NRC Research Press

ARTICLE

DNA barcoding as an aid for species identification in austral black flies (Insecta: Diptera: Simuliidae)

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Abstract: In this paper, the utility of a partial sequence of the COI gene, the DNA barcoding region, for the identification of species of black flies in the austral region was assessed. Twenty-eight morphospecies were analyzed: eight of the genus *Austrosimulium* (four species in the subgenus *Austrosimulium* s. str., three species in the subgenus *Novaustrosimulium*, and one species unassigned to subgenus), two of the genus *Cnesia*, eight of *Gigantodax*, three of *Paracnephia*, one of *Paraustrosimulium*, and six of *Simulium* (subgenera *Morops*, *Nevermannia*, and *Pternaspatha*). The neighbour-joining tree derived from the DNA barcode sequences grouped most specimens according to species or species groups recognized by morphotaxonomic studies. Intraspecific sequence divergences within morphologically distinct species ranged from 0% to 1.8%, while higher divergences (2%–4.2%) in certain species suggested the presence of cryptic diversity. The existence of well-defined groups within *S. simile* revealed the likely inclusion of cryptic diversity. DNA barcodes also showed that specimens identified as *C. dissimilis*, *C.* nr. *pussilla*, and *C. ornata* might be conspecific, suggesting possible synonymy. DNA barcoding combined with a sound morphotaxonomic framework would provide an effective approach for the identification of black flies in the region.

Key words: DNA barcoding, black flies, Simuliidae, Australia, New Zealand, Argentina.

Résumé : Dans ce travail, les auteurs ont évalué l'utilité d'une séquence partielle du gène COI, la région du code à barres, pour l'identification des espèces au sein des mouches noires de la région australe. Vingt-huit espèces morphologiques ont été analysées dont 8 espèces au sein du genre *Austrosimulium* (4 espèces du sous-genre *Austrosimulium* s. str., 3 du sous-genre *Novaustrosimulium*, et 1 espèce non encore assignée à un sous-genre), 2 au sein du genre *Cnesia*, 8 *Gigantodax*, 3 *Paracnephia*, 1 *Paraustrosimulium* et 6 *Simulium* (sous-genres *Morops*, *Nevermannia* et *Pternaspatha*). Un arbre de type neighbour-joining a été produit à l'aide des séquences des codes à barres et a permis de grouper la plupart des spécimens en fonction de leur espèce ou groupe d'espèces définies sur la base d'études morpho-taxonomiques. Les divergences intraspécifiques au sein d'espèces morphologiquement distinctes variaient entre 0 et 1,8 %, tandis que des divergences plus importantes au sein de certaines espèces (2–4,2 %) suggèrent la présence de diversité cryptique. L'existence de groupes bien définis au sein du *S. simile* suggère également la présence de diversité cryptique. Les codes à barres de l'ADN ont également révélé que des spécimens d'espèces identifiées comme étant *C. dissimilis*, *C.* nr. *pusilla* et *C. ornata* pourraient s'avérer conspécifiques et possiblement synonymes. Le codage à barres de l'ADN, combiné à un cadre morpho-taxonomique solide, fournirait une approche efficace pour identifier les mouches noires au sein de la région. [Traduit par la Rédaction]

Mots-clés : codage à barres de l'ADN, mouches noires, Simuliidae, Australie, Nouvelle-Zélande, Argentine.

Introduction

Black flies (Diptera: Simuliidae) comprise 26 genera and an estimated 2189 species (2177 living and 12 fossil) (Adler and Crosskey 2015*a*). In most species, the female requires a blood meal for egg maturation, and it is this requirement that makes members of this family important biting pests and vectors for the transmission of parasites of the blood and skin of humans and other warm-blooded

Received 20 November 2015. Accepted 4 November 2016.

Corresponding Editor: Ian Hogg.

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animals (Hernández-Triana 2011; Hernández-Triana et al. 2012, 2015; Shelley et al. 2010). The most important simuliid-transmitted parasites of humans are the nematodes *Onchocerca volvulus* (Leuckart), the cause of onchocerciasis or "river blindness", and *Mansonella ozzardii* Manson, which causes mansonelliasis or "serous cavity filariasis", primarily in Latin America (Shelley et al. 2010). Recently, it has been hypothesized that certain species of black flies in onchocerciasis endemic areas may also transmit a neurotropic virus that may be an endosymbiont of the microfilariae that causes nodding syndrome and epilepsy without nodding (Colebunders et al. 2014).

Simuliids are also of concern because they transmit protozoans such as *Leucocytozoon* to both domestic and wild birds and can cause mortality, loss of weight gain, reduced milk production, malnutrition, and impotence in cattle, pigs, and sheep (Adler et al. 2004; Currie and Adler 2008). In Latin America, some species of Simuliidae are thought to be responsible for outbreaks of endemic pemphigus foliaceus in Brazil (Eaton et al. 1998) as well as the etiological agent of Altamira haemorrhagic syndrome (Pinheiro et al. 1986). In addition to their medical importance, black flies are environmentally important because of their role as "keystone" organisms in the ecology of freshwater ecosystems. Simuliid larvae consume dissolved organic matter in the water, making it subsequently available to the food chain (Currie and Adler 2008; Malmqvist et al. 2001, 2004), and they are also an important food source for fishes and invertebrates (Currie and Adler 2008). In addition, black flies are important as indicators of freshwater contamination and stream degradation, because their immature stages are susceptible to both organic and inorganic pollution (e.g., Feld et al. 2002; Pramual and Kuvangkadilok 2009). Because of their medical, veterinary, and environmental importance, black flies are one of the groups targeted for the development of a DNA barcode reference library based upon specimens identified through morphology to support species identification (Barcode of Life Data, Ratnasingham and Hebert 2007).

There has been little research on Simuliidae from the southern hemisphere in recent years, except for the review of Craig et al. (2012) on the New Zealand fauna and the cladistic analysis of Gil-Azevedo and Maia-Herzog (2007). In Argentina, Simuliidae are well characterized mainly because of the efforts of Coscarón (1987, 1991), Coscarón and Coscarón-Arias (2007), Coscarón and Wygodzinsky (1972), Coscarón-Arias (1989, 1998, 2002), and Wygodzinsky and Coscarón (1973, 1989) (reviewed in Hernández et al. 2009), while the monographs of Dumbleton (1963, 1972), Mackerras and Mackerras (1948), and Tonnoir (1925) on the Australian Simuliidae fauna and the genus *Austrosimulium* Tonnoir are still pivotal in our understanding of the zoogeographical relationships of southwestern Pacific simuliid fauna. Molecular investigation of Simuliidae taxonomy in the austral region has been sporadic, although Moulton (1997, 2000, 2003) explored relationships within the family, and further information has been provided by Adler et al. (2004). In 1994, Ballard showed that evidence from the 12S ribosomal RNA gene could resolve relationships in *Austrosimulium*; more recently, Craig and Cywinska (2012) investigated the relationships of New Zealand *Austrosimulium* species using DNA sequences from three regions of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene in combination with morphological characters.

In the present study, we aimed to develop a COI DNA barcoding library for the poorly studied black fly fauna of the austral region (Argentina, Patagonia, Australia, and New Zealand) as an aid for species identification. In addition, we assessed the barcode variability within and between morphospecies to reveal hidden diversity in the species we analyzed.

Material and methods

Collection of specimens

Standardized collection protocols implemented at the Natural History Museum were used in this study (Hernández 2007; Hernández-Triana 2011; Hernández-Triana et al. 2012, 2014). Larvae, pupae, and link-reared adults were collected in rivers and streams across the black fly species distribution range in Nahuel Huapi National Park (see Brooks et al. 2009; Hernández et al. 2009). Material from Australia and New Zealand was collected in a similar way by Douglas Craig and Shelley McMurtrie, especially at or near the type locality for each species. Efforts were also made to collect females of species known to bite humans (see species list in Table 1).

Specimens were preserved in 95% ethanol and were held at –5 °C until molecular analysis was begun. The alcohol was changed once before storing the vials at –5 °C. Dried pinned specimens (human-biting females or link-reared adults) were kept at room temperature in insect drawers without naphthalene.

DNA extraction, PCR, and sequencing

Larvae of species collected for molecular analyses did not have their digestive tract disturbed to reduce the possibility of contamination (Hernández-Triana et al. 2012; Rivera and Currie 2009). Larval specimens had a long strip of the posterior abdominal wall removed as a source for DNA extraction; the remainder of the body was retained as a voucher following the protocols of the Canadian Centre for DNA Barcoding (CCDB; http://www.dnabarcoding.ca). When pupae were selected for analysis, most of the thorax, gill, and cocoon were retained as a voucher, while the pupal abdomen and the region around the legs were used for DNA extraction. In the case of adults preserved in alcohol or pinned, two to three legs were removed from the specimen for DNA extraction, while the remainder of the specimen was retained as a voucher. In the case of pinned material, a yellow label stating "legs removed for DNA barcoding" was attached to the pin as recommended by Golding et al. (2009).

Table 1. List of black fly species, country of collection, and number of specimens with DNA barcodes.

Species	Collection country	*n*	Mean (%)
Austrosimulium (*Austrosimulium* s. str.) *australense*	New Zealand	125	0.3
Austrosimulium (*Austrosimulium* s. str.) *montanum*	Australia	14	0
Austrosimulium (*Austrosimulium* s. str.) *campbellense*	New Zealand	5	0
Austrosimulium (*Austrosimulium* s. str.) *cornutum*	Australia	7	0
Austrosimulium (*Novaustrosimulium*) *furiosum*	Australia	22	0.6
Austrosimulium (*Novaustrosimulium*) *torrentium*	Australia	2	—
Austrosimulium (*Novaustrosimulium*) *victoriae*	Australia	3	1.1
Austrosimulium colboi	Australia	3	1.1
Cnesia dissimilis	Argentina	58	See Table 3
Cnesia ornata	Argentina	2	0
Gigantodax antarcticus	Argentina	6	0
Gigantodax chilensis	Argentina	5	0
Gigantodax dryadicaudicis	Argentina	1	—
Gigantodax femineus	Argentina	10	See Table 3
Gigantodax igniculus	Argentina	8	0.9
Gigantodax marginalis	Argentina	9	0
Gigantodax rufescens	Argentina	8	0
Gigantodax shannoni	Argentina	12	0
Paracnephia aurantiaca	Australia	4	0
Paracnephia fergusoni	Australia	4	See Table 3
Paracnephia orientalis	Australia	2	—
Paraustrosimulium anthracinum	Argentina	19	0.8
Simulium (*Pternaspatha*) nr. *albilineatum*	Argentina	19	0.7
Simulium (*Pternaspatha*) *nemorale*	Argentina	48	1.2
Simulium (*Pternaspatha*) *simile***	Argentina	25	See Table 3
Simulium (*Morops*) *melatum*	Australia	1	—
Simulium (*Morops*) *torresianum*	Australia	1	—
Simulium (*Nevermannia*) *ornatipes* s.l.*	Australia	3	0

Note: Mean (%) intraspecific values of sequence divergence (Kimura 2-parameter) are shown, with missing entries indicating that fewer than two specimens were analyzed. Species complexes (*) and taxa with deep splits (**) in the neighbour-joining tree are marked with asterisks.

Forceps used for dissection were flame-sterilized between specimens to avoid transfer of DNA (Hernández-Triana et al. 2012, 2014; Rivera and Currie 2009).

The tissue sample from each specimen was deposited into one of the wells of a 96-well plate for cell lysis and subsequent DNA extraction. A digital image of each specimen was taken at the Biodiversity Institute of Ontario using a Leica compound microscope equipped with a Z-stepper and digital camera. Detailed specimen records, sequence information (including trace files), and digital images were uploaded to the Barcode of Life Database (BOLD; http://www.boldsystems.org) and can be found within the Working Group 1.4 Initiative "Human Pathogens and Zoonoses". The Digital Object Identifier for the project is dx.doi.org/10.5883/DS-AUSIM. All sequences have also been submitted to GenBank (accession numbers KU566570 to KU566745). Individual records can be found in the following projects in BOLD: "[VTKSM] Vectors Blackflies-Australia and New Zealand_2012"; "[NPSIM] Blackflies of Nahuel Huapi National Park, northern Patagonia, Argentina (Diptera, Simuliidae)_2009"; and "[NHSIM] Blackflies of Nahuel Huapi National Park, northern Patagonia, Argentina (Diptera, Simuliidae)_2012". Sequences of *Austrosimulium australense* (BOLD project "[ACBZ] New Zealand *Austrosimulium*") were included in the current study because this species is the type species of the subgenus *Austrosimulium* s. str. (see Craig and Cywinska 2012; Adler and Crosskey 2015).

DNA extraction, PCR amplification, and sequencing were performed according to CCDB protocols (www.ccdb.ca; www.dnabarcoding.ca). In brief, extractions were automated using a 96-channel Biomek NX robotic liquid handler (Beckman Coulter Inc., Mississauga, Ont., Canada) with a Thermo Scientific Cytomat hotel. PCR primers were those developed by Folmer et al. (1994) (LCO1490, HCO2198), which are considered standard to amplify the 658-bp target region of the COI gene (Hebert et al. 2003*a*, 2003*b*). Samples that did not yield PCR product with the Folmer primers were re-amplified using primers that amplify two short overlapping fragments of the COI DNA barcode region: LepF1 (5′-ATTCAACCA ATCATAAAGATATTGG-3′) with MLepR1 (5′-GTTCAWCCW GTWCCWGCYCCATTTTC-3′) and MLepF1 (5′-GCTTTCCCA CGAATAAATAATA-3′) with LepR1 (5′-TAAACTTCTGGATG TCCAAAAAATCA-3′) (Hajibabaei et al. 2006; Hebert et al. 2013). Both forward and reverse strands were sequenced using BigDye Terminator (version 3.1) and an ABI PRISM 3730XL capillary sequencer (Applied Biosystems). All DNA extraction, PCR amplification, and sequencing protocols are available at www.ccdb.ca.

Sequence analysis

Paired bidirectional sequence traces were combined to produce a single consensus sequence (e.g., the full-length 658-bp barcode sequence). Individual forward and reverse traces were oriented, edited, and aligned using the Sequencer (v.4.5; Genes Codes Corporation, Ann Arbor, Mich., USA), GenDoc (v.2.6.02), and ClustalX sequence analysis programs.

The full data set was also analyzed in MEGA v.6 (Tamura et al. 2013). A neighbour-joining (NJ) tree analysis was carried out using the Kimura 2-parameter distance metric to represent the clustering pattern; bootstrap values were calculated to test the robustness of the tree and were obtained by conducting 1000 pseudoreplicates. NJ trees were exported as JPG files in Adobe Acrobat 8 Professional and then edited using Adobe Photoshop CS3 (v.10.0.1). Only groups with more than 70% bootstrap support are shown in the partially collapsed NJ tree (see Fig. 1) (Hernández-Triana et al. 2012, 2014, 2015). A detailed NJ tree showing all individuals is provided in the supplementary information (Fig. S1[1]).

After their upload to BOLD, most barcode sequences larger than 500 bp were assigned a Barcode Index Number (BIN), an interim taxonomic system that segregates similar barcode sequences into a BIN (Ratnasingham and Hebert 2013). An NJ tree was generated in BOLD and all BINs for each morphospecies were mapped (Fig. 1, Fig. S1[1]). We analyzed the taxonomic discordance in our data set using BOLD capabilities, which provide a means of confirming the concordance between barcode sequence clusters and species designations. The BOLD system performs this validation by comparing the taxonomy on input records against all others in the same BINs, including those submitted and managed by other users.

Results

A total of 28 morphospecies of *Austrosimulium* (eight species) (subgenera *Austrosimulium* s. str. and *Novaustrosimulium*), *Cnesia* (two species), *Gigantodax* (eight species), *Paracnephia* (three species), *Paraustrosimulium* (one species), and *Simulium* (six species) (subgenera *Pternaspatha*, *Morops*, and *Nevermannia*) (see Adler and Crosskey 2015; Craig et al. 2012) were included in the analysis (Tables 1, 2). Three or more representative specimens were available for 12 morphospecies (Table 1). In total, we analyzed 415 individuals, of which 22 yielded a barcode sequence length between 280 and 514 bp. The remaining 393 specimens yielded sequences longer than 554 bp.

In most cases, individuals of the same morphospecies grouped together even when samples were geographically distant (see Fig. 1). For example, specimens identified as *Cnesia dissimilis* or *Cnesia* nr. *pussilla* clustered together, along with *Cnesia ornata*, with high bootstrap support. This is not surprising, as these species are morphologically similar (Coscarón 1991; Coscarón-Arias 1989, 1998; Wygodzinsky and Coscarón 1973). *Cnesia pussilla* was described by Wygodzinsky and Coscarón (1973) from two reared females, two reared males, and an undisclosed number of pupae and larvae from Rio Negro and Neuquén provinces. The authors stated that *C. pussilla* may be difficult to separate from *C. dissimilis* because it differs only by its smaller size at all life stages, the black scutum of the female, and the "comparatively" small membranous area at the insertion of the spermathecal duct. *Cnesia ornata* might be separated by the black scutum of the male and the absence of platelets in the pupa. In 1991, Coscarón reviewed the three species and provided a key to separate the female, male, and pupal stage based on the aforementioned characters. The first and second authors of the present paper visited the type locality of *C. pussilla* (3 km from Bariloche airport, Argentina) and collected numerous specimens identified as *C. dissimilis* across Nahuel Huapi National Park, Patagonia; we also collected specimens of *C. ornata* in the same localities. The coloration of the female and male of *C. dissimilis* varies from pale brown to dark brown and is often black, which falls within the variation found in adults of *C. pussilla* and *C. ornata*. Although the species cannot be separated based on pupal gill configuration, *C. ornata* have tubercles on the thorax (Coscarón 1991). In the present study, black topotype males identified as *C. pussilla* grouped together with *C. dissimilis* with bootstrap values of 100% (Fig. S1[1]), and with *C. ornata* with values of 99%, and all sequences had the same BIN number. This suggests that *C. pussilla* and *C. ornata* might be junior synonyms of *C. dissimilis*. Specimens of *A. australense* seemed to form two separate clusters in the NJ tree (Fig. S1[1]). They showed a low genetic divergence (0.3%, Table 1) and while this finding was not well supported by bootstrap values (Fig. S1[1]), it is in agreement with a suggestion of two cryptic species by Craig et al. (2012) for this species.

Levels of sequence divergence were variable across taxa. Thus, while conspecific individuals collected from a single site often exhibited zero or very low divergence, other specimens exhibited higher divergence (e.g., *Gigantodax femineus*) (Table 1). Intraspecific divergence averaged 1.39% (range 0%–1.8%) (Table 1), while interspecific divergence averaged 17.5% (range 1.72%–30%) (Table 2). Genetic divergence values were higher between species from different genera or subgenera as recognized by Adler and Crosskey (2015). The most divergent pairs were *Simulium* (*Pternaspatha*) *nemorale* and *Gigantodax igniculus*, *Austrosimulium* (*Novaustrosimulium*) *victoriae* and *Paracnephia aurantiaca*, and *S. nemorale* and *Simulium* (*Morops*) *torresianum* (30%). As expected, smaller divergence values were

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2015-0168.

Fig. 1. Partially collapsed neighbour-joining tree of COI DNA barcodes (658 bp) for species of austral Simuliidae. A divergence of >2% is indicative of separate operational taxonomic units. Bootstrap values >70% are shown at each node.

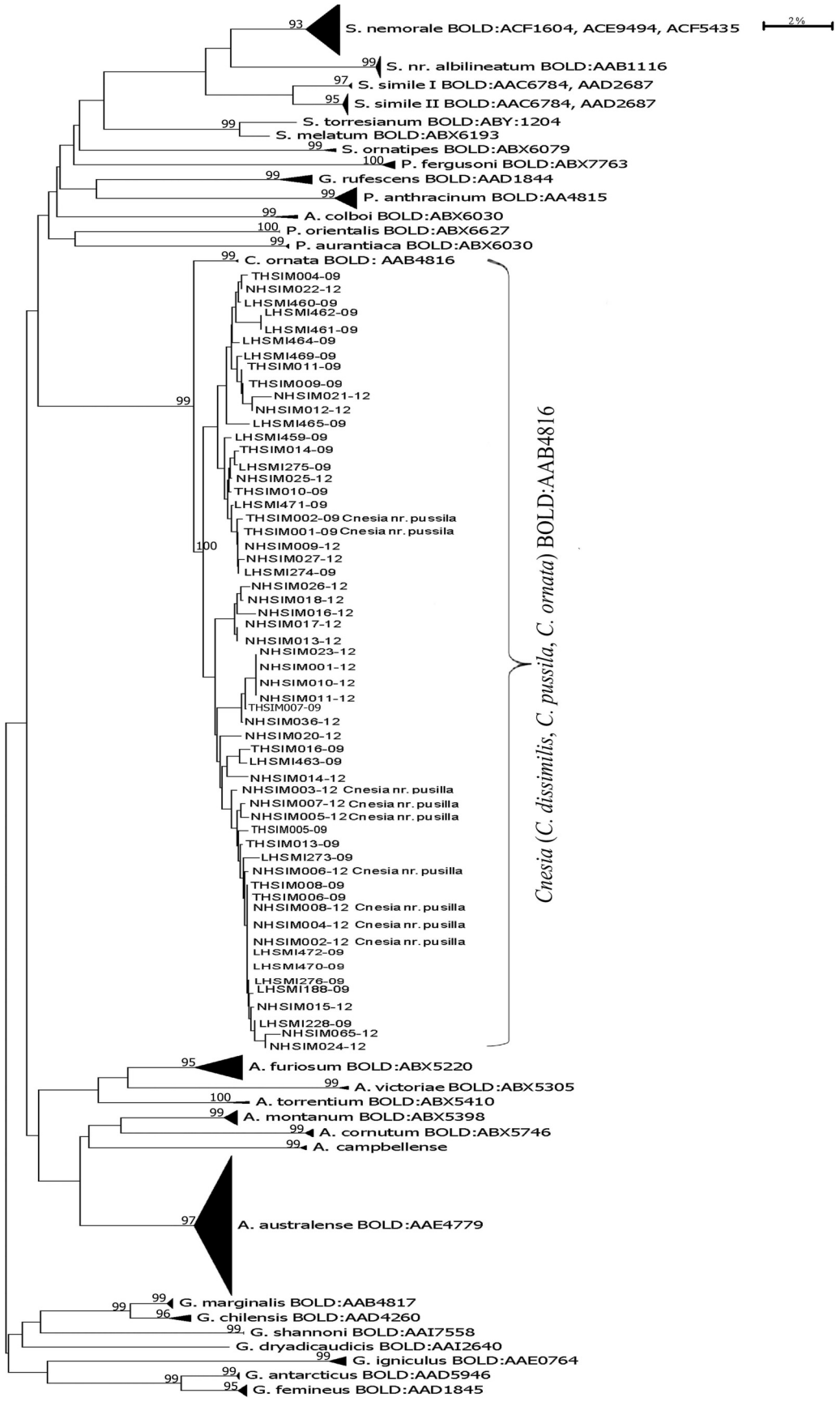

Table 2. Interspecific (between group) pairwise Kimura 2-parameter genetic divergence of unique DNA barcodes (658 bp) representing 28 species in six genera of Simuliidae.

	torres.	aur.	fur.	torren.	fem.	ori.	vic.	mel.	cor.	mon.	col.	orn.	fer.	cam.	ign.	ant.	ruf.	dis.	nem.	mar.	sim.	nr. alb	ant.	chi.	orn.	sha.	dr.
S. torresianum																											
P. aurantiaca	0.22																										
A. furiosum	0.22	0.24																									
A. torrentium	0.19	0.24	**0.07**																								
G. femineus	0.26	0.17	0.14	0.18																							
P. orientalis	0.20	0.13	0.24	0.20	0.18																						
A. victoriae	**0.27**	**0.30**	**0.08**	0.11	0.18	0.26																					
S. melatum	**0.02**	0.20	0.20	0.17	0.24	0.17	0.25																				
A. cornutum	0.24	0.15	0.15	0.13	0.19	0.16	0.16	0.22																			
A. montanum	0.19	0.17	0.11	**0.09**	0.16	0.15	0.12	0.17	**0.05**																		
A. colboi	0.15	0.24	0.15	0.21	0.16	0.17	0.19	0.17	0.24	0.17																	
S. ornatipes	0.13	0.15	0.18	0.19	0.24	0.17	0.23	0.11	0.15	0.15	0.20																
P. fergusoni	0.23	0.26	0.28	0.23	0.31	0.19	0.27	0.25	0.22	0.21	**0.30**	0.27															
A. campbellense	0.24	0.15	0.19	0.15	0.14	0.17	0.21	0.22	0.11	**0.09**	0.19	0.19	0.29														
G. igniculus	0.18	0.19	0.21	0.19	0.15	0.21	0.22	0.21	0.14	0.14	0.21	0.25	0.28	0.16													
G. antarcticus	0.24	0.23	0.15	0.19	**0.06**	0.22	0.18	0.26	0.22	0.17	0.13	0.27	0.29	0.19	0.16												
G. rufescens	**0.27**	0.15	0.17	0.15	0.14	0.13	0.19	0.24	0.11	**0.09**	0.24	0.17	0.24	0.13	0.12	0.15											
C. dissimilis	0.18	0.21	0.12	0.11	0.17	0.17	0.15	0.16	0.19	0.15	0.13	0.14	0.28	0.19	0.21	0.18	0.17										
S. nemorale	0.26	0.21	0.18	0.22	0.17	0.21	0.25	0.23	0.26	0.23	0.22	0.16	0.27	0.21	**0.30**	0.19	0.16	0.17									
G. marginalis	0.21	0.17	0.15	0.15	0.16	0.17	0.17	0.19	0.18	0.20	0.19	0.15	0.24	0.20	0.21	0.17	0.13	0.14	0.19								
S. simile	**0.30**	0.25	0.20	0.23	0.19	**0.28**	0.27	0.28	0.23	0.18	0.23	0.20	0.29	0.20	0.25	0.20	0.18	0.17	0.11	0.22							
S. nr. *albilineatum*	0.20	0.13	0.20	0.19	0.14	0.14	0.24	0.18	0.21	0.18	0.20	0.14	0.19	0.19	0.24	0.15	0.12	0.13	**0.07**	0.13	0.14						
P. anthracinum	0.20	0.11	0.20	0.18	0.11	0.10	0.21	0.18	0.12	0.11	0.18	0.18	0.24	0.11	0.17	0.13	**0.08**	0.15	0.15	0.18	0.21	**0.08**					
G. chilensis	0.18	0.22	0.16	0.18	0.20	0.22	0.18	0.16	0.23	0.25	0.22	0.16	0.25	0.24	0.25	0.21	0.17	0.18	0.19	**0.04**	0.25	0.15	0.22				
C. ornata	0.19	0.22	0.13	0.13	0.18	0.18	0.16	0.17	0.19	0.15	0.15	0.15	0.29	0.21	0.18	0.19	0.13	**0.04**	0.19	0.11	0.18	0.14	0.16	0.15			
G. shannoni	0.21	0.11	0.21	0.26	0.16	0.22	0.25	0.24	0.19	0.24	0.17	0.20	0.31	0.17	0.21	0.17	0.26	0.20	0.26	0.15	0.28	0.20	0.17	0.19	0.21		
G. dryadicaudicis	0.20	0.15	0.22	0.22	0.24	0.17	0.25	0.22	0.11	0.15	0.22	0.13	0.25	0.21	0.14	0.22	0.13	0.22	0.20	0.24	0.26	0.18	0.13	0.24	0.24	0.22	
A. australense	0.15	0.13	0.15	0.13	0.17	0.11	0.17	0.13	0.11	0.07	0.13	0.11	0.26	0.11	0.14	0.19	**0.09**	0.11	0.19	0.15	0.21	0.14	0.10	0.20	0.11	0.19	0.13

Note: Highest pairwise distances (most divergent taxa) are highlighted in bold and underlined. Lowest pairwise distances are highlighted in bold. Full species name are found in Table 1.

Table 3. Level of genetic divergence in suspected species complexes and number of individuals per species.

Species complex status	Country	*n*	Percent of divergence (max.)
With level of genetic divergence near or above 2%			
Cnesia dissimilis	Argentina	58	2.7
Gigantodax femineus	Argentina	10	4.2
Paracnephia fergusoni	Australia	4	1.8
With deep splits in the NJ tree (>70% bootstrap values)			
Simulium simile	Argentina	25	1.3

Note: NJ, neighbour-joining.

found among species within the same genus or subgenus, for example *Gigantodax chilensis* and *G. marginalis* (0.4%) and *Austrosimulium* (*Austrosimulium*) *montanum* and *A. cornutum* (0.5%) (Table 2).

In this study we analyzed only *Simulium* (*Nevermannia*) *ornatipes* s.l. as a known (or suspected) species complex, but the three specimens we studied originated from the same locality. Therefore, we detected no genetic diversity. However, not all morphospecies clustered as expected. Certain species exhibited higher levels of divergence, at or above 2% (see Table 3 and Fig. 1, Fig. S1[1]). Intraspecific genetic divergence averaged 2.7% for *C. dissimilis*, 4.2% for *G. femineus*, and 1.8% for *Paracnephia fergusoni*. Surprisingly, *Simulium* (*Pternaspatha*) *simile* showed a deep split in the NJ tree with two distinct groups, I and II (Fig. 1, Fig. S1[1]), with a divergence of 1.3% and more than 95% bootstrap support. Interspecific divergence between species with similar deep splits in the NJ tree ranged from 1.7% to 29% (Table 2). Lower values of divergence were found between *C. dissimilis* and *S. simile* (1.7%), while other species from different genera and (or) subgenera had higher values, for example *P. fergusoni* and *C. dissimilis* (28%) and *P. fergusoni* and *S. simile* (29%).

The BIN count in our data set of 474 barcode records was higher than the species count (28 species). In general, 402 barcodes were assigned BIN numbers representing 29 BINs; 9 BINS were discordant (287 records), 16 BINs were taxonomically concordant (111 records), and 4 BINS were singletons. Most of the discordant BINs occurred at the species level, mainly because the taxonomic species list within BOLD accounts for different species, for example *S. nemorale* and *S.* nr. *nemorale*. In one case, BIN AAB4815 (*Paraustrosimulium anthracinum*), there was a discrepancy for one specimen at the genus level (*Simulium minusculum*, Process ID: SIM-CANADA-391). A closer look at this record revealed that the sequence identified as *S. minusculum* might be a contamination within BOLD. BIN splits were detected in *S. simile* (two BINs) and *S. nemorale* (three BINs) (Fig. 1, Fig. S1[1]). BIN merges were uncommon and occurred only in *Cnesia* (BIN AAB4816, identified as *C. dissimilis*, *C. ornata*, and *C. pussilla*) (Fig. 1, Fig. S1[1]).

Discussion

Hernández-Triana (2011) and Hernández-Triana et al. (2012, 2014, 2015) have discussed the use of COI DNA barcoding in Simuliidae and also reviewed the controversies that this approach has generated in recent years.

In this paper, nearly all well-established morphospecies formed well-defined groups using NJ analysis based on DNA barcodes (Fig. 1), supporting the value of this approach as a tool for species identification. Genetic divergence between morphospecies averaged 17. 5% (range 1.72%–30%), whereas intraspecific genetic divergence within morphologically distinct species averaged 1.39% (range 0%–1.8%) (Table 1). Most of the specimens within a morphospecies were resolved in the NJ tree, although individuals identified as *Cnesia dissimilis*, *C.* nr. *pussilla*, and *C. ornata* clustered together, indicating that they might be conspecific. These taxa are difficult to separate, and variation in the colour of the female and in male thoracic morphology occurs along their distribution range. Therefore, it is proposed that other molecular markers such as the internal transcribed spacer and other genes such as the fast-evolving ECP1 gene (Senatore et al. 2014), in combination with further cytotaxonomic study, should be used to challenge their specific status.

Craig and Cywinska (2012) produced a detailed revision of the genus *Austrosimulium* in New Zealand, in which they proposed a phylogeny based on morphological traits and explored the relationships using molecular data. They found a lack of resolution within the *tillardianum* species group, but in general there was a strong concordance in their tree topology based on morphology and COI gene, and they concluded that the mitochondrial COI gene was also of phylogenetic value. In our study, specimens of *A. australense* appeared to form two groups (Fig. S1[1]), but these groups were not supported by bootstrap values in the NJ tree. The lack of support in our data set for this species is an indication that further research is needed on the use of COI barcoding in this species group, perhaps using more informative markers. The presence of well-supported subgroups in certain species, such as *C. dissimilis*, *G. femineus*, *S. simile*, and *P. fergusoni*, would suggest the presence of cryptic diversity (Fig. 1, Fig. S1[1]; Tables 2, 3). Divergence values in these cases of potential cryptic species are within the range for closely related species of Neotropical and Nearctic black flies (e.g., Rivera and Currie 2009; Hernández-Triana 2011; Hernández-Triana et al. 2012) and Nearctic mosquitoes (Cywinska et al. 2006). The taxonomy of Australian black fly species is in need of revision, and there is ongoing controversy with regard to the classification of "Australian *Cnephia*" for some authors and *Paracnephia* for others (Adler and Crosskey 2015; Craig et al. 2012). Nonetheless, all specimens of the three species of *Paracnephia* we identified (Table 1) grouped together with 99% to 100% bootstrap values in the NJ COI tree, which supports the current species identifications.

Although sibling species were not cytotyped in this study, it would be expected that genetic variation between random individuals from sibling species complexes would be higher, on average, than that between individuals from morphospecies that are not known to be sibling species complexes (Hernández-Triana et al. 2012, 2014, 2015; Rivera and Currie 2009). If correct, this pattern would be revealed in the NJ tree by relatively deeply divergent groups within species complexes. In general, certain species showed high intraspecific genetic divergences, such as *C. dissimilis*, *G. femineus*, *P. fergusoni*, and *S. simile*, which might indicate the presence of hidden diversity, although further work is needed to confirm this. Hernández-Triana et al. (2012, 2014, 2015) have discussed DNA barcoding data for many medically important species of Simuliidae. The variation in intra- and interspecific genetic values found in the present paper falls within the ranges in the aforementioned papers.

Because of the strong correspondence between BINs and traditionally recognized species (e.g., Ratnasingham and Hebert 2013), the splits found in *S. simile* and *S. nemorale* may represent the presence of hidden diversity, although other explanations might be possible. The detection of a BIN merge for specimens morphologically identified as separate species (*C. dissimilis*, *C. pussilla*, and *C. ornata*) confirms that these taxa might be synonyms. Nonetheless, a revision of the genus *Cnesia* in combination with other molecular markers is needed to test this hypothesis.

The present study provides COI data to support species identification in the large and understudied fauna of Argentinian populations of the genera *Cnesia* (three species), *Gigantodax* (eight species), and *Paraustrosimulium* (one species) and anthropophilic species of *Simulium* (three species). It also augments data for species diversity of *Austrosimulium* (six species), *Paracnephia* (three species), and *Simulium* (three species) in Australia and the population of *Austrosimulium campbellense* in New Zealand (Campbell Islands, Honey Falls, and Tucker Stream). Even though the volume of DNA barcode data in BOLD and GenBank is increasing rapidly, much work is still required to populate these databases with respect to the global simuliid fauna. Ongoing research is augmenting BOLD by targeting adults reared from a single pupa (link-reared adults) upon which morphological identification can reliably be achieved in most species (see also Hernández-Triana et al. 2014; Shelley et al. 2010). As a result, it is envisaged that the barcoding library can be used to aid the identification of immature larvae collected during biodiversity inventories of aquatic ecosystems (e.g., Pramual and Wongpakam 2014) or for the identification of biting females in closely related species of medical importance, for example the Amazonicum group in Brazil (Shelley et al. 2010). With regard to the species complexes, little is known about the DNA barcode profile of each of the main vector complexes in combination with their chromosomal banding pattern across their distribution range (Hernández-Triana 2011; Hernández-Triana et al. 2012, 2015). This highlights the continuing need for research using an integrated taxonomic approach on the Simuliidae on a worldwide basis.

In this study, the COI DNA barcoding region correctly distinguished nearly all morphologically distinct species we examined from Patagonia, Australia, and New Zealand, demonstrating its value for species identification, which agrees with other findings in Europe (e.g., Day et al. 2008, 2010; Kúdela et al. 2014; Ilmonen et al. 2009) and the Oriental region (e.g., Pramual and Adler 2014; Pramual and Kuvangkadilok 2009; Pramual et al. 2011). It has also been demonstrated that the COI barcoding region is a useful tool for revealing levels of genetic diversity in poorly known taxa, for example *C. dissimilis*, *G. femineus*, *P. fergusoni*, and *S. simile*. However, it is uncertain whether this level of genetic divergence is indicative of the presence of species complexes. Very few studies employing molecular and cytogenetic methods have been published on austral Simuliidae (see Adler and Crosskey 2015*b*). Therefore, we advocate for further integrated research on known pest species or taxonomically problematic taxa as endorsed by Adler et al. (2004), Adler and Crosskey (2015*b*), Craig et al. (2012), Low et al. (2016), and Shelley et al. (2010). Integrated research on pest species or taxonomically problematic taxa would have a direct impact on ecological and control strategies and (or) studies on disease transmission by supporting correct species identification.

Acknowledgements

This research was enabled by grants from Genome Canada through the Ontario Genomics Institute, the Canada Foundation for Innovation, the Ontario Ministry of Research and Innovation, the Natural Sciences and Engineering Research Council of Canada, and the Gordon and Betty Moore Foundation. LMH-T would like to thank the Darwin Initiative, United Kingdom, for providing funds to carry out fieldwork in Nahuel Huapi National Park, Patagonia, Argentina (project 15/025). Special thanks is also given to Susana Seijas, Maria Sage, and the staff at the headquarters of Nahuel Huapi National Park, Bariloche, for their logistical support. Thanks are given to the curators of Diptera (Zoe Adams, Erica McAlister, and Theresa Howard) at the Natural History Museum, London, UK, for providing space and facilities in aid of the study. We thank Doug Craig (Professor Emeritus, University of Alberta, Canada) for providing material from Australia and New Zealand, and Nick Johnson (Animal and Plant Health Agency, UK) for critical review of the manuscript. SMcM would like to thank the 50 Degrees South Trust for their support of the Campbell Island Bicentennial Expedition (www.campbellisland.org.nz), during which time she collected the *A. campbellense* specimens.

References

Adler, P.H., and Crosskey, R.W. 2015*a*. World blackflies Diptera: Simuliidae: a fully revised edition of the taxonomic and geographical inventory. Available from http://entweb.clemson.edu/biomia/pdfs/blackflyinventory.pdf [accessed 27 April 2015].

Adler, P.H., and Crosskey, R.W. 2015*b*. Cytotaxonomy of the Simuliidae (Diptera): a systematic and bibliographic conspectus. Zootaxa, **3975**: 1–111. doi:10.11646/zootaxa.3975.1.1. PMID: 26249931.

Adler, P.H., Currie, D.C., and Wood, D.M. 2004. The Black Flies Simuliidae of North America. Cornell University Press, Ithaca, New York.

Ballard, J.W.O. 1994. Evidence from 12S ribosomal RNA sequences resolves a morphological conundrum in *Austrosimulium* (Diptera: Simuliidae). Aust. J. Entomol. Soc. **33**: 131–135. doi:10.1111/j.1440-6055.1994.tb00938.x.

Brooks, S.J., Hernández, L.M., Massaferro, J., Spinelli, G.R., and Penn, M. 2009. Capacity building for freshwater insect studies in northern Patagonia, Argentina. Rev. Soc. Entomol. Argent. **68**: 145–154.

Colebunders, R., Hendy, A., Nanyunja, M., Wamala, J.F., and van Oijen, M. 2014. Nodding syndrome — a new hypothesis and new direction for research. Int. J. Infect. Dis. **27**: 74–77. doi:10.1016/j.ijid.2014.08.001.

Coscarón, S. 1987. El género *Simulium* Latreille en la Región Neotropical: Análisis de los Grupos Supraspecíficos, Especies que los Integran y Distribución Geográfica (Simuliidae, Diptera). Mus. Paraense Emílio Goeldi, 112 pp.

Coscarón, S. 1991. Fauna de Agua Dulce de la República Argentina. 38. Insecta, Diptera. 2. Simuliidae. Fundación para la Educación, la Ciencia y la Cultura, Buenos Aires.

Coscarón, S., and Coscarón-Arias, C.L. 2007. Aquatic biodiversity in Latin America. Vol. 3. *In* Neotropical Simuliidae (Diptera: Insecta). *Edited by* J. Adis, J.G. Arias, J.G. Rueda-Delgado, and R.W. Wantzen, Pensoft. Moscow, Sofia.

Coscarón, S., and Wygodzinsky, P. 1972. Notas sobre simúlidos neotropicales. Sobre tres especies de jejenes con hembras de color claro del S. E. del Paraguay y del N. E. de Argentina Simuliidae-Diptera. Rev. Mus. La Plata N.S. Sec. Zool. **11**: 203–231.

Coscarón-Arias, C.L. 1989. Estudios citotaxonómicos y bioecológicos de Simuliidae (Diptera, Insecta) de Argentina. Tésis doctoral, Facultad de Ciencias Naturales y Museo, Universidad Nacional de la Plata.

Coscarón-Arias, C.L. 1998. The polytene chromosomes of *Cnesia dissimilis* (Edwards) and three species of *Gigantodax* Enderlein (Diptera: Simuliidae) from Lanin National Park Argentina. Mem. Inst. Oswaldo Cruz, **93**: 445–458. doi:10.1590/S0074-02761998000400006.

Coscarón-Arias, C.L. 2002. Los simúlidos de Patagonia Simuliidae, Diptera, Insecta. *In* Actualizaciones en Antropología Sanitaria Argentina. Serie de Enfermedades Transmisibles, Publicación Monográfica 2, O.D. Salomón Comp., Fundación Mundo Sano, Buenos Aires.

Craig, D.A., and Cywinska, A. 2012. Molecular analysis of New Zealand *Austrosimulium* Diptera: Simuliidae species. *In* Fauna of New Zealand. Simuliidae Insecta: Diptera. Vol. 68. *Edited by* D.A. Craig, R.E.G. Craig, and T.K. Crosby. 336 pp.

Craig, D.A., Craig, R.E.G., and Crosby, T.K. 2012. Simuliidae Insecta: Diptera. *In* Fauna of New Zealand. Vol. 68. 336 pp.

Currie, D.C., and Adler, P.H. 2008. Global diversity of black flies (Diptera: Simuliidae) in freshwater. Hydrobiologia, **595**: 469–475. doi:10.1007/s10750-007-9114-1.

Cywinska, A., Hunter, F.F., and Hebert, P.D.N. 2006. Identifying Canadian mosquito species through through DNA barcodes. Med. Vet. Entomol. **20**: 413–424. doi:10.1111/j.1365-2915.2006.00653.x. PMID:17199753.

Day, J.C., Goodall, T.I., and Post, R.J. 2008. Confirmation of the species status of the blackfly *Simulium galeratum* in Britain using molecular taxonomy. Med. Vet Entomol. **22**: 55–61. doi:10.1111/j.1365-2915.2008.00719.x. PMID:18380654.

Day, J.C., Mustapha, M., and Post, R.J. 2010. The subgenus *Eusimulium* (Diptera: Simuliidae: *Simulium*) in Britain. Aquat. Insects, **32**: 281–291. doi:10.1080/01650424.2010.533130.

Dumbleton, L.J. 1963. The classification and distribution of the Simuliidae Diptera with particular references to the genus *Austrosimulium*. N.Z. J. Sci. **3**: 320–357.

Dumbleton, L.J. 1972. The genus *Austrosimulium* Tonnoir (Diptera: Simuliidae) with particular reference to the New Zealand fauna. N.Z. J. Sci. **15**: 480–584.

Eaton, D.P., Díaz, L.A., Hans-Filho, G., Santos, V.D., Aoki, V., Friedman, H., et al. 1998. The Cooperative Group on Fogo Selvagem Research. 1998. Comparison of Blackflies species (Diptera: Simuliidae) on an Amerindian reservation with a high prevalence of fogo selvagem to neighboring disease free sites in the state of Mato Grosso do Sul, Brazil. J. Med. Entomol. **35**: 120–131. doi:10.1093/jmedent/35.2.120. PMID:9538571.

Feld, C.K., Kiel, E., and Lautenschläger, M. 2002. The indication of morphological degradation of streams and rivers using Simuliidae. Limnologica, **32**: 273–288. doi:10.1016/S0075-9511(02)80033-0.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. **3**: 294–299. PMID:7881515.

Gil-Azevedo, L.H., and Maia-Herzog, M. 2007. Preliminary considerations on the phylogeny of Simuliidae genera from Southern Hemisphere (Insecta: Diptera). Zootaxa, **1643**: 39–68.

Golding, G.B., Hanner, R., and Hebert, P.D.N. 2009. Special issue on Barcoding Life. Mol. Ecol. Res. **1**(Suppl.): 1–257. doi:10.1111/j.1755-0998.2009.02654.x.

Hajibabaei, M., Smith, A.M., Janzen, D.J., Rodríguez, J.J., Whitefield, J.M., and Hebert, P.D.N. 2006. A minimalist barcode can identify a specimen whose DNA is degraded. Mol. Ecol. Notes, **6**: 959–964. doi:10.1111/j.1471-8286.2006.01470.x.

Hebert, P.D.N., Cywinska, A., Ball, S.L., and DeWaard, J.R. 2003*a*. Biological identifications through DNA barcodes. Proc. R. Soc. B Biol. Sci. **270**: 313–321. doi:10.1098/rspb.2002.2218.

Hebert, P.D.N, Ratnasingham, S., and deWaard, J.R. 2003*b*. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc. R. Soc. B Biol. Sci. **270**: S96–S99. doi:10.1098/rsbl.2003.0025.

Hebert, P.D.N., deWaard, J.R., Zakharov, E.V., Prosser, S.W.J, Sones, J.E., McKeown, J.T.A., et al. 2013. A DNA 'Barcode Blitz': Rapid digitization and sequencing of a natural history collection. PLoS ONE, **8**: e68535. doi:10.1371/journal.pone.0068535. PMID:23874660.

Hernández, L.M. 2007. Recommendations by The Natural History Museum on methods for collecting, preserving, rearing and mailing of simuliid specimens. Available from http://www.blackflies.info [accessed 12 July 2014].

Hernández, L.M., Montes de Oca, M., Penn, M., Massaferro, J., Garré, A., and Brooks, S.J. 2009. "Jejénes" (Diptera: Simuliidae) of Nahuel Huapi National Park, Patagonia, Argentina: Preliminary results. Rev. Soc. Entomol. Argentina, **68**: 193–200.

Hernández-Triana, L.M. 2011. Systematics of the blackfly subgenus *Trichodagmia* Enderlein (Diptera: Simuliidae: *Simulium*) in the New World. Ph.D. thesis, Wageningen University, Wöhrmann Printing Services, Holland.

Hernández-Triana, L.M., Crainey, J.L., Hall, A., Fatih, F., Mackenzie-Dodds, J., Shelley, A.J., et al. 2012. The utility of

DNA barcoding for species identification within the blackfly subgenus *Trichodagmia* Enderlein (Diptera: Simuliidae: *Simulium*) and related taxa in the New World. Zootaxa, **3514**: 43–69.

Hernández-Triana, L.M., Prosser, S.W., Rodríguez-Pérez, M.A, Chaverri, L.G., Hebert, P.D.N., and Gregory, R.T. 2014. Recovery of DNA barcodes from blackfly museum specimens (Diptera: Simuliidae) using primer sets that target a variety of sequence lengths. Mol. Ecol. Res. **14**: 508–518. doi:10.1111/1755-0998.12208.

Hernández-Triana, L.M., Prosser, S.W., Rodríguez-Pérez, M.A, Chaverri, L.G, Hebert, P.D.N., Gregory, R.T., and Johnson, N. 2015. DNA barcoding Neotropical black flies Diptera: Simuliidae: Species identification and discovery of cryptic diversity in Mesoamerica. Zootaxa, **3936**: 93–114. doi:10.11646/zootaxa.3936.1.5.

Imonen, J., Adler, H.A., Malqvist, B., and Cywinska, A. 2009. The *Simulium vernum* group (Diptera: Simuliidae) in Europe: multiple character sets for assessing species status. Linn. Soc. (Lond.), **156**: 847–863. doi:10.1111/j.1096-3642.2009.00500.x.

Kúdela, M., Brúderová, T., Jedlička, L., Bernotienė, R., Celec, P., and Szemes, T. 2014. The identity and genetic characterization of *Simulium reptans* (Diptera: Simuliidae) from central and northern Europe. Zootaxa, **3802**: 301–317. doi:10.11646/zootaxa.3802.3.1.

Low, V.L., Pramual, P., Adler, P.H., Ya'cob, Z., Huang, Y.T., Da Pham, X., and Sofian-Azirun, M. 2016. Delineating taxonomic boundaries in the largest species complex of black flies (Simuliidae) in the Oriental Region. Scientific Reports, **6**: 1–8. doi:10.1038/srep20346.

Mackerras, I.M., and Mackerras, M.J. 1948. Revisional notes on Australasian Simuliidae (Diptera). Proc. Linn. Soc. N. S. W., **73**: 429–439.

Malmqvist, B., Wotton, R.S., and Zhang, Y. 2001. Suspension feeders transform massive amounts of seston in large northern rivers. Oikos, **92**: 35–43. doi:10.1034/j.1600-0706.2001.920105.x.

Malmqvist, B., Adler, P.H., Kuusela, K., Merritt, R.W., and Wotton, R.S. 2004. Black flies in the boreal biome, key organisms in both terrestrial and aquatic environments: a review. Ecoscience, **11**: 187–200. doi:10.1080/11956860.2004.11682824.

Moulton, J.K. 1997. Molecular systematic of the Simuliidae (Diptera: Culicomorpha). Ph.D. thesis, The University of Arizona, USA.

Moulton, J.K. 2000. Molecular sequence data resolves basal divergences within Simuliidae. Syst. Entomol. **25**: 95–113. doi:10.1046/j.1365-3113.2000.00097.x.

Moulton, J.K. 2003. Can the current molecular arsenal adequately track rapid divergence events within Simuliidae (Diptera)? Mol. Phylogenet. Evol. **27**: 45–57. doi:10.1016/S1055-7903(02)00397-4. PMID:12679070.

Pinheiro, F.P., Bensabath, G., Freitas, R.B., and Costa, D. 1986. Fundação Serviços de Saúde Pública. Instituto Evandro Chagas: 50 anos de contribuição às ciências biológicas e à medicina tropical. Fund. Serv. Saúd. Púb. **2**: 795–798.

Pramual, P., and Adler, P.H. 2014. DNA barcoding of tropical black flies (Diptera: Simuliidae) of Thailand. Mol. Ecol. Resour. **14**: 262–271. doi:10.1111/1755-0998.12174.

Pramual, P., and Kuvangkadilok, C. 2009. Agricultural land use and blackflies (Diptera, Simuliidae) species richness and species assemblages in tropical streams, Northern Thailand. Hydrobiologia, **625**: 173–184. doi:10.1007/s10750-009-9706-z.

Pramual, P., and Wongpakam, K. 2014. Association of black fly (Diptera: Simuliidae) life stages using DNA barcode. J. Asia-Pac. Entomol. **17**: 549–554. doi:10.1016/j.aspen.2014.05.006.

Pramual, P., Wongpakam, K., and Adler, P.H. 2011. Cryptic biodiversity and phylogenetic relationships revealed by DNA barcoding of Oriental black flies in the subgenus *Gomphostilbia* (Diptera: Simuliidae). Genome, **54**(1): 1–9. doi:10.1139/G10-100. PMID:21217800.

Ratnasingham, S., and Hebert, P.N.D. 2007. BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Mol. Ecol. Notes, **7**: 355-364. doi:10.1111/j.1471-8286.2007.01678.x. PMID:. PMID:18784790.

Ratnasingham, S., and Hebert, P.D.N. 2013. A DNA-based registry for all animal species: The Barcode Index Number (BIN) System. PLoS ONE, **8**: e66213. doi:10.1371/journal.pone.0066213. PMID:23861743.

Rivera, J., and Currie, D. 2009. Identification of Nearctic blackflies using DNA barcodes (Diptera: Simuliidae). Mol. Ecol. Res. **9**: 224–236. doi:10.1111/j.1755-0998.2009.02648.x.

Senatore, G.L., Alexander, E.A., Adler, P.H., and Moulton, J.K. 2014. Molecular systematics of the *Simulium jenningsi* species group (Diptera: Simuliidae), with three new fast-evolving nuclear genes for phylogenetic inference. Mol. Phylogenet. Evol. **75**: 138–148. doi:10.1016/j.ympev.2014.02.018. PMID:24602987.

Shelley, A.J., Hernández, L.M., Maia-Herzog, M., Luna Dias, A.P.A., and Garritano, P.R. 2010. The Blackflies of Brazil (Diptera, Simuliidae). *Edited by* J. Adis, J. Arias, S. Golovatch, K.M. Mantzev, G. Rueda-Delgado, and E. Domínguez. Aquatic Biodiversity in Latin America ABLA Series, Vol. 6. Pensoft, Sofia-Moscosw.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. **30**: 2725–2729. doi:10.1093/molbev/mst197. PMID:24132122.

Tonnoir, A.L. 1925. Australasian Simuliidae. Bull. Entomol. Res. **15**: 213–255. doi:10.1017/S0007485300046198.

Wygodzinsky, P., and Coscarón, S. 1973. A review of the Mesoamerican and South American black flies of the tribe Prosimuliini Simuliinae, Simuliidae. Bull. Am. Mus. Nat. Hist. **151**: 129–200.

Wygodzinsky, P., and Coscarón, S. 1989. A revision of the blackfly genus *Gigantodax* Enderlein (Simuliidae, Diptera, Insecta). Bull. Am. Mus. Nat. Hist. **189**: 1–269.