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 analysed, 8 in the genus *Austrosimulium* (4 species in the subgenus *Austrosimulium* s.str., 3 in the subgenus *Novaustrosimulium*, and 1 species unassigned to subgenus), 2 of the genus *Cnesia*, 8 of *Gigantodax*, 3 of *Paracnephi*a, 1 *Paraustrosimulium* and 4 species to *Simulium,* subgenera (*Morops, Nevermannia*, and *Pternaspatha*). The Neighbour Joining tree derived from the DNA barcodes 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 of species 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.

INTRODUCTION

Black flies (Diptera: Simuliidae) comprise 26 genera and an estimated 2,189 species (2, 177 living and 12 fossil) (Adler and Crosskey 2015a). 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 in the transmission of parasites, pathogens of the blood, skin of humans, and other warm-blooded animals (Hernández-Triana et al. 2011, 2012, 2015; Shelley et al. 2010). The most important simuliid-transmitted parasites of humans are the nematodes *Onchocerca volvulus* (Leuckart), the cause 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 which 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 protozooans 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 Pemphigous Foliaceus in Brazil (Eaton et al. 1998), as well as the etiological agent of the 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; Malmquivist 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).

Research on the Simuliidae from the southern hemisphere has seen little investigation 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 Herzog (2007). In Argentina, Simuliidae are well characterised mainly due to the efforts of Coscarón (1987; 1991), Coscarón and Coscarón-Arias (1989; 1998; 2002), Coscarón and Wygodzinsky (1972) and Wygodzinsky and Coscarón (1973; 1989) (reviewed in Hernández et al. 2009), while the monographs of Dumbleton (1963, 1973), 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 south-western 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 have been provided by Adler et al. (2004). In 1994, Ballard showed evidence from the 12S ribosomal gene could resolve relationships in *Austrosimulium*; more recently, Craig and Cywinska (2012) investigated the relationships of New Zealand *Austrosimulium* employing DNA sequences from three regions of the mtCOI gene in combination with morphological characters.

In the present paper, we aim 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 assess 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 et al. 2011, 2012, 2014). Larvae, pupae and link-reared adults were collected in rivers and streams across the black flies species distribution range in Nahuel Huapi National Park (see Brooks et al. 2009; Hernández et al. 2009). Material from Australia and New Zealand were 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 -5ºC until molecular analysis was begun. The alcohol was changed once before storing the vials at -5ºC. 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 track disturbed to reduce the possibility of contamination (Hernández-Triana et al. 2012; Rivera and Currie 2009). Larval specimens had a long strap of the posterior abdominal wall removed as a source for DNA extraction; the remainder of the body was retained as 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/or 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). 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 in a 96 well plate for DNA lysis and subsequent DNA extraction. A digital image of each specimen was taken at BIO 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 (DOI) for the project is as follows: 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 paper because it 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 of the specimens followed the CCDB protocols (Ivanova, deWaard and Hebert - www.dnabarcoding.ca). In brief, extractions were automated using a 96 multichannel Biomek NX robotic liquid handler (Beckman Coulter Inc.) with a Thermo Cytomat hotel. Polymerase chain reaction 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. 2003a,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'-ATTCAACCAATCATAAAGATATTGG-3') with MLepR1(5'-GTTCAWCCWGTW CCWG CYCCATTTTC-3') and MLepF1 (5'GCTTTCCCACGAATAAATAATA-3') with LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-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 Biosystematics). All DNA extractions, PCR amplification conditions and sequencing protocols are available at www.dnabarcoding.ca/ CCDB_sequencing.pdf.

Sequence analysis

Paired bi-directional sequence traces were combined to produce a single consensus sequence (e.g., the full length 658 bp barcode sequence). To achieve this, individual forward and reverse traces were oriented, edited, and aligned using the Sequencer (v.4.5; Genes Codes Corporation, Ann Harbour, MI), GenDoc (v. 2.6.02) and ClustalX sequence analysis programs. Published sequences of *Austrosimulium*

(*Austrosimulim s.str.*) *australense* were used because this species is the type species of the subgenus *Austrosimulium* (Adler and Crosskey 2015; Craig et al. 2012).

The full data set was also analyzed in MEGA v.6 (Tamura et al. 2013). A NJ tree analysis was carried out using the K2P distance metric to represent their 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 Adobe Photoshop CS3 (v. 10.0.1) was used to edit them. 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).

After their upload to BOLD, most barcode sequences larger than 500 bp were assigned a Barcode Index Number (BIN), an interim taxonomic systems that segregates similar barcode sequences into a BIN (Ratnasingham and Hebert 2013). A NJ was generated in BOLD and we mapped all BINs for each morphospecies (Fig. 1 and Fig. S1). We analyzed the taxonomic discordance in our dataset by using BOLD capabilities, which provides a means of confirming the concordance between barcode sequence clusters and species designations. The report 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* (8 species) (Subgenera *Austrosimulium* s.str. and *Novaustrosimulium*), *Cnesia* (2 species), *Gigantodax* (8 species), *Paracnephia* (3 species), *Paraustrosimulium* (1 species) and *Simulium* (6 species) (subgenera *Pternaspatha*, *Morops* and *Nevermannia*) (see Adler and Crosskey 2015; Craig et al. 2012) were included in the analysis (Tables 1 and 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 280-514 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, although this was not the case for all taxa (see Fig. 1). For example, specimens identified as *Cnesia dissimilis* and *C. near pussilla* clustered together, and also *Cnesia ornata*, all supported with high bootstrap values. 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 undisclosed number of pupae and larvae, from Rio Negro and Neuquén provinces. The authors stated that *C. pussilla* may not be easy to separate from *C. dissimilis* differing only by its smaller size in 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 this 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*. Neither species can be separated based on pupal gill configuration, except for *C. ornata* which have tubercles in the thorax (Coscarón 1991). In the present study, topotype black males identified as *C. pussilla* all grouped together with *C. dissimilis* with bootstrap values of 100% (Fig. S1), and *C. ornata* with 99%, all having the same BIN number. This suggests that *C. pussilla* and *C. ornata* might be a junior synonym of *C. dissimilis*. Specimens of *Austrosimulium australense* seem to form two separate clusters in the NJ tree (Fig. S1). They showed a low genetic divergence (0.3%, Table 1) and while not well supported by bootstrap values (Fig. S1), this finding 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 the taxa. Thus, while conspecific individuals collected from a single site often exhibited zero or very low divergence values, other specimens exhibited higher values (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% to 30%) (Table 3). 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 nemorale*/*Gigantodax igniculus, Austrosimulium victoriae/Paracnephia aurantiaca* and *Simulium* (*Pternaspatha*) *nemorale/Simulium* (*Morops*) *torresianum* (30%). As expected, smaller values were found among species within the same genus or subgenus, for example *Gigantodax chilensis/G. marginalis* (0.4%), and *Austrosimulium montanum/A. cornutum* (0.5%) (Table 2).

In this study we only analysed *S. ornatipes* as a known (or suspected-to-be) 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). Intraspecific genetic divergence averaged 2.7% for *C. dissimilis*, 4.2% for *G. femineus*, and 1.8% for *P. fergunsoni*. Surprisingly, *S. simile* showed a deep split in the NJ with two well distinct groups, I and II (Fig. 1, Fig. S1) with a divergence of 1.3% with 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 *Paracnephia fergunsoni* and *Cnesia dissimilis* (28%) and *Paracnephia fergunsoni* and *S. simile* (29%).

The BIN count in our dataset of 474 barcode records was higher than the species count (28 species). In general 402 barcodes were assigned a BIN number, which represented 29 BINs; nine BINS were discordant (287 records), 16 BINs were taxonomically concordant (111 records), while four BINS were singletons. Most of the discordant BINs occurred at the species level, mainly because of the taxonomic species list within BOLD account for different species, for example *Simulium nemorale* and *Simulium* nr. *nemorale*. In one case, BIN AAB4815 (*Paraustrosimulium anthracinum*), there was a discrepancy in one specimen at the genus level (*S. 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). BIN merges were

uncommon and occurred only in *Cnesia* (BIN AAB4816, identified as *C. dissimilis*, *C. ornata* and *C. pussilla*) (Fig. 1, Fig. S1).

Discussion

Hernández-Triana et al. (2011, 2012, 2014, 2015) have discussed the use of COI DNA barcoding in Simuliidae, and also reviewed the controversies that this approach has generated in the 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 male thoracic morphology occurs along their distribution range. Therefore, it is proposed that other molecular markers such as the internal transcribed spacer marker (ITS) 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 & 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 relationship 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, and concluded that the mtDNA COI gene was also of phylogenetic value. In our study, specimens of *A. australense* appeared to form two groups (Fig. S1), but these groups were not supported by bootstrap values in the NJ. The lack of support in our dataset 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 sub-groups in certain species, such as *C. dissimilis*, *G. femineus*, *S. simile*, and *P. fergunsoni* would

suggest the presence of cryptic diversity (Fig. 1, Fig. S1; 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 et al. 2011, 2012) and Nearctic mosquitoes (Cywinska et al. 2006). The taxonomy of Australian black flies species is in need of a revision, and there is still an ongoing controversy with regards 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 support the current species identification.

Although sibling species were not cytotyped in this study, it would be expected that genetic variation between random individuals from sibling species complexes would show, on average, higher levels than that between individuals from those 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. fergunsoni* and *S. simile*, which might indicate the present 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 this paper fall 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 *Simulium simile* and *S. nemorale* may represent the presence of hidden diversity, although other explanation might be possible. The detection of a BIN merge in specimens morphologically identified as separate species *C. dissimilis*, *C. pussilla* and *C. ornata* confirms that these taxa might be synonyms. Nonetheless, a revision of this genus in combination with other molecular markers is needed to test this hypothesis.

The present study provides COI data to support species identification in a large and understudied fauna for Argentinian populations of the genera *Cnesia* (3 species), *Gigantodax* (8 species), *Paraustrosimulium* (1 species), and anthropophilic species of *Simulium* (3 species). It also augments data

for species diversity of *Austrosimulium* in Australia (6 species), *Paracnephia* (3 species) and *Simulium* (3 species), and for the population of *A. 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 on aquatic ecosystems (e.g. Pramual &, Wongpakam, 2014), or for the identification of biting females in closely related species of key medically 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 et al. 2011, 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 in revealing levels of genetic diversity in poorly known taxa, for example *C. dissimilis*, *G. femineus*, *P. fergunsoni*, and *S. simile*. However, it is uncertain whether this level of genetic divergence is indicative of the presence of species complex. Very few studies employing molecular and cytogenetic methods have been published on Austral Simuliidae (see Cytotaxonomic Conspectus of the Simuliidae by Adler & Crosskey, 2015b). Therefore, we advocate for further integrated research on known pest species or taxonomically problematic taxa as endorsed by Adler et al. (2004), Adler & Crosskey (2015b), Craig et al. (2012), Low et al. (2016), and Shelley et al. (2010). The 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 the correct species identification.

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TABLE 1. List of black fly species, country of collection, and number of specimens with DNA barcodes. Mean (%) intraspecific values of sequence divergence (K2P) are shown with missing entries indicating that less than two specimens were analyzed. Species complexes (*) and taxa with deep splits (**) in the Neighbor Joining tree are marked with asterisks.

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	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	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	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	Table 3
Simulium (Morops) melatum	Australia	1	—
Simulium (Morops) torresianum	Australia	1	—
*Simulium (Nevermannia) ornatipes s.l. **	Australia	3	0

TABLE 2. Interspecific (between groups) pairwise K2P genetic divergence of unique DNA barcodes (658 bp) representing 28 species in six genera of Simuliidae. 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.

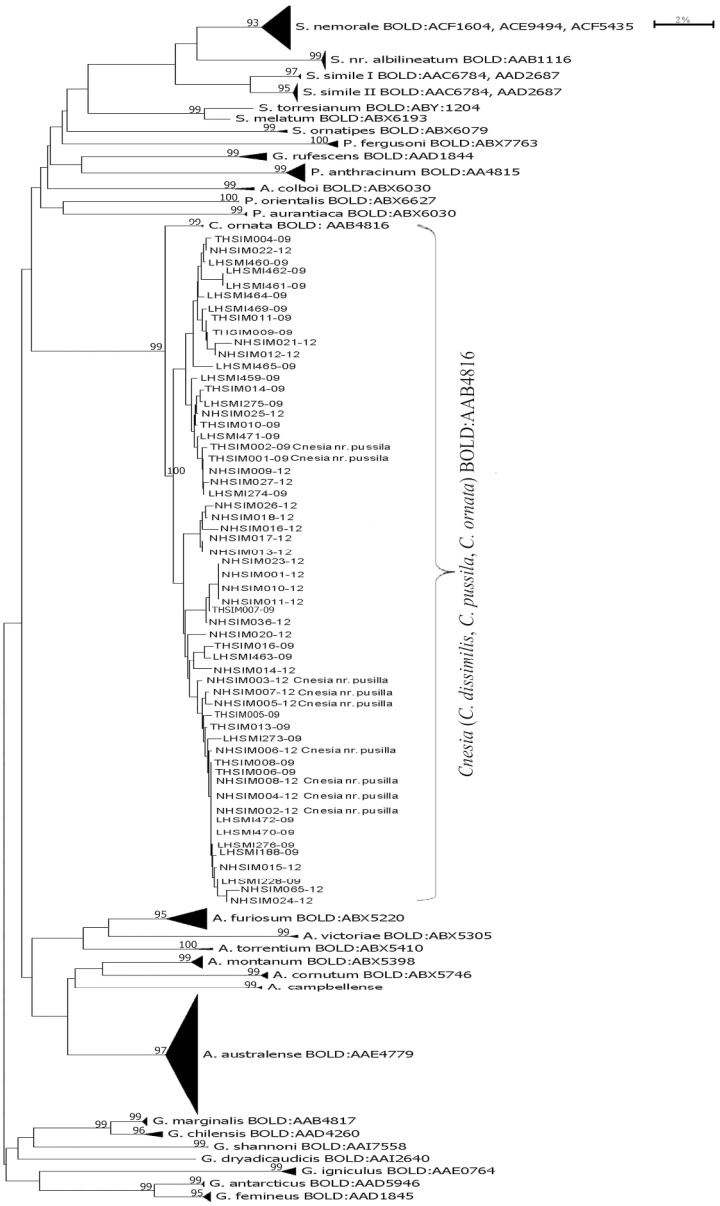
	torres.	aur.	fur.	torren.	fem.	ori.	vic.	mel.	cor.	mon.	col.	orn.	fer.	cam.	ign.	ant.	ruf.	dis.	nem.	mar.	sim.	nr. alb	ant.	chi.	orn.	sha.	dr.
S. torresianum																											
P. aurantiaca	0.22																										
A. furiosum	0.22	0.24																									
A. torrentium	0.19	0.24	**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	**0.0**4	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

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

Species complex status	Country	*n*	% 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

CAPTION FOR ILLUSTRATIONS

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. Bootstrapped values >70% are shown at each node.



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