interspecific variation in the genitalic morphology (Ekrem and Reiss, 1999). Despite this drawback, recent years have seen increased interest concerning the taxonomy of the group in the Neotropical region (Sanseverino and Trivinho-Strixino, 2010; Sanseverino *et al.*, 2010; Trivinho-Strixino *et al.*, 2015).

Orthocladiinae

Orthocladiinae is the second largest subfamily, with the greatest diversity of ecological requirements (Ashe and Cranston, 1990). There is no accepted division of Orthocladiinae into tribes. In our study, the subfamily was represented by 18 genera, comprising at least 39 species. *Cricotopus*, represented by five morphospecies, was the most widespread orthoclad. The genus is one of the largest in the Orthocladiinae, comprising five subgenera, with worldwide distribution (Cranston *et al.*, 1989; Ashe and O'Connor, 2009). Another commonly found Orthocladiinae was *Corynoneura*, represented by four morphospecies recorded from several localities. The genus belongs to the *Corynoneura* group, which is a large and ecologically diverse clade containing over 180 species of non-biting midges worldwide and at least 42 in the Neotropical region (Silva and Wiedenbrug, 2014). Additionally, it is important to point out the first record of *Lipumetriocnemus* in southern South America. This genus was described from St. Vincent and St. Lucia in the British West Indies and later recorded from Brazil and North America (Andersen *et al.*, 2016). In our study, *Lipumetriocnemus* was recorded from four localities in Chile, representing the southernmost occurrence of this genus.

Podonominae

Podonominae is a subfamily distributed worldwide, and currently represented by 15 genera. In our study, Podonominae was represented by *Parochlus araucanus* Brundin, and *Podonomus pepinellii* Roque and Trivinho-Strixino. Four larval morphospecies of *Podonomus* could not be ascribed to any of the previously described species, which is expected since only few larval associations have been made. So far, *P. pepinellii* is the only species described from high mountains in Brazil, which can be attributed to the largely cold-stenothermic preference exhibited by most podonomines. *Parochlus* is the most widespread and rich genus within Podonominae. The genus is very diverse in the southern temperate zone, from sub- and true Antarctic, and in Australia, New Zealand and South America. According to Cranston *et al.* (2010), *Parochlus* originated in low latitudes, moving into the Andes as they rose. The migration of a single widespread species, *P. kiefferi*, into the Northern Hemisphere may be attributable to dispersal events from the Neotropical region through the nearly continuous chain of mountains that extend from southern Chile to Arctic Canada (Cranston *et al.*, 2010). Therefore, it is expected that evidence of dispersal in *Parochlus* will be reflected as a series of closely related species with older lineages found toward the south, with progressively younger lineages present further north, with *P. kiefferi* as sister to the northernmost Neotropical *Parochlus* (Cranston *et al.*, 2010).

Prodiamesinae

Prodiamesinae is a small subfamily comprising four genera: *Compteromesa*, *Monodiamesa*, *Odontomesa* and *Prodiamesa*. Here Prodiamesinae was represented only by one species from Chile, *Monodiamesa mariae* Andersen. No genera of Prodiamesinae are known to have a worldwide distribution (Ashe *et al.*, 1987), but *Monodiamesa* is the most widespread and rich in species genus. The presence of *Monodiamesa*, as well as *Prodiamesa* in the Neotropical Region may be attributable to dispersal events from the Northern Hemisphere through the North American Rockies and the mountains of Central America, and down the Andes Mountains (Andersen, 1996). In contrast to *Parochlus* (Podonominae), it is expected that dispersal in *Monodiamesa* will be reflected as a sequence of closely related species, with older lineages found towards the north, and progressively younger lineages present further south.

Tanypodinae

Tanypodinae is the third most speciose subfamily in the Chironomidae, with species distributed widely across most of the globe, occupying numerous habits including small streams and ponds to lakes and bays (Silva *et al.*, 2011).

Table 3. Variable and informative sites, and average nucleotide composition in the aligned COI gene sequences.

Nucleotide position	Variable site (%)	Informative site (%)	T (%)	C (%)	A (%)	G (%)	AT (%)	GC (%)
1st	21.6	22.3	27.4	16.3	27.4	28.9	54.8	45.2
2nd	6.8	4.0	43.1	26.8	13.9	16.2	57.0	43.0
3rd	72.4	73.7	46.3	7.6	44.0	2.1	90.3	9.7
All	45.6	92.7	38.9	16.9	28.4	15.7	67.3	32.7

Tanypodinae were represented by at least 12 genera, comprising at least 51 species. *Labrundinia* was the richest in species, with at least 16 species. The genus is a large group, predominantly Neotropical, with immature stages inhabiting a variety of aquatic systems (Silva and Fonseca-Gessner, 2009; Silva *et al.*, 2011, 2014c). Silva *et al.* (2015), based on phylogenetic inference, suggested that *Labrundinia* may have had its initial diversification in warmer waters in the Neotropical region and that current presence in the Nearctic region and southern South America is due to subsequent dispersal. Another commonly found Tanypodinae was *Ablabesmyia*; with 13 species, it was the most widespread tanypodine. *Ablabesmyia* is a remarkably species-rich genus of the Tanypodinae, composing one of the most distinctive and well-defined clade within the tribe Pentaneurini (Silva and Ekrem, 2016). The highest diversity of *Ablabesmyia* seems to be in the tropical and warm-temperate belts.

Doubtful records

Larvae of *Labrundinia separata* (Edwards) differ from other *Labrundinia* species by the surface of head capsule covered with spinules, lateroventral and posteroventral spine groups absent; subbasal seta of posterior parapod simple; bifid claw with V-shaped lower indentation (Silva *et al.*, 2014c). So far, *L. separata* is distributed exclusively in southern South America in Argentina and Chile. During sampling in a small stream in Central-West Brazil, a fourth-instar larva fitting the diagnostic criteria of *L. separata* was collected. However, in absence of association between larval and adult specimens, this record is kept as doubtful. Similarly, larvae of *L. pilosella* (Loew) were collected at the same locality. This species is one of the most widespread within *Labrundinia*, with the distribution ranging from northern Canada to Central America. For the same reason as given for *L. separata*, the record of *L. pilosella* in Central-West Brazil is considered uncertain. However, based on its wider distribution, *L. pilosella* is likely to occur in South America.

Molecular analyses

Material suitable for genetic analyses was obtained for 120 specimens belonging to *Ablabesmyia*, *Chironomus*, *Labrundinia* and *Polypedilum*. The aligned COI sequences were 658 bp long with 300 variable sites (45.6%), of which 278 (92.7%) were potentially parsimony informative

(Table 3). Most variable sites occurred in the third codon position. The sequences were somewhat AT-biased, especially in the third position (Table 3). A NJ tree demonstrated that a substantial barcode divergence exists between all putative species. Bootstrap supports were 99% or higher for all species clusters.

Based on clustering of specimens in the NJ analysis, we were able to distinguish: 13 species of *Ablabesmyia*, 11 species of *Chironomus*, 15 species of *Labrundinia* and 27 species of *Polypedilum*. Maximum intraspecific divergence was observed in *Chironomus* sp. 1 (range: 3.5–5.3%, average 4.4%), followed by *Polypedilum* sp. 1 (range: 3.3–3.7%, average 3.4%), *Polypedilum* sp. 14 (2.4%) and *Labrundinia* sp. 6 (2.2%). The lowest interspecific distances were found between *Chironomus* sp. 10 and *Chironomus* sp. 11 (3.7%), followed by *Ablabesmyia* sp. 2 and *Ablabesmyia* sp. 3 (range: 5.8–6.3%, average 6.1%), *Ablabesmyia* sp. 2 and *Ablabesmyia* sp. 4 (average 6.3%), and *Polypedilum* sp. 6 and *Polypedilum* sp. 7 (6.4%).

The specimens identified morphologically as *Chironomus* sp. 1 were divided into two distinct clusters, which may represent cryptic species. Nucleotide sequences of these specimens differed by a minimum of 3.4% and in up to 41 nucleotide sites, but there were no observable morphological characters that differentiated the specimens belonging to these clusters. Specimens of *Chironomus* sp. 1 allocated to each clusters were collected from five localities in Argentina and Chile (Table 2). The two groups exhibited geographical structure in the clustering, since they were collected in localities more than 750 km distant from each other.

Regarding the geographical variation among populations, a distinct phylogeographic pattern was detected. *Ablabesmyia* sp. 5 and sp. 6, both sampled from southern South America, clustered together and apart from those from northern South America. Similar patterns may be noticed in the specimens composing the cluster *Chironomus* sp. 1. Although geographical distribution in *Polypedilum* was not fully structured, DNA barcodes indicated that several species from southern South America represented close relatives (*e.g.*, *Polypedilum* sp. 1, sp. 3 and sp. 4, and *Polypedilum* sp. 23 and sp. 24), not refuting the aforementioned pattern noted for *Ablabesmyia* and *Chironomus* (Fig. 2). Unfortunately, no information on geographical structuring could be inferred from *Labrundinia*. Up to now, *L. separata* Edwards is the only species recorded from southern South America. Here, *L. separata* was sampled solely in northern South America, as well as other *Labrundinia*, and its record remains doubtful (see above).

Fig. 2. NJ tree of the genera *Ablabesmyia*, *Chironomus*, *Labrundinia* and *Polypedilum*, based on partial COI sequences (DNA barcodes) and the K2P model. Numbers on branches are bootstrap values > 80%. Red numbers indicate sampling localities. See Table 1 for more details.

Biogeographers have documented similarity between the austral biotas for more than a century (e.g., [Erichson, 1842](#); [Cranston and Naumann, 1991](#)), even before understanding how these patterns might have arisen ([Krosch and Cranston, 2013](#)). The vicariance model replaced a paradigm of successive colonization by dispersal from the north ([Darlington, 1965](#)). Regarding Chironomidae, the distribution of each subfamily, within its geographical range, is primarily driven by the availability of water ecologically suited to the requirements of the larvae ([Oliver and Roussel, 1983](#)). Nevertheless, in extreme environments, the ecological requirements of the adults seem to be the regulating factor in most of the cases. At the

Numerous taxa within Chironomidae are considered to represent “classic” Gondwanan relicts (Krosch *et al.*,

2011). Lars Brundin recognized within the subfamilies Aphroteniinae, Diamesinae and Podonominae putative ancient groups with disjunct austral distributions (Brundin, 1963, 1966). Based on morphological data, Brundin reconstructed phylogenies for these hypothetical Gondwanan clades, which indicated congruent patterns of relationships among austral clades that were consistent with the geological consensus of the Gondwanan-breakup (Brundin, 1965, 1966). Recently, taxonomic descriptions and generic revisions of non-biting midges belonging to the subfamily Orthocladiinae have recognized similar disjunct taxa (e.g., *Barbadocladus*, *Botryocladus*, *Ferringtonia*, *Limnophyes* and *Pirara*) of potential Gondwanan origin (Sæther, 1990; Boothroyd and Cranston, 1995; Cranston and Edward, 1999; Sæther and Andersen, 2010; Cranston and Krosch, 2011). Similar patterns may be expected among several groups from southernmost South America toward the temperate-tropical tier, where Pan-American warm-adapted taxa may predominate.

The Neotropical region of South America comprises most of the continent, with the exception of the Andean portion (Amorim and Pires, 1996; Morrone, 1999, 2002). According to Halffter (1964, 1987), during the pre-Quaternary, the South American Neotropical biota expanded northward to Central America and Mexico, and southward to the South American transition zone and the Andean region (Morrone and Lopretto, 1994; Maury *et al.*, 1996). In this regard, the Neotropical component includes a fauna occurring in lowlands, essentially warm-acclimated (Watson and Heyn, 1992), with either limnetic or rheophilic species (Reiss and Sublette, 1985). Within this component, genera such as *Axarus*, *Beardius*, *Caladomyia*, *Coelotanypus*, *Djalmabatista*, *Goeldichironomus*, *Labrundinia*, *Lipurometriocnemus*, *Lopescladius*, *Nilothauma*, *Pentaneura* and *Polypedilum* (*Asheum*) possess a Pan-American distribution, having secondarily dispersed into the southern Nearctic via Central America or the Caribbean (Reiss and Sublette, 1985). *Endotribelos*, *Ichthyocladus*, *Oukuriella*, *Pelomus* and *Ubatubaneura* have so far presented endemic distribution in the Neotropical region. The Nearctic component includes genera such as *Cryptochironomus*, *Kiefferulus*, *Monodiamesa* and *Orthocladus*, those taxa are highly diverse and widespread in the Holarctic region, and apparently dispersed into South America via the Antilles or Mexico. *Ablabesmyia*, *Cladotanytarsus*, *Clinotanypus*, *Corynoneura*, *Chironomus*, *Cricotopus*, *Larsia*, *Polypedilum* and *Tanytarsus* are examples of genera with worldwide distributions.

For a long time, most of the studies on South American entomofauna remained restricted to the Andean and Patagonian fauna (e.g., Edwards, 1931; Brundin, 1966; Reiss, 1972). Schuh and Stonedahl (1986) performed a cladistic biogeographic analysis of some plant bugs belonging to the family Miridae (Hemiptera). Their results recovered a general-area cladogram indicating two major biotic components, Laurasia and Gondwana. Within the first, a clade involves the Indo-Pacific (tropical Africa

plus the Oriental region) and tropical America, while the latter one comprises the southern temperate areas (South Africa, southern South America and Australia). More recently, intercontinental relationships based on a biogeographic analysis of the Anisopodoidea (Diptera, Bibionomorpha) were proposed by Amorim and Tozoni (1994). In addition, Lamas *et al.* (2014) found evidence that an ancient Caribbean event was mainly responsible for the diversification of major lineages of *Heterostylum* (Diptera: Bombyliidae).

Here we tried to sample in as many different localities and aquatic systems as possible. We also visited some of the most neglected areas in terms of chironomid studies, as well as other Diptera, such as northeast and northwest Brazil. However, due to the limited duration and extension, we believe that the species richness documented here represents only a small range of the true richness of chironomid fauna in South America. A further limitation stems from the incomplete knowledge of the full life history, which, associated with the lack of descriptions and keys to a local fauna, prevents species identifications. Comparing our results with Spies and Reiss (1996), who catalogued roughly 110 genera in the Neotropical Region, we found more than half of the forms recorded at that time in the continent. It is expected that further collecting campaigns will find some widespread Neotropical genera, for example *Antilocladus*, *Fissimentum*, *Procladius*, *Stictocladus*, *Tanypus*, *Zavreliella* and *Zavreliomyia*, surprisingly not collected at this time.

The advantage of working with megadiverse taxa is that they most likely exhibit many repeated patterns, which may provide evidence of underlying processes (Coscaron *et al.*, 2009). Genera such as *Ablabesmyia*, *Chironomus*, *Labrundinia* and *Polypedilum*, examined here in detail, are largely widespread and rich in species, and, therefore, may be considered as megadiverse. However, as a result of this greater diversity, their adult identification, based mostly on genitalic structures, is usually challenging and demands time-consuming genitalic dissections. Moreover, the identification of adults of the Chironomidae is very often further complicated by a lack of diagnostic morphological features to distinguish several species. Additionally, identification of some species can be achieved only by individual rearing of larvae and collecting larval and pupal skins to establish the associations between life stages (Silva and Wiedenbrug, 2014). In this context, DNA barcodes seem to be particularly useful for taxonomic groups that are diverse and difficult to identify using morphology (Hebert *et al.*, 2004; Krosch and Cranston, 2013; Silva *et al.*, 2013).

Partial COI gene sequences have been shown to be appropriate to delimit and identify species, as well as associate life stages in Chironomidae (Carew *et al.*, 2003; Ekrem *et al.*, 2007, 2010). In the Neotropical region, DNA barcoding has been used successfully for species delimitation and solving taxonomic conflicts in the *Corynoneura* group (Orthocladiinae), from the Atlantic Forest, Brazil (Silva and Wiedenbrug, 2014). In addition, Silva *et al.* (2013) were able to associate different life stages and

delimitate species of *Labrundinia* (Tanypodinae) using the standard barcode fragment of COI amplified with the universal primers. Taking into account a biogeographic framework, the inclusion of molecular data into rigorous analyses permits biogeographers to create statistically testable hypotheses of dispersal and range expansion within the hypothetico-deductive framework (Mantooth and Riddle, 2011). Despite this advantage, there are some drawbacks regarding the use of DNA barcodes in biogeographic analyses. Cryptic speciation, high intraspecific nucleotide diversity and/or population structuring driven by geographical or biological factors may generate differences in DNA barcodes (Carew and Hoffmann, 2015). For example, processes such as incomplete mitochondrial lineage sorting may cause DNA barcodes to become dissociated with species divergences established by morphology and/or nuclear sequences (Alexander *et al.*, 2009; Dai *et al.*, 2012).

Our results support DNA-barcoding as an excellent tool for species delimitation in groups where taxonomy by means of morphology is challenging or even impossible. Regardless of the promising results, the inclusion of multiple nuclear sequences (Carew and Hoffmann, 2015) and further collections, considering the geographic and ecological landscapes from which sequences were previously sampled, coupled with the relationships among lineages, are valuable for species delimitation and may strengthen results, making it easier to classify the uniqueness of populations and species (Mantooth and Riddle, 2011).

Conclusions

Although our results document a considerable chironomid diversity in South America, we believe that sampling in different periods (including both the rainy and dry seasons) and broadening the variety of habitats and geographic area will reveal much greater numbers of species than currently detected. While efforts to detect and describe the diversity of the chironomid fauna in South America have increased lately, knowledge of their biology and ecology, as well as their biogeography and phylogeny, remains fragmentary and incomplete. Thus, additional inventories are required to obtain robust estimates of local species richness, and discover and analyze possible areas of endemism in the Neotropical region. Moreover, future chironomid inventories should also allocate more sampling effort to collecting floating pupal exuviae; this technique has been proven to provide a simple and safe means of obtaining abundant and reliable data on diversity. Furthermore, trace DNA has been successfully extracted from pupal skins, which increases the effectiveness of pupal exuviae as a resource in biodiversity monitoring and conservation programs (Krosch and Cranston, 2012; Kranzfelder *et al.*, 2016). Finally, the most promising aspect of DNA barcoding is that it constitutes an effective union between the goals of systematists, biogeographers and ecologists. For purposes of conservation biogeography, DNA barcoding will likely have its greatest impact by

filling in some of the details of species diversity in lesser-known and morphologically enigmatic taxa, and distributions of taxa.

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