Systematics of the *Culex coronator* complex (Diptera: Culicidae): morphological and molecular assessment

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The *Culex coronator* complex of the mosquito subgenus *Culex* includes five currently recognized species: *Cx. camposi*, *Cx. coronator*, *Cx. ousqua*, *Cx. usquatissimus* and *Cx. usquatus*. Because of the confusing taxonomic history of the complex, we aimed to clarify the specific status of these nominal forms based on an examination of holotypes and lectotypes and molecular data from other specimens. Critical assessment of published descriptions and study of type specimens revealed that the known distributions of the five species overlap considerably and exhibit biotic sympatry in some areas. Sequences from the *COI* barcode region and complete mitochondrial genomes were used to assess the relationships and degree of genetic divergence of the species and two newly discovered morphological forms, *Cx. coronator* Forms 1 and 2. Genetic distances in the *COI* dataset varied from 0.00 to 2.67%, with the largest relative divergence being 4.41 between specimens of *Cx. coronator* and *Cx. coronator* Form 1. Bayesian Poisson tree process analysis of the *COI* barcode region also failed to provide support for the nominal species. Evidence from the morphological and molecular data thus leads us to conclude (at least provisionally) that the *Cx. coronator* complex is a single polymorphic species. The forms constitute a monophyletic group but there is no support for the specific status of the five nominal forms.

ADDITIONAL KEYWORDS: *COI* sequences – *Culex camposi* – *Culex ousqua* – *Culex usquatissimus* – *Culex usquatus* – genetic divergence – mitochondrial genomes – morphology.

INTRODUCTION

The *Culex coronator* complex of subgenus *Culex* Linnaeus (Diptera: Culicidae: Culicinae: *Culex*) includes five species: *Cx. camposi* Dyar, *Cx. coronator* Dyar & Knab, *Cx. ousqua* Dyar, *Cx. usquatissimus* Dyar and *Cx. usquatus* Dyar (Forattini, 2002; Harbach, 2017), which exhibit the following diagnostic features. Male genitalia: subapical lobe slightly produced, seta *g* absent, ventral arm of the phallosome dentiform and bent at a right angle, lateral arm with 5–14 teeth, paraproct glabrous. Larvae: siphon with subapical spines, siphon index greater than 5.

Complexes of morphologically similar or isomorphic species are common among invertebrates, and especially in Culicidae, for example, in the genera *Anopheles* Meigen (Foster *et al.*, 2013) and *Sabethes*

Robineau-Desvoidy (Pedro, Sallum & Butlin, 2008). More than 40 species complexes are recognized in the genus *Culex* (Harbach, 2011), including four within the subgenus *Culex*, that is, the *Cx. coronator*, *Cx. pipiens*, *Cx. restuans* and *Cx. salinarius* complexes. Three of the five species of the *Cx. coronator* complex, *Cx. coronator*, *Cx. usquatissimus* and *Cx. usquatus*, are known to occur in Argentina (Rossi, 2015), and two other morphological forms (herein designated *Cx. coronator* Forms 1 and 2) have been found in the country during the revision of entomological collections and entomological surveys that cannot be identified as one or other of the currently recognized species of the complex.

Members of species complexes share morphological traits in most life stages, making identification difficult. For this reason, molecular methods need to be integrated with morphological taxonomy to more accurately identify specimens, resolve nomenclatural problems and elucidate phylogenetic relationships

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among cryptic species (Harbach, 2007). A fragment of the cytochrome *c* oxidase subunit I (*COI*) mitochondrial gene has been used extensively as a DNA barcode for species identification (Hebert *et al.*, 2003a) and to evaluate genetic distances among closely related species (Hebert *et al.*, 2003b). Mitochondrial markers are preferable to nuclear markers due to their abundance (relative to most nuclear genes), lack of introns, limited exposure to recombination, haploid mode of inheritance (Saccone *et al.*, 1999) and more rapid evolution, resulting in the accumulation of differences between closely related species (Brown, George & Wilson, 1979). Hebert *et al.* (2004) suggested a standard sequence threshold high enough to separate specimens that belong to different species – the mean interspecific genetic divergence should be at least ten times greater than the average intraspecific genetic distance. Particularly for mosquitoes, a mean intraspecific Kimura two-parameter (K2P) divergence varying from 0.2 to 1.4% and a mean interspecific variation between 2.0 and 5.6% were proposed by Ruiz-Lopez *et al.* (2012). Mitochondrial *COI* barcode sequences have been used to identify mosquito species in Canada (Cywinska, Hunter & Hebert, 2006), India (Kumar *et al.*, 2007), China (Wang *et al.*, 2012), Argentina and Brazil (Laurito *et al.*, 2013), Pakistan (Ashfaq *et al.*, 2014), Sweden (Engdahl *et al.*, 2014), Belgium (Versteirt *et al.*, 2015) and Australia (Batovska *et al.*, 2016). They have also been used to reveal species complexes within the subgenus *Nyssorhynchus* Blanchard of *Anopheles* in the Neotropical Region (Ruiz-Lopez *et al.*, 2012; Bourke *et al.*, 2013; Foster *et al.*, 2013). However, recent studies have provided conflicting results on the efficacy of *COI* barcodes for species delimitation within the Culicidae (Laurito *et al.*, 2013; Versteirt *et al.*, 2015; Batovska *et al.*, 2016). Advances in sequencing technology have made *de novo* sequencing readily accessible and complete mitochondrial genomes have been utilized as diagnostic markers across a wide range of taxa (Coissac *et al.*, 2016).

Because of the complex and confusing taxonomic history of the *Cx. coronator* complex (see details in the 'Discussion' section), the aim of the present study, far from conducting exhaustive taxonomic descriptions, was to clarify the specific status of the five nominal forms based on a critical examination of type specimens (holotypes and lectotypes) and molecular data derived from specimens that share the diagnostic male genitalia features of the primary types. Sequence data contained in the *COI* barcode region of mtDNA, as well as the full mitochondrial genome, was used to assess the degree of genetic divergence of the five nominal species and two previously unknown forms of the complex.

MATERIAL AND METHODS

MOSQUITOES

The mosquitoes examined during the morphological study were obtained during field studies and from entomological collections (Supporting Information, Table S1) held in the National Museum of Natural History (NMNH), Washington, DC, Centro de Investigaciones Entomológicas (Córdoba), Fundación e Instituto Miguel Lillo (San Miguel de Tucumán) and the Instituto de Biología de la Altura (San Salvador de Jujuy). Specimens were identified to species based on features of the male genitalia in comparison with primary name-bearing type specimens (holotypes and lectotypes) on loan from the NMNH.

DNA EXTRACTION, SEQUENCING, ASSEMBLY AND ANNOTATION

Total genomic DNA (gDNA) was extracted from nine whole specimens from across the *Cx. coronator* complex using the Bioline Genomic II Isolate kit (Bioline, London, UK) (see Supporting Information, Table S1). Following quantification of double-stranded DNA with a Qubit fluorometer 2.0 (Invitrogen, Waltham, MA), indexed libraries were constructed with the TruSeq nano library kit (Illumina, San Diego, CA) for each specimen and subsequently sequenced on $1/20^{th}$ of an Illumina MiSeq flowcell, version 3 chemistry, 600 cycle paired-end. Reads for each specimen were trimmed using default settings in Geneious v. 8.1.7 (http://www.geneious.com, Kearse *et al.*, 2012) and assembled to the complete *COI* gene of the mitochondrial genome of *Cx. quinquefasciatus* Say (Behura *et al.*, 2011), GenBank accession NC014574. Unassembled reads were then iteratively mapped and reassembled to the putative *COI* sequences until the resulting contigs could be circularized. Gene boundaries were annotated using MITOS (Bernt *et al.*, 2013) and verified by visualization of open reading frames and comparison to alignments of culicine mitochondrial genes.

BARCODE CLUSTERS, NEIGHBOR-JOINING AND MAXIMUM PARSIMONY

The mitochondrial *COI* sequences of 41 morphologically identified specimens of the *Cx. coronator* complex (Supporting Information, Table S2) were used for molecular analysis, including nine generated during the present study from specimens collected in Argentina (four *Cx. coronator*, two *Cx. coronator* Form 1 and three *Cx. coronator* Form 2) and 32 obtained from GenBank for specimens of *Cx. usquatus* from Argentina (one), Brazil (11) and Ecuador (two), and *Cx. coronator* (13), *Cx. camposi* (three) and *Cx. usquatissimus* (two)

from Brazil. Four additional sequences for *Cx. coronator* listed on the BOLD (Barcode of Life Data System) website were not used because they were obtained from females, whose morphological identity cannot be confirmed. The outgroup comprised sequences obtained from GenBank for *Anopheles darlingi* Root (JF923695) and 14 Neotropical species of the subgenus *Culex*: *Cx. acharistus* Root (KF919245), *Cx. apicinus* Philippi (KF919251), *Cx. bidens* Dyar (KF919201), *Cx. bilineatus* Theobald (KF919219), *Cx. brethesi* Dyar (KF919207), *Cx. chidesteri* Dyar (KF919243), *Cx. declarator* Dyar & Knab (KF919211), *Cx. dolosus* Lynch Arribálzaga (KF919215), *Cx. lygrus* Root (KF919221), *Cx. mollis* Dyar & Knab (KF919255), *Cx. nigripalpus* Theobald (KF919227), *Cx. pipiens* Linnaeus (KF919189), *Cx. quinquefasciatus* (KF919188) and *Cx. tatoi* Casal & García (KF919234).

Nucleotide sequences were aligned using the Muscle algorithm (Edgar, 2004) in SeaView v. 4 (Gouy, Guindon & Gascuel, 2010). Pairwise nucleotide sequence divergences and mean intraspecific and interspecific distances for the entire dataset (*Cx. coronator* complex and the 14 Neotropical *Culex* species) were estimated for the *COI* barcode region using K2P distance (Kimura, 1980), implemented in MEGA v. 6 (Tamura *et al.*, 2013). Of the 41 sequences from specimens of the *Cx. coronator* complex, 39 unique haplotypes were recovered in DAMBE v. 5 (Xia, 2013), which subsequently were used to generate neighbor-joining (NJ) and maximum parsimony (MP) trees. The NJ analysis was conducted using the K2P model in MEGA v. 6 (Tamura *et al.*, 2013) to evaluate the clustering pattern between the nominal species. The MP analysis of the molecular data, implemented in TNT v. 1.5 (Goloboff & Catalano, 2016) using equal weighting and gaps treated as missing data, was conducted to corroborate the topology of the NJ results. The search for Wagner trees was conducted using a series of 10 000 random addition sequences, retaining up to 100 trees per replication, and tree bisection and reconnection (TBR) as branch rearrangements followed by a second heuristic search. The resulting trees were swapped with another round of TBR. Statistical support for groups in the NJ and MP trees was estimated using bootstrap values (BSV) obtained from 1000 bootstrap replicates.

SLIDING WINDOW ANALYSIS

To assess levels of nucleotide divergence across the full mitochondrial genomes, sliding window analysis was implemented using DnaSP v. 5.10.01 (Librado & Rozas, 2009) with window sizes of 300 bp and step sizes of 10 bp. The analysis was performed using the nine complete mitochondrial genome sequences generated from this study (GenBank accessions MF509887-95; see

Supporting Information, Table S2) and subsequently reanalysed to include the mitochondrial genomes of the following five specimens of the *Cx. coronator* complex sequenced by Demari-Silva *et al.* (2015): *Cx. camposi* MS04-38 (MF040164), *Cx. usquatus* SP29-156 (MF040161), *Cx. coronator* RS10-109 (MF040162), *Cx. usquatissimus* AC16-101 (MF040165) and *Cx. usquatissimus* RO25-19 (MF040163).

PHYLOGENETIC ANALYSIS OF MITOCHONDRIAL GENOMES

Bayesian phylogenetic analysis was carried out using the concatenated sequences of the 13 protein coding genes of the nine complete mitochondrial genome sequences generated in this study, the mitochondrial genomes of the five specimens of the *Cx. coronator* complex sequenced by Demari-Silva *et al.* (2015) listed above and the mitochondrial genomes of four species available from GenBank, *Aedes albopictus* (NC_006817), *A. darlingi* (NC_014275), *Cx. pipiens* (HQ724616; HQ724615) and *Cx. quinquefasciatus* (HQ724617).

Nucleotide sequences were aligned using Clustal W (Thompson, Higgins & Gibson, 1994). Aligned sequences were concatenated, verified by eye and subjected to phylogenetic analysis using Mr Bayes v. 3.1.2 and partitioned by gene. The best-fit model of nucleotide substitution for each gene was estimated in jModelTest v. 2.1.7 (Darriba *et al.*, 2012), under Bayesian Information Criterion (BIC). Two independent runs with one cold and three heated chains each were implemented for 1 000 000 generations with trees sampled every 100 generations; the consensus topology was generated after a burn-in of 25% of the retained trees following Demari-Silva *et al.* (2015).

BAYESIAN SPECIES DELIMITATION

Bayesian implementation of the Poisson tree process (PTP) methodology (Zhang *et al.*, 2013) was used to check the number of distinct species that could be identified within the *Cx. coronator* complex. The dataset used for the NJ and MP analyses was used to construct a phylogenetic tree for Bayesian Poisson tree process (bPTP) analysis. The best-fit partitioning schemes and models of molecular evolution for each partition selected by the BIC in PartitionFinder v. 1.0.1 (Lanfear *et al.*, 2012) are presented as follows: for position 1, JC (Jukes & Cantor, 1969) evolution model was preferred; for position 2, F81 (Felsenstein, 1981) was the selected model and for position 3, the TrN + I (invgamma distribution) (Tamura & Nei, 1993) model was used. Using Mr Bayes v. 3.2.2 (Ronquist & Huelsenbeck, 2003), six independent MCMC (Markov

Chain Monte Carlo) chains (temperature = 0.15) were run simultaneously for three million generations. Trees were sampled every 2000 generations with the first 250 sampled trees discarded as burn-in. The standard deviation of the split frequencies between runs (<0.01) and the effective sample size were monitored to ensure stationarity, convergence and correct mixing of the chains. Bayesian bPTP analysis was performed using the bPTP webserver (accessed 2016: species.h-its.org) using default options and including the outgroup taxa.

RESULTS

MORPHOTAXONOMY

Based on critical review of published descriptions and examination of primary type specimens, the features of the male genitalia that characterize the nominal species of the *Cx. coronator* complex are summarized in Table 1. The cluster of setae at the apex of the gonocoxite is borne on the ventral side (pre-rotation sense), near the base of the gonostylus (Fig. 1), and these setae are distinct from the dorsal apical setae that are always short. The cluster is defined as short if the setae do not reach the mid-length of the gonostylus and are defined as long when they reach or extend beyond the midpoint. Ornamentation of the subapical lobe consists of rod-like setae (inappropriately dubbed 'rods' in previous studies), which are generally stouter and more rigid than filiform (more flexible, filament-like) and flattened blade-like setae (Fig. 1). Setae classified as either rod-like or filiform may be of equal length and thickness or subequal, normally stouter and longer when borne on the proximal part of the subapical lobe.

Three species of the *Cx. coronator* complex are recorded in Argentina, that is, *Cx. coronator*, *Cx. usquatissimus* and *Cx. usquatus* (Fig. 2). All specimens of the complex collected in Argentina and the specimens we examined from Ecuador, except specimens 13/01 and 13/02 (Supporting Information, Table S1) due to the way in which the genitalia were mounted, have an additional seta borne on a small tubercle located on the ventrolateral margin of the gonocoxite at or just above the level of the distal margin of the subapical lobe (seta labelled vlSe in Figs 1C, 3C, 4C). This seta, termed here the 'ventrolateral seta', has not been noticed or mentioned previously. Based on comparative morphological study of Argentinian specimens and primary type specimens, *Cx. coronator*, *Cx. usquatus* and two previously unknown forms occur in the country. The two new forms are diagnosed as follows (see Supporting Information, Table S1, for specimen collection data).

Culex coronator Form 1 (Fig. 3): subapical lobe undivided or very slightly divided, proximal part with two or three subequal rod-like setae (Fig. 3A, B, E–H), distal part of lobe with two or three narrow foliform setae (Fig. 3A, B) and one to three blade-like or lanceolate setae (Fig. 3D), or a variable combination of those setal types (three to five setae in total) (Fig. 3E, F), distal part with more than four subequal filiform setae (Fig. 3A,

Table 1. Male genitalia characters of nominal species of the *Culex coronator* complex based on primary type specimens (holotype or lectotype)

Form	Type specimen	Cluster of setae at apex of gonocoxite	Subapical lobe			
			Proximal division		Distal division	
			Shape	Ornamentation	Shape	Ornamentation
camposi	Holotype	Long	N/A	Three equal + two shorter rod-like setae	N/A	Single stout seta
coronator	Lectotype	Long	N/A	Three or four subequal rod-like setae	N/A	Three filiform setae
ousqua	Holotype	Short	Conical	13 filiform setae	Columnar	Five filiform setae (one stout, four subequal)
usquatissimus	Lectotype	Long	N/A	Three equal rod-like setae	N/A	Three flattened blade-like setae
usquatus	Lectotype	Long	N/A	Five subequal rod-like setae	N/A	Five or more subequal setae

N/A, not applicable.
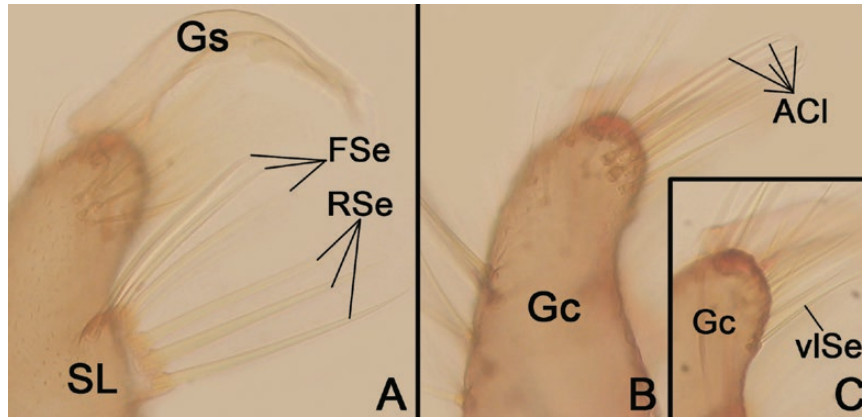
Figure 1. Gonocoxopodite of a specimen of *Culex usquatissimus* Dyar collected in San Salvador de Jujuy, Jujuy Province, Argentina (Ju-14-01 in Supporting Information, Table S1). A, dorsal aspect. B, ventral aspect. C, ventrolateral aspect. Abbreviations: ACl, apical cluster of setae; FSe, filiform setae; Gc, gonocoxopodite; Gs, gonostylus; RSe, rod-like setae; SL, subapical lobe; vlSe, ventrolateral seta.

B, D–H); apical cluster of gonocoxite comprising short setae (Fig. 3A, B, D–F), ventrolateral seta present (Fig. 3C). One specimen collected in Catamarca Province (specimen Cat-12-12 in Supporting Information, Table S1) has two foliform setae on the left gonocoxite and filiform setae on the right one (Fig. 3G, H). The apical cluster of setae on the gonocoxite is short as in *Cx. ousqua* but the shape and ornamentation of the subapical lobe are completely different. The foliform setae resemble those of *Cx. usquatissimus* but the setae of the apical cluster are short rather than long. The shape and ornamentation of the subapical lobe is similar in *Cx. coronator* and *Cx. usquatus* but the setae of the apical cluster are long in those species.

Culex coronator Form 2 (Fig. 4): subapical lobe undivided (Fig. 4A), slightly divided in some specimens from Catamarca Province (Fig. 4B), proximal part of lobe with two or three subequal rod-like setae, distal part with more than six subequal filiform setae (Fig. 4A, B); apical cluster of gonocoxite comprising short setae (Fig. 4B), ventrolateral seta present (Fig. 4C). This form has rod-like setae as in *Cx. coronator* but differs in having a short apical cluster of setae and more filiform setae on the distal part of the subapical lobe. It resembles *Cx. usquatus* in having more than six filiform setae but it has fewer rod-like setae and the setae of the apical cluster are short. Also, the shape and ornamentation of the subapical lobe differs from that of *Cx. ousqua*.

MOLECULAR ANALYSES

A pairwise and means matrix of K2P distances between and within groups was constructed for the 55 *COI* barcode sequences of the *Cx. coronator* complex (41 specimens) and the other 14 *Culex* (*Culex*) species. The K2P distances between each nominal species and forms of the *Cx. coronator* complex are shown in Supporting Information, Tables S3 and S4. The K2P distances within the complex vary from 0.00 to 2.67%. As expected, the minimum genetic divergence occurs between sequences corresponding to specimens of the same morphological form: *Cx. coronator* (specimen numbers Ju-14-01 and Ju-14-05) and *Cx. usquatus* (KF671026 and KF919242). Curiously, this lack of divergence also occurs between specimens identified as different morphological forms, as follows: *Cx. coronator* Form 1 (Mis-14-04) and *Cx. coronator* Form 2 (Mis-14-07), *Cx. coronator* Form 1 (Mis-14-01) and *Cx. usquatus* (KJ812977), *Cx. coronator* Form 2 (Ju-14-30) and *Cx. coronator* (KJ812987), *Cx. coronator* (KJ812989) and *Cx. usquatus* (KJ812977), *Cx. usquatus* (KJ812980) and *Cx. coronator* (KF919199), *Cx. usquatus* (KJ812980) and *Cx. camposi* (KF919208), and *Cx. coronator* (KF919199) and *Cx. camposi* (KF919208) (Supporting Information, Table S3). The greatest genetic distance (2.67%) is between two sequences that correspond to morphologically identified specimens of *Cx. coronator* from São Paulo State, Brazil (KJ812988 and KJ812991). Interspecific distances range from 0.47 to 0.98%, values which in many cases are lower than the intraspecific divergence (Supporting Information, Table S4). The interspecific distance between *Cx. coronator* and *Cx. coronator* Form 1 is only 4.41 times greater than the average variation within *Cx. coronator* Form 1, the largest relative divergence in the dataset. Mean K2P distances between each nominal species and form of the complex and the other *Culex* (*Culex*) species (not included herein) range from 3.92% (between *Cx. coronator* Form 1 and *Cx. brethesi*) and 7.56% (between *Cx. coronator* Form 2 and *Cx. apicinus*). Considering the *Cx. coronator* complex
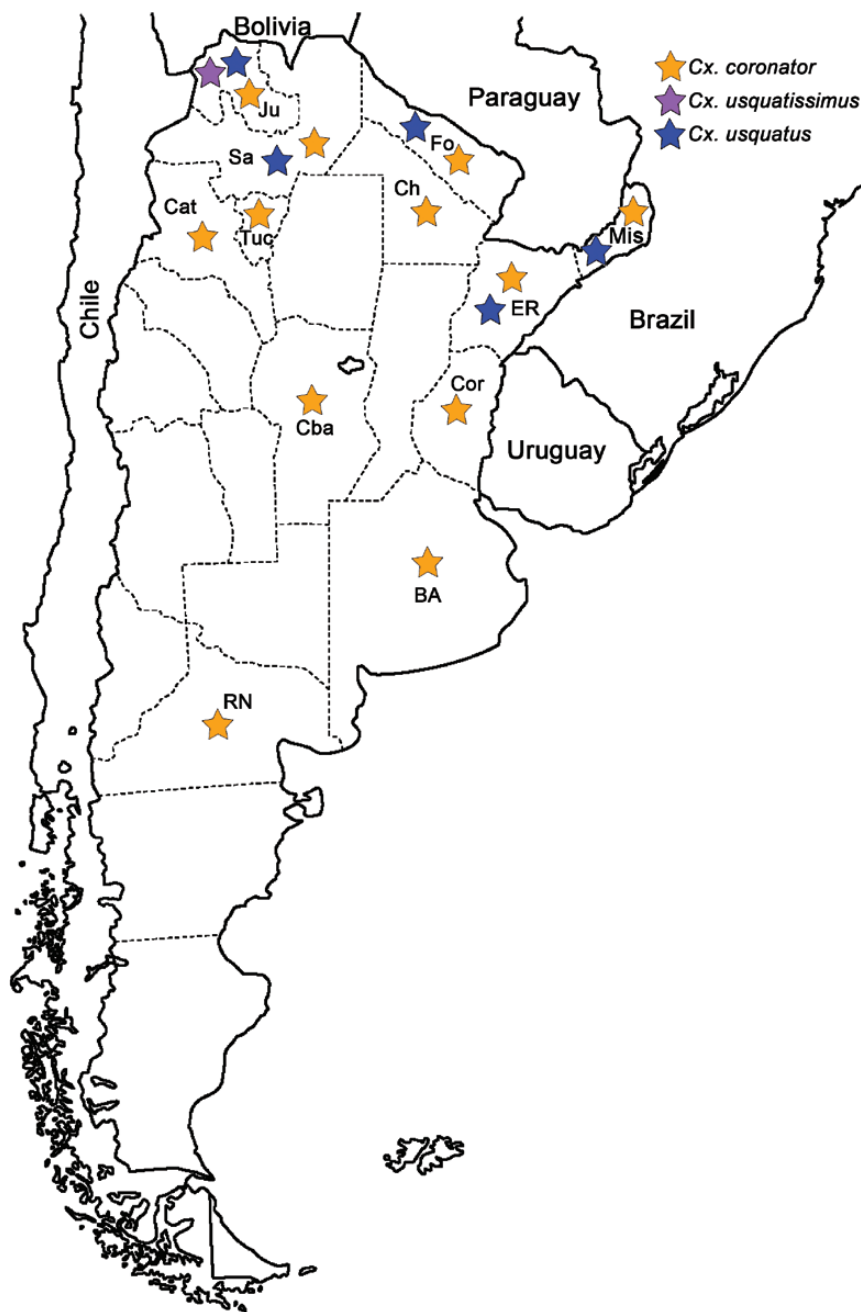
Figure 2. Localities in Argentina where *Culex coronator*, *Cx. usquatissimus* and *Cx. usquatus* have been collected. Abbreviations: BA, Buenos Aires Province; Cat, Catamarca Province: Cba, Córdoba Province; Ch, Chaco Province; Cor, Corrientes Province; ER, Entre Ríos Province; Fo, Formosa Province; Ju, Jujuy Province; Mis, Misiones Province; RN, Río Negro Province; Sa, Salta Province; Tuc, Tucumán Province.

as a whole, genetic divergence varied from 4.04%, with *Cx. brethesi*, to 7.44%, with *Cx. apicinus*, being 0.85% of the mean divergence within the complex.

Because the topologies of the NJ and MP trees are consistent, only the latter is described here. The analyses yielded 360 most parsimonious trees of 313 steps. A strict consensus is shown in Figure 5. The

well-supported clade comprising all sequences of the *Cx. coronator* complex, with a BSV of 99%, is in a terminal relationship to a pectinate series of clades comprising the other *Culex* (*Culex*) species. The three well-supported clades within the complex are recovered in both analyses, with similar BSV. Three *COI* lineages are recovered in the MP analysis,
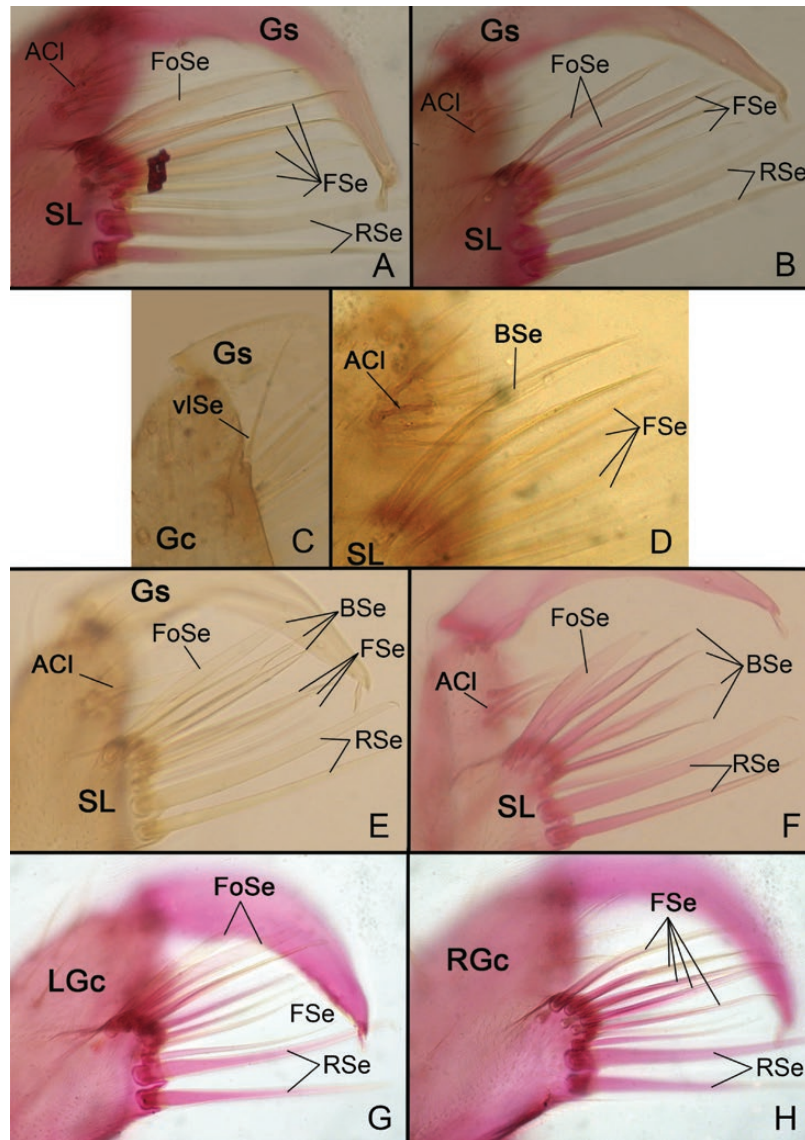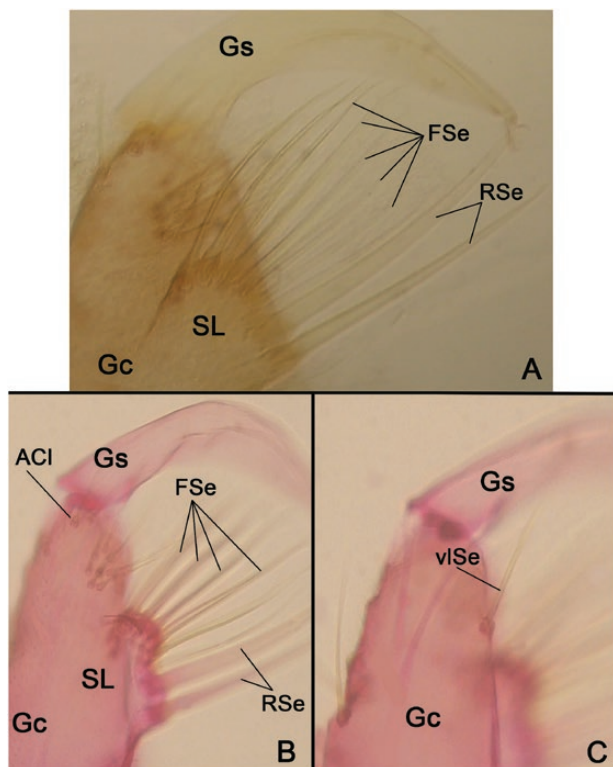
Figure 3. Gonocoxopodites of specimens of *Culex coronator* Form 1 collected in Argentina. A, specimen from San Pedro, Misiones Province (Mis-12-04 in Supporting Information, Table S1). B, specimen from Puerto Iguazú, Misiones Province (M079 in Supporting Information, Table S1). C, D, specimen from San Isidro de Lules, Tucumán Province (M064 in Supporting Information, Table S1). E, specimen from Las Juntas, Catamarca Province (Cat-12-32 in Supporting Information, Table S1). F, specimen from San Fernando del Valle de Catamarca, Catamarca Province (Cat-12-38 in Supporting Information, Table S1). G, H, specimen from El Rodeo, Catamarca Province (Cat-12-12 in Supporting Information, Table S1). Abbreviations: ACl, apical cluster of setae; BSe, blade-like seta; FSe, filiform setae; FoSe, foliform setae; Gc, gonocoxopodite; Gs, gonostylus; LGc, left gonocoxopodite; RGc, right gonocoxopodite; RSe, rod-like setae; SL, subapical lobe; vlSe, ventrolateral seta.

which does not always corroborate previously identified species and forms. The clade of two specimens morphologically identified as *Cx. coronator* consists of a well-supported pair of sequences (BSV 73%). The clade of specimens identified as *Cx. usquatus* is moderately supported by a BSV of 61%. Sequences from other specimens identified as *Cx. usquatus* are not grouped together. Lastly, the clade comprising a polytomy of four taxa, supported by BSV 86%, includes sequences derived from specimens identified as *Cx. coronator* and *Cx. usquatus* (from Brazil) and *Cx. coronator* Form 2 (from Argentina). All other groupings are weakly supported in both trees and a large polytomy of unresolved relationships results when all branches with BSV less than 60% are collapsed.

Figure 4. Gonocoxopodites of specimens of *Culex coronator* Form 2 collected in Argentina. A, specimen from Timbó Viejo, Tucumán Province (M057 in Supporting Information, Table S1). B, C, ventral aspect (B) and dorso-ventral aspect (C) of specimen from Las Juntas, Catamarca Province (Cat-12-08 in Supporting Information, Table S1). Abbreviations: ACl, apical cluster of setae; FSe, filiform setae; Gc, gonocoxopodite; Gs, gonostylus; RSe, rod-like setae; SL, subapical lobe; vlSe, ventrolateral seta.

The tree resulting from the Bayesian analysis of the *COI* mtDNA sequences is shown in Figure 6. Sequences from individuals of *Cx. coronator*, *Cx. camposi*, *Cx. usquatus*, *Cx. usquatissimus* and *Cx. coronator* Forms 1 and 2 comprise a strongly supported (Bayesian posterior probability = 1) but unresolved clade that falls within a polytomy with other species of subgenus *Culex*. The unresolved relationships within the clade comprising members of the *Cx. coronator* complex neither support nor refute the specific status of the nominal species and forms.

The Bayesian PTP analysis (Fig. 7) identified three possible species within the *Cx. coronator* complex, with a range of 9–18 species across all taxa. The individual Bayesian support values are low for two of the groups (0.36 and 0.37), but a group comprising three mosquitoes (GenBank accessions KJ812984, KJ812993 and KJ812991) exhibited relatively high support (0.85), although this was not reflected in the support values for the original Bayesian tree.

The complete mitochondrial genomes of the nine specimens belonging to the *Cx. coronator* complex ranged from 15 572 bp (*Cx. coronator*) to 15 576 bp (*Cx. coronator* Form 2) in length. The variation in length was found to be exclusively contained within the non-coding AT-rich control region in homopolymer repeats, and thus could be a result of sequencing error. All mitogenomes coded for the typical 13 protein-coding genes, 22 tRNAs, and the large and small rRNA genes as well as a large A-T–rich non-coding region. Gene order was conserved among all specimens and showed the same basic pattern as other culicids. Sliding window analysis (Fig. 8) showed consistently low levels of nucleotide variation across all gene-coding regions of the mitochondrial genome (98.7–99.9%) with the highest level of variation in the *ND5* gene, as found by Demari-Silva *et al.* (2015), suggesting that the *COI* barcode region is not a suitable marker for distinguishing members of the complex. Phylogenetic analysis of the mitochondrial protein-coding genes also failed to provide support for recognizing the five nominal forms of the complex as separate species (Fig. 9). Contrary to Demari-Silva *et al.*, the inclusion of additional taxa shows that *Cx. camposi* is sister to other members of the complex, with strong support. Although two additional clades comprising specimens from Argentina, one containing specimens Ju-14-01, Ju-14-04, Ju-14-05 and Ju-14-12 and the other containing Mis-14-01, Mis-14-04, Mis-14-07 and Ju-14-30, also exhibited strong support, the other relationships could not be adequately resolved.

DISCUSSION

TAXONOMIC HISTORY OF THE *Culex coronator* COMPLEX

Dyar & Knab (1906) described *Cx. coronator* as a new species based on a larva (Fig. 10A) with a long siphon (index 9) bearing pecten spines with basal denticles on the proximal 0.4 and a number of strong preapical spines, and the saddle of segment X with distinct spicules on the posterolateral margins (Fig. 10B). The subapical spines ('crown of spikes') was described as 'usually well developed, sometimes nearly obsolete', denoting a high degree of variation. The male genitalia were not described until Howard, Dyar & Knab (1915), who characterized the subapical lobe of the gonocoxite as a 'quadrate lobe at the outer third, bearing a row of eight rods' that are progressively smaller distally (Fig. 11A); gonostylus 'bearing a small terminal claw and two minute setae situated on inner face before the tip'; ventral arm of the phallosome 'long and curved' and the lateral arm with 'several large lamellæ [i.e. teeth], with angular rounded corners' (Fig. 11B). Howard *et al.* illustrated (pl. 17, fig. 216) but did not
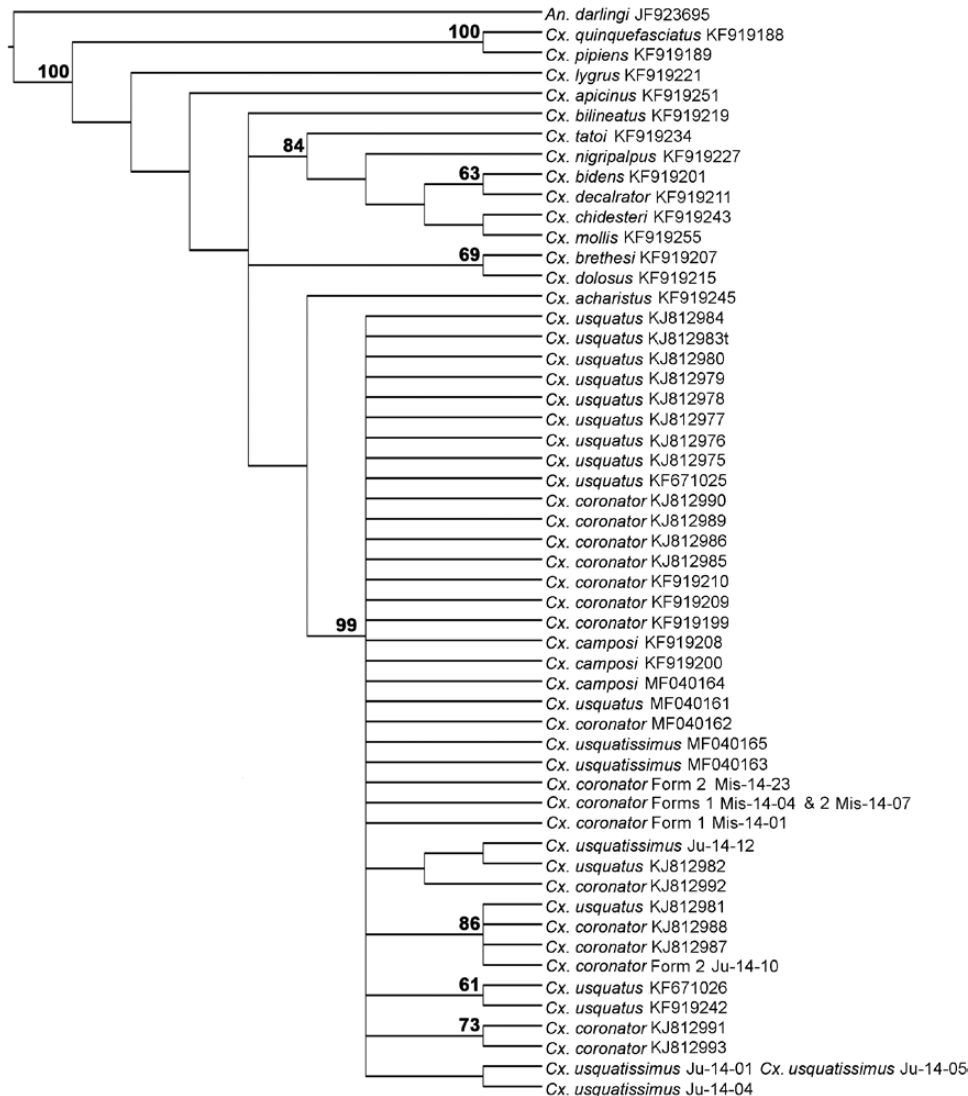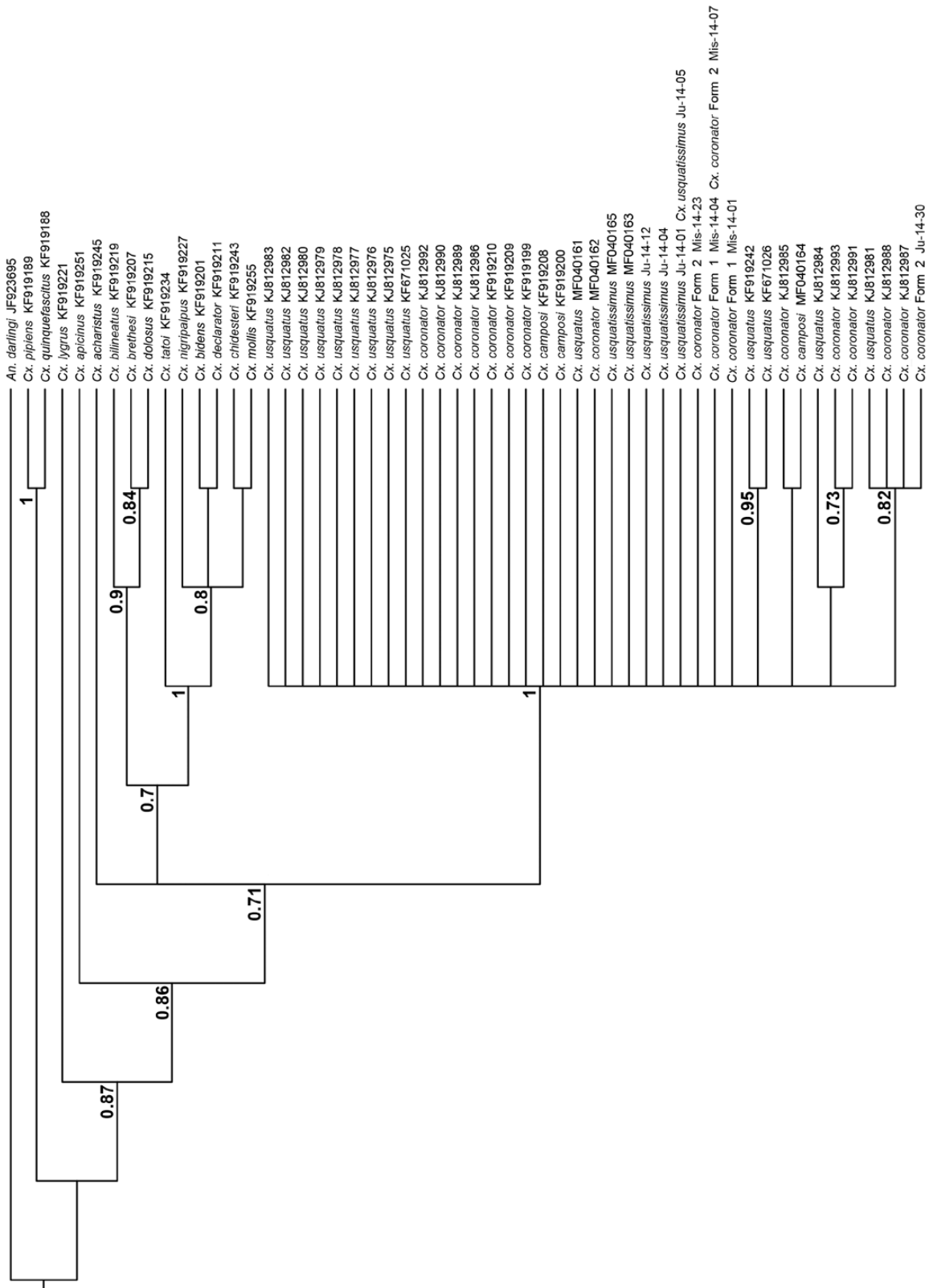
Figure 5. Strict consensus tree of 360 equally weighted most parsimonious trees of the *COI* mtDNA sequences from specimens of the *Culex coronator* complex collected in Argentina, Brazil and Ecuador, and the outgroup comprising 14 species of *Culex* (*Culex*) and *Anopheles darlingi* from the Neotropical Region. Bootstrap support values are indicated in bold (values less than 60% are not shown). See Supporting Information, Table S2, for GenBank accession numbers for the nine specimens of the *Cx. coronator* complex sequenced during the present study.

mention the cluster of small setae borne at the apex of the gonocoxite, which were also illustrated (but not mentioned) as being much longer (nearly half as long as the gonostylus) by Carpenter & LaCasse (1955: fig. 233). Because the specimens examined by Howard *et al*. are no longer available, we dissected the genitalia of the lectotype male of *Cx. coronator* (Fig. 12A) from St. Joseph, Trinidad and mounted them on a microscope slide in Canada balsam (Fig. 12B). The genitalia are described as follows: subapical lobe of the gonocoxite more or less divided with four similar stout rod-like setae on the proximal part of the lobe (toward the base of the gonocoxite) and several subequal filiform setae on the distal part of the lobe (toward the apex of the gonocoxite); gonocoxite with an apical cluster of fine setae that extend to mid-length of the gonostylus; the gonostylar claw is of the usual type for *Culex* (Fig. 12C); the dorsal arms of the phallosome are broad, flattened in the distal third and markedly longer than the lateral plates, the ventral arms are curved laterally and the lateral arms each have five to eight laterally bent, apically blunt, sclerotized teeth (Fig. 12D).

Figure 6. Bayesian tree of the *COI* mtDNA sequences from specimens of the *Culex coronator* complex collected in Argentina, Brazil and Ecuador, and the outgroup comprising 14 species of *Culex* (*Culex*) and *Anopheles darlingi* from the Neotropical Region. The data were partitioned by codon position. Numbers at branches indicate Bayesian posterior probabilities (>0.7). See Supporting Information, Table S2, for GenBank accession numbers for the nine specimens of the *Cx. coronator* complex sequenced during the present study.
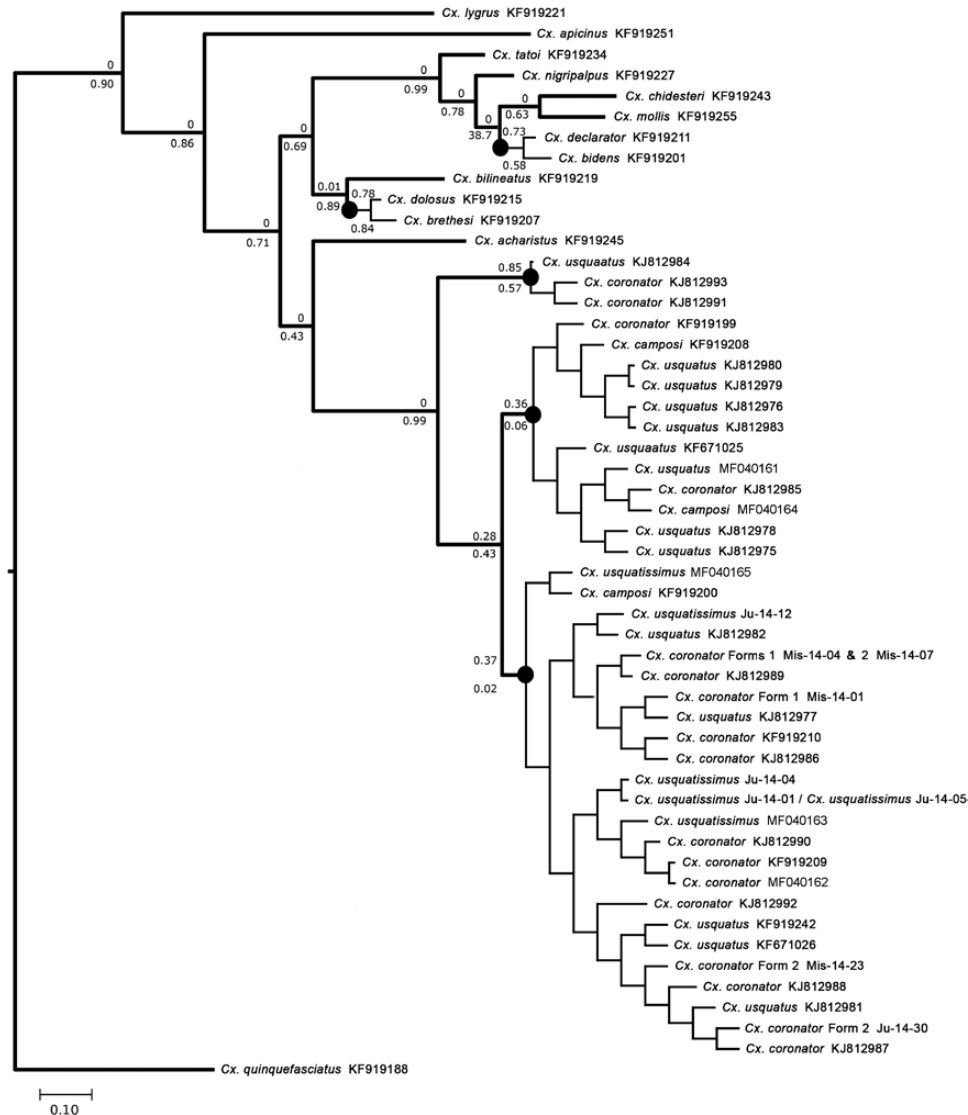
Figure 7. Bayesian Poisson tree process (bPTP) analysis. The 36 *COI* barcode sequences on a Mr Bayes metric gene tree showing the bPTP solution with the highest support recognizing 14 prospective species, including the outgroup. Values above each node represent bPTP support values indicating that all daughter sequences belong to a single species population. Values below the nodes represent Bayesian posterior probabilities indicating branch support. See Supporting Information, Table S2, for GenBank accession numbers for the nine specimens of the *Culex coronator* complex sequenced during the present study.

Culex ousqua was originally described and named by Dyar (1918a) as a variety of *Cx. coronator*. The larva of this form (Fig. 13A) has several subapical spines on one side of the siphon (Fig. 13B). The subapical lobe of the male genitalia (Fig. 14A) was characterized as consisting of a conical inner or proximal portion with 13 filiform setae and a smaller columnar outer or distal part with five setae (Fig. 14B). Dyar used the development of setae on the proximal part to distinguish *ousqua* from *coronator*, which has three rod-like setae as opposed to 13 filiform setae. He also mentioned that the dorsal and lateral arms of the phallosome were similar in the two varieties (Fig. 15). He did not mention the cluster of setae at the apex of the gonocoxite, which is much shorter in *ousqua*, shorter than half as long as the gonostylus. In the same year, Dyar (1918b) described *Cx. usquatus* as a new species stating explicitly that the adult is like the adults of *coronator* and *ousqua* and the larva has a long, slender siphon with two or three subapical spines much as in *ousqua*. He defined the species based on features of the male genitalia (Fig. 16), but did not illustrate them. He noted that the lateral plate was like the lateral plate of *Cx. coronator* illustrated by Dyar (1918a) and described
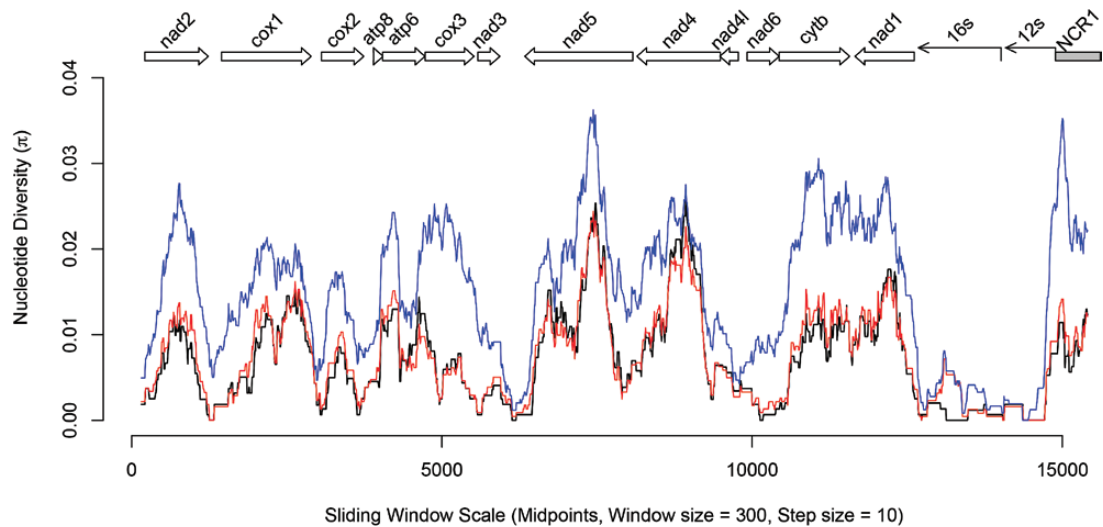
Figure 8. Sliding window analysis of the complete mitochondrial genomes from 14 specimens belonging to the *Culex coronator* complex and *Cx. quinquefasciatus*. Arrows indicate positions and directions of genes; only protein-coding and ribosomal gene locations are labelled. The black line depicts levels of nucleotide variation between specimens sequenced as part of this study. The red line depicts nucleotide variation between specimens from this study and those from Demari-Silva *et al*. (2015). The blue line shows levels of nucleotide variation between all 14 specimens of the *Cx. coronator* complex and *Cx. quinquefasciatus* (NC014574).
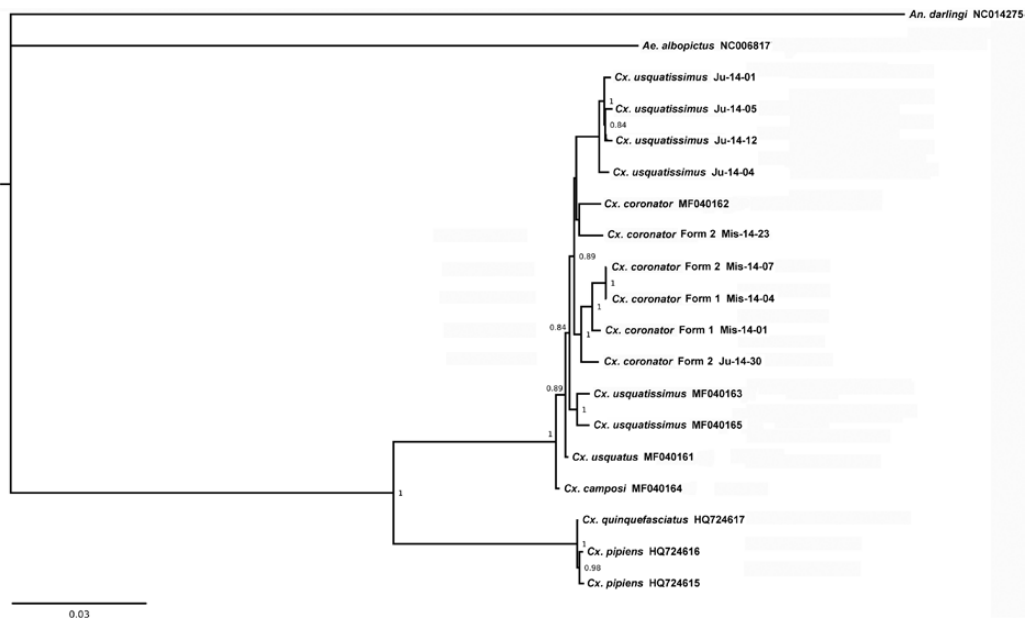

Figure 9. Bayesian tree from analysis of protein-coding genes of 14 specimens belonging to the *Culex coronator* complex plus *Cx. pipiens* and *Cx. quinquefasciatus*. The mitochondrial genomes of *Aedes albopictus* and *Anopheles darlingi* were used as outgroup. Only nodes supported by posterior probabilities greater than 0.75 are annotated. See Supporting Information, Table S2, for GenBank accession numbers for the nine specimens of the *Cx. coronator* complex sequenced during the present study.

the subapical lobe as follows: 'the lobe of the side-piece [i.e. gonocoxite] is scarcely divided, having outwardly [i.e. distal part] a group of five spines, inwardly [i.e. proximal part] the three usual rods, but supplemented basally by a stout spine and a seta; the tip of the side-piece bears a dense group of about eight stout setae, situated upon a rounded prominence'. Word choice and phraseology aside, examination of the genitalia
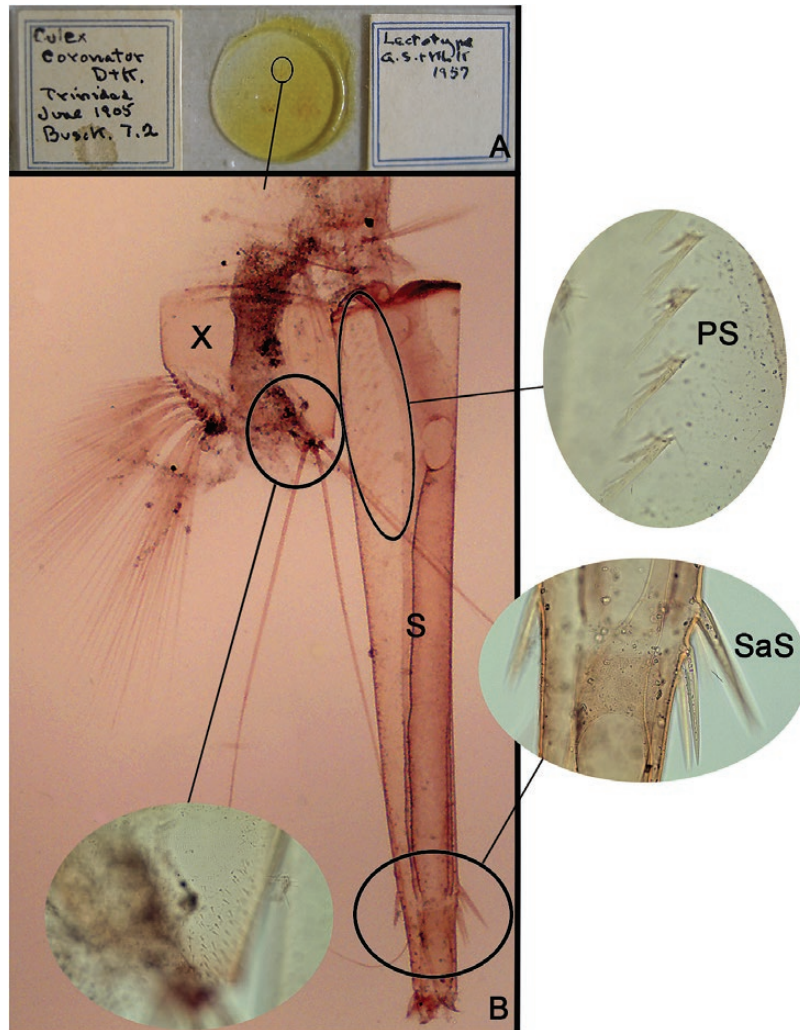
Figure 10. Lectotype of *Culex coronator* Dyar & Knab. A, microscope slide bearing the larval exuviae. B, detail of segment X and subapical spines of the siphon. Abbreviations: S, siphon; SaS, subapical spines; X, segment X.
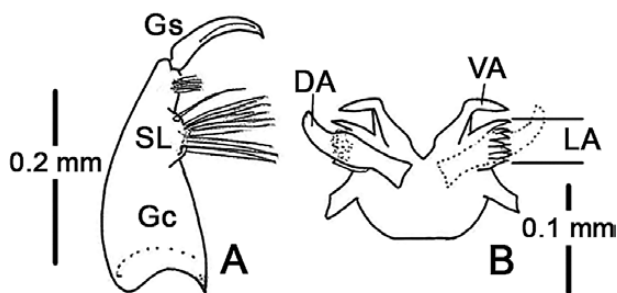

Figure 11. Male genitalic structures of *Culex coronator* Dyar & Knab (after Forattini, 2002). (A) Gonocoxopodite. (B) Phallosome. Abbreviations: DA, dorsal arm; Gc, gonocoxite; Gs, gonostylus; SL, subapical lobe; VA, ventral arm.

of the lectotype male (Fig. 16A) and the gonocoxite illustrated by Dyar (1922: fig. 2) corroborates Dyar's (1918a) description: subapical lobe scarcely divided (Fig. 16B), proximal part with five rod-like setae and distal part with five or more subequal filiform setae; apex of gonocoxite with a prominence bearing a tuft of setae that extend to mid-length of the gonostylus (Fig. 16B).

Dyar (1922) described *Cx. usquatissimus* as a 'new form' but listed it as a binomen, thus constituting the name of a species (Fig. 17). The setae of the cluster at the apex of the gonocoxite are very long, extending beyond mid-length of the gonostylus, the subapical lobe bears three rod-like setae proximally and distomesally three flattened blade-like setae on a small swelling distinctly separated from the proximal part (Fig. 17B, C). The flattened blade-like form of the three distal setae has not been noted before now.

Dyar (1922) observed that 'the mesosomal [i.e. phallosomal] structures of the male genitalia are identical.
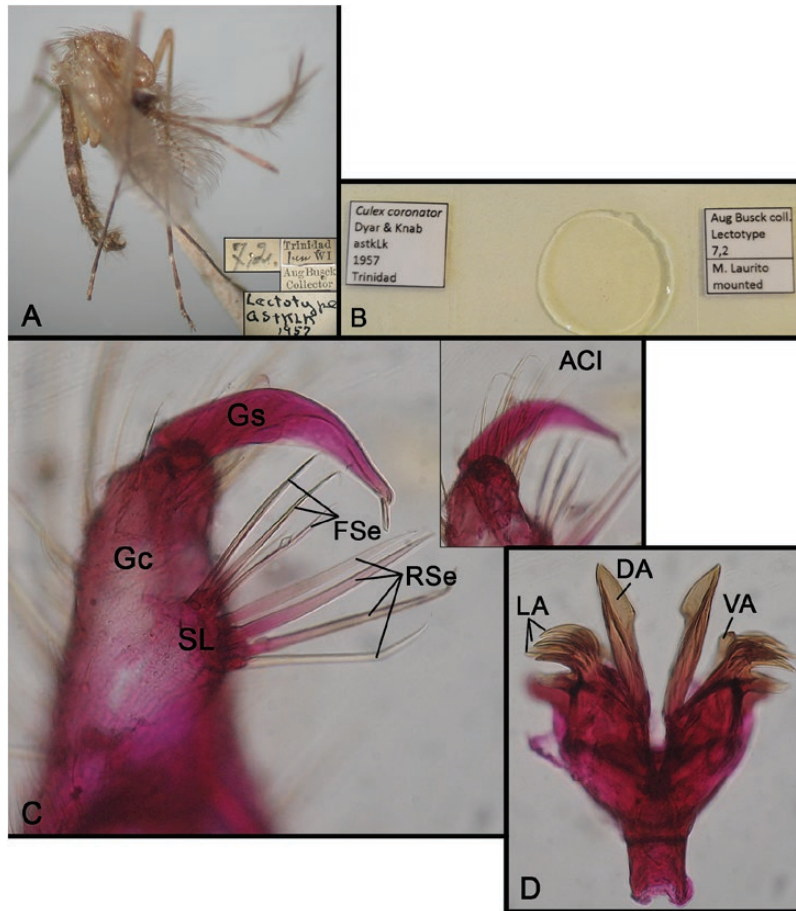
Figure 12. Lectotype male of *Culex coronator* Dyar & Knab. A, pin-mounted specimen from St. Joseph, Trinidad before dissection of its genitalia. B, microscope slide bearing the dissected genitalia. C, detail of the gonocoxopodite. D, detail of the lateral plate of the phallosome. Abbreviations: ACl, apical cluster of setae; DA, dorsal arm; FSe, filiform setae; Gc, gonocoxopodite; Gs, gonostylus; LA, lateral arm; RSe, rod-like setae; SL, subapical lobe; VA, ventral arm.

Variation occurs in the setae of the lobe of the side-piece [i.e. gonocoxite] and the apex'. He did not note the degree of separation of the proximal and distal setae of the subapical lobe. This feature, however, is not useful for identification of the nominal species because it is highly variable, with evidence of gradation between males of the same species reared from larvae collected together from the same habitat. It is noteworthy that 'transitional forms' between *Cx. ousqua* and *Cx. usquatus* have been recognized in Suriname and Venezuela (Bonne & Bonne-Wepster, 1925).

Culex camposi was originally described as a race of *Cx. coronator* (Dyar, 1925a). The description was based on a single male from Ecuador, with dissected genitalia (Fig. 18A, B). The proximal part of the subapical lobe bears five slender rod-like setae, two shorter than the other three. The distal part, a small prominence located well distomesad from the proximal part, bears a single strong seta (Fig. 18C). Dyar assumed

that the specimen was the 'normal form' of *Cx. coronator* in Ecuador. Although not mentioned, the cluster of setae at the apex of the gonocoxite extends beyond mid-length of the gonostylus (Fig. 18C).

After further study and consideration, Dyar (1925b) synonymized *Cx. camposi*, *Cx. ousqua*, *Cx. usquatus* and *Cx. usquatissimus* with *Cx. coronator* stating that 'The names in synonymy indicate various forms of the male hypopygium [i.e. genitalia]'. Bonne & Bonne-Wepster (1925), in their treatment of the mosquitoes of Suriname, listed the latter three as synonyms of *Cx. coronator* (*Cx. camposi* was not included, probably because the authors were not aware that it was described in the same year their article was published, or because it was not known to occur in Suriname). They stated that 'Dyar gives these forms [including *coronator*] sub-specific names' and provided a comparative listing of the characteristics of setae on the proximal and distal parts of the subapical lobe (as inner and outer divisions respectively) and at the apex
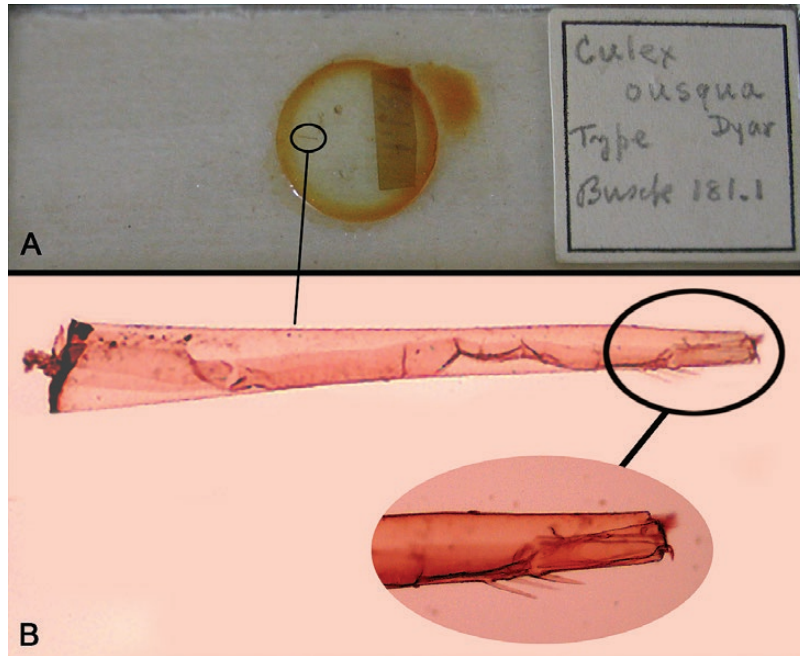
Figure 13. Holotype male of *Culex ousqua* Dyar. A, microscope slide bearing the larval exuviae. B, detail of spicules on one side of the siphon.

of the gonocoxite for the four forms. They did not note differences in the separation of the proximal and distal parts of the subapical lobe. They described the female, male, male genitalia and larva of the polymorphic *Cx. coronator*, which reveals some inconsistencies with the original diagnoses of the species, possibly because their study did not include the examination of type material.

Another contribution to the recognition of variant forms of *Cx. coronator* was made by Root (1927), who found two forms in Brazil ('typical *coronator*' and 'atypical *coronator*') 'agreeing exactly in adult coloration and in the structure of the mesosome [i.e. phallosome], but differing decidedly in the structure of the lobe of the side-piece [i.e. gonocoxite]'. He further stated that the lobe 'is so variable in *coronator* that it seems unnecessary to designate the two forms by different names, but the facts are perhaps worth recording, since no intermediate forms were seen'. He described the 'typical' form as having the subapical lobe 'distinctly divided into two portions', the proximal portion with one slender and two stout rod-like setae and the distal portion with a 'large group of setae'. He described the apical cluster of setae on the gonocoxite as 'a small patch of short hairs, about half as long as the setae of the lobe' and the siphon of the larva as having 'a considerable number of spines in the "crown", just before the tip'. In contrast, Root described the subapical lobe of the 'atypical' form as 'curved or horse-shoe-shaped, with the upper [distal]

arm nearly vertical and the lower [proximal] one transverse', bearing a line of 'long, stout setae [that] runs all around the curve, but no definite rods and no division of the lobe'. He described the cluster of setae at the apex of the gonocoxite as being longer, about as long as the setae of the subapical lobe, and borne 'more apically'. Root had a single larval exuviae of this form, with a single subapical spine on the posterior surface. The 'atypical' males were collected at five localities on different dates in Río de Janeiro State, highlighting its distinction from the 'typical' form.

Dyar (1928), while recognizing *Cx. coronator* as a single species, noted that the subapical lobe varies from being 'entire[ly] or indistinctly divided'. Lane (1953) agreed with Dyar that variation and gradation in the number and positions of setae on the subapical lobe supported the existence of a single species. He consequently recognized *camposi* as a subspecies of *Cx. coronator* based on the reduced number of setae and considered *Cx. ousqua*, *Cx. usquatissimus* and *Cx. usquatus*, as well as *Cx. albertoi* Anduze (described in Anduze, 1943), as synonyms of the nominotypical form. The following year, Vargas & Martinez Palacios (1954) described *Cx. coronator mooseri* as a new subspecies.

Culex coronator was subsequently considered as a single, widely distributed species until Bram (1967), who 'critically examined' and associated the characteristics of male genitalia with different geographic locations. He concluded: 'Consideration of the
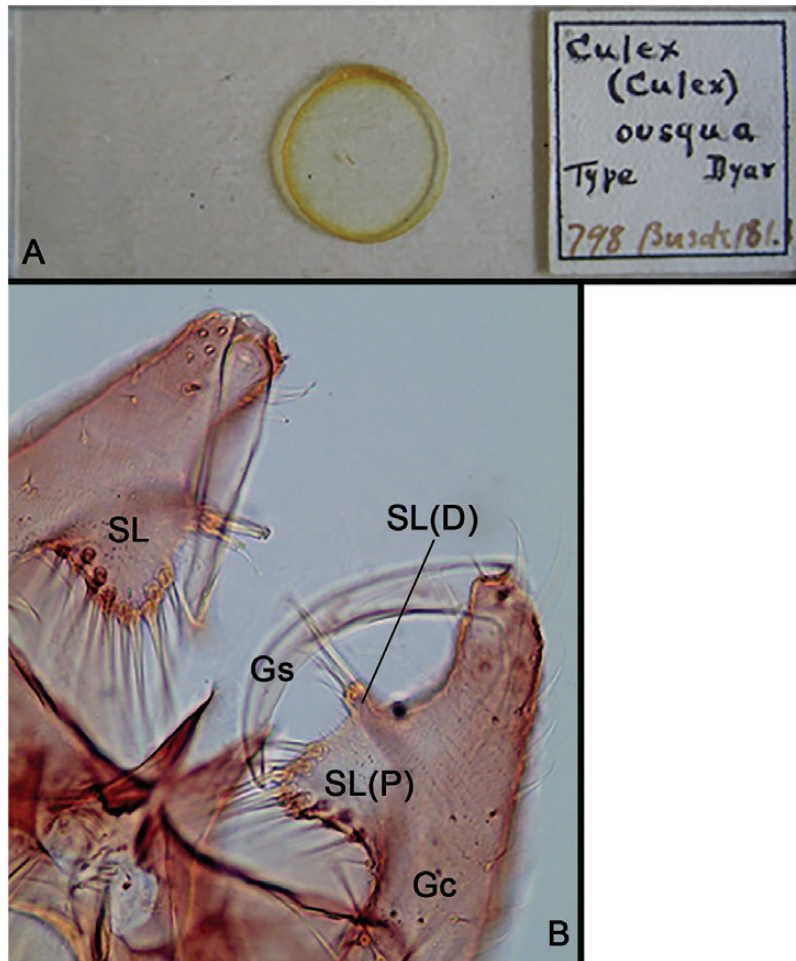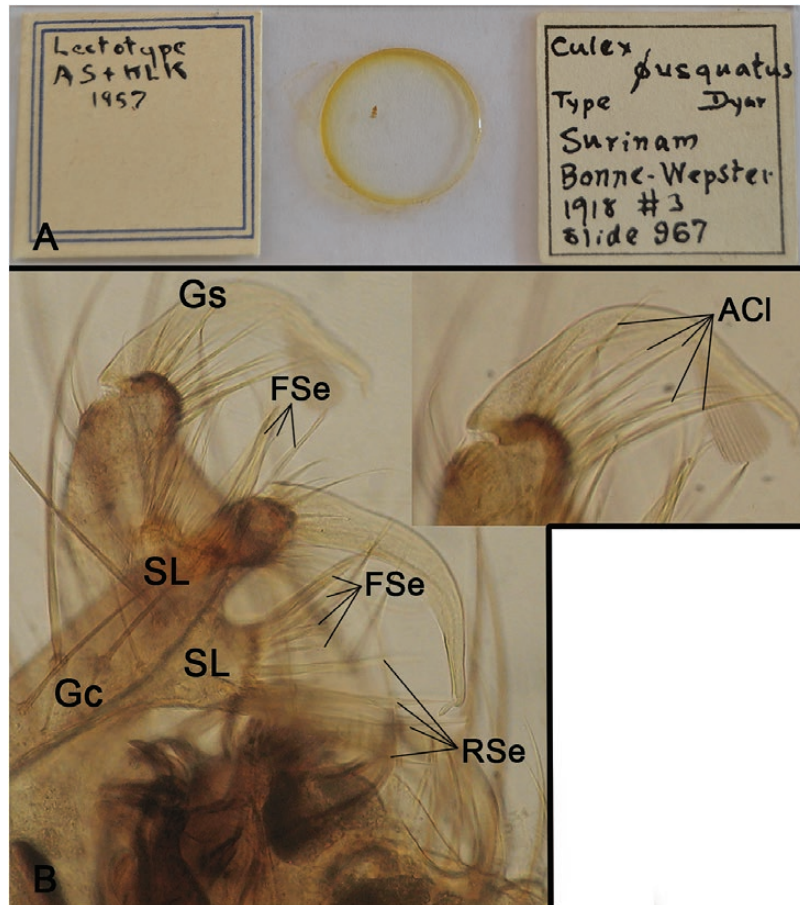
Figure 14. Holotype male of *Culex ousqua* Dyar. A, microscope slide bearing the dissected genitalia. B, detail of the gonocoxopodite. Abbreviations: Gc, gonocoxite; Gs, gonostylus; SL, subapical lobe; SL(D), subapical lobe (distal part); SL(P), subapical lobe (proximal part).
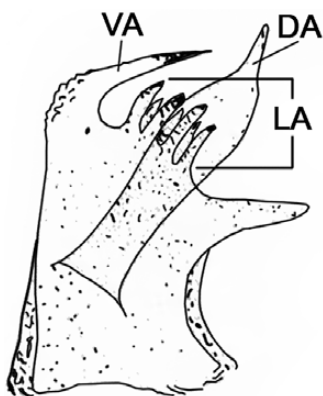
Figure 15. Lateral plate of *Culex coronator* Dyar (after Dyar, 1918a: pl. IV, fig. 9). Abbreviations: DA, dorsal arm; LA, lateral arm; VA, ventral arm.

morphological differences and the geographical distribution of each taxon reveals that the various forms are not randomly distributed throughout the areas, but assume discrete distributional patterns. All forms are sympatric in area IV (Colombia), but in no other collection area have all forms been found. Thus, the morphological and distributional data suggest that speciation has occurred, and it is for this reason' that he reinstated *Cx. camposi*, *Cx. ousqua*, *Cx. usquatissimus* and *Cx. usquatus* as valid species of the 'Culex coronator Complex', with *Cx. coronator mooseri* and *Cx. albertoi* as synonyms of *Cx. coronator* and *Cx. ousqua*, respectively. As noted above, Forattini (2002) distinguished the species of the *Cx. coronator* complex based on the length of the setal cluster at the apex of the gonocoxite and the ornamentation of the subapical lobe. He agreed with Bram that the division of the lobe is not a
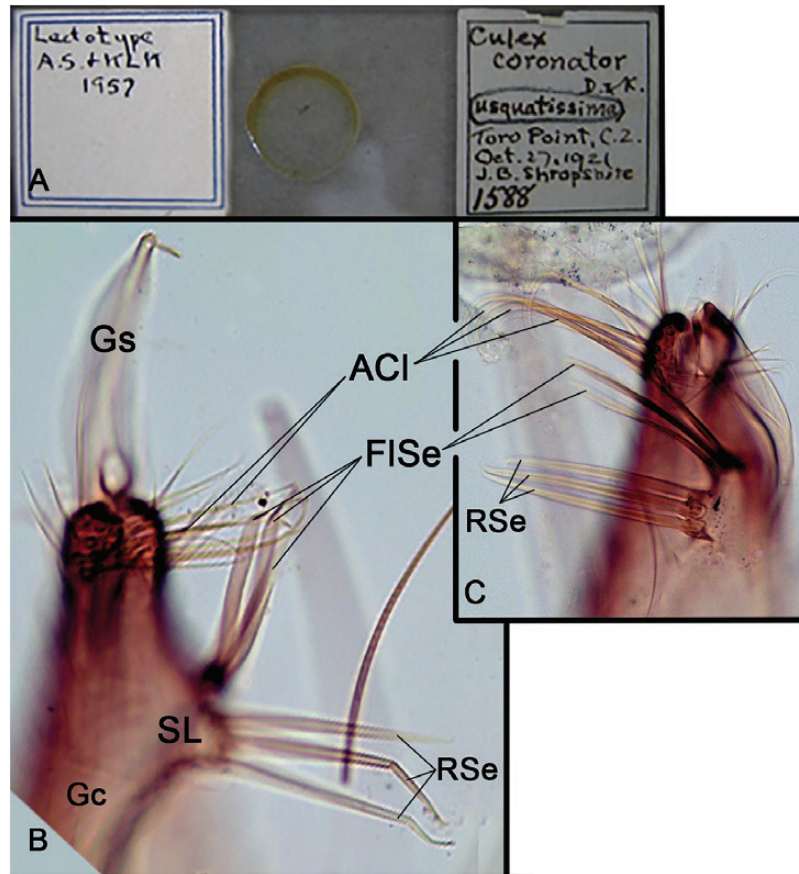
Figure 16. Lectotype male of *Culex usquatus* Dyar. A, microscope slide bearing the dissected genitalia. B, detail of the gonocoxopodite (lateral aspect). Abbreviations: ACl, apical cluster of setae; FSe, filiform setae; Gc, gonocoxopodite; Gs, gonostylus; SL, subapical lobe; RSe, rod-like setae.

relevant trait due to high variability. Neither Forattini nor later authors have contributed new information about the distributions of the species; however, it is noteworthy that Linton *et al.* (2013) collected larvae of *Cx. usquatus* and *Cx. camposi* from the same habitat in Amazonian Ecuador. The two species were identified based on the male genitalia of specimens reared from the larvae.

Our critical assessment of published descriptions and study of type material revealed the following errors and problems attributable to Bram (1967): (1) He characterized *Cx. coronator* as having a short cluster of setae at the apex of the gonocoxite, resulting in misidentifications by other authors. The setal cluster is long in *Cx. coronator*. (2) He examined the male genitalia of 227 specimens but did not mention, and there is no evidence, that he examined type specimens. (3) Bram states that rod-like setae are absent in *Cx. usquatus* and only a group of 10–15 subequal setae are borne on the subapical lobe, which disagrees with the description of Dyar (1918b). (4) He used locality data

for the 227 specimens to plot 'probable' distributions, which he used in concert with morphological features of the male genitalia to formally recognize *Cx. camposi*, *Cx. coronator*, *Cx. ousqua*, *Cx. usquatissimus* and *Cx. usquatus* as valid species. He maintained that the morphological differences and the provenance of the material examined indicated the species have 'discrete distributional patterns', noting that all forms were sympatric in Colombia but 'in no other collection area have all forms been found'. This latter statement is misleading because Bram's distribution maps (his fig. 11B–F) show that the 'probable distribution of species', extrapolated from ten areas where collections had been made (Fig. 19), overlap one or other in various countries. Of the ten collection areas (Fig. 19), *Cx. coronator* is the only species that occurs in area I (southern Texas, USA); two or more species have overlapping distributions in each of the other nine collection areas. Two or more of the five nominal forms, as well as *Cx. coronator* Forms 1 and 2 described above, not only have overlapping distributions in Colombia
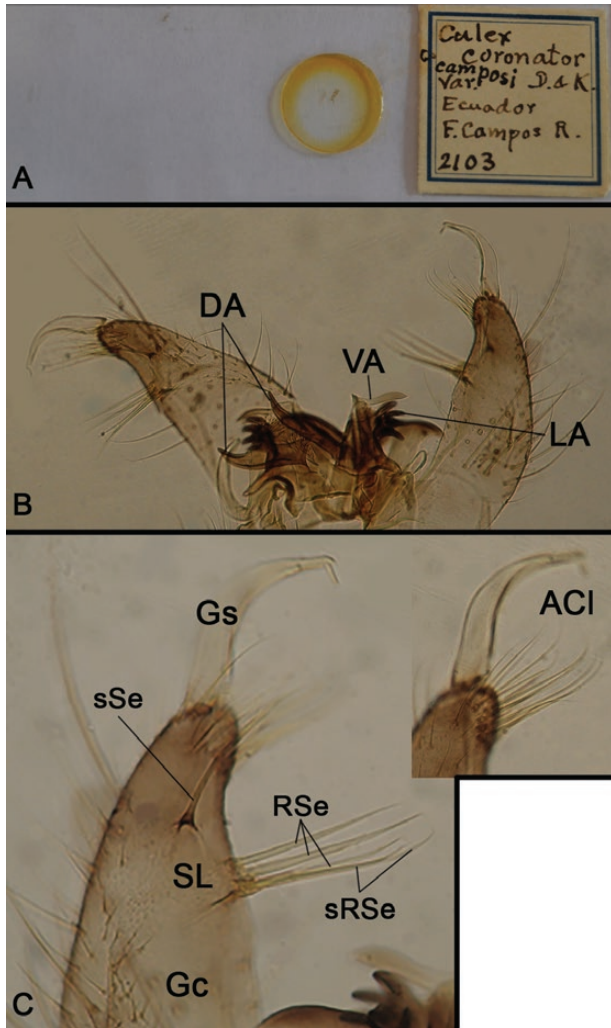
Figure 17. Lectotype male of *Culex usquatissimus* Dyar. A, microscope slide bearing the dissected genitalia. B, detail of the gonocoxopodite (lateral aspect). C, detail of the gonocoxite (medial aspect). Abbreviations: ACl, apical cluster of setae; FlSe, flattened blade-like setae; Gc, gonocoxopodite; Gs, gonostylus; RSe, rod-like setae; SL, subapical lobe.

(Bram, 1967), Ecuador (Linton *et al.*, 2013) and Argentina (Fig. 20), but also exhibit biotic sympatry, that is, the immature stages are found together in the same habitat (Supporting Information, Table S5).
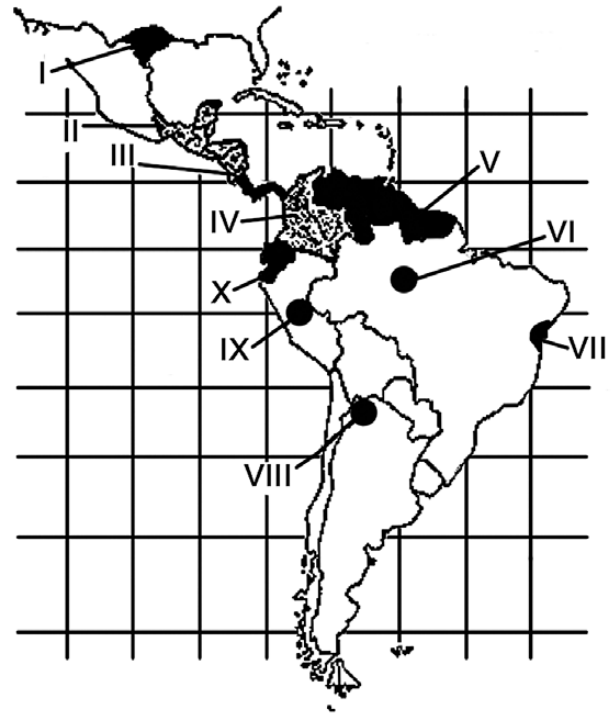
SPECIES OR SPECIES COMPLEX?

Ruiz-Lopez *et al.* (2012) considered K2P values of 0.2–1.4% as an indicator of intraspecific variation and K2P values greater than 2.0% as a measure of interspecific divergence between members of the *Anopheles albitarsis* group of the subgenus *Nyssorhynchus*. Similar values have been used as a measure of intraspecific variation in other mosquito studies, for example, 0.00–2.58% for the *Anopheles strodei* subgroup of subgenus *Nyssorhynchus* (Bourke *et al.*, 2013), 0.00–2.40% for 32 species in Pakistan (Ashfaq *et al.*, 2014), 0.00–2.52% for 24 species in Belgium (Versteirt *et al.*, 2015), 0.00–2.30% for 13 *Culex* species in Turkey (Gunay *et al.*, 2015) and particularly for species of subgenus *Culex*, 0.00–2.80% (Tahir, Kanwal & Mehwish, 2016). In the present study, the K2P values for the entire

COI dataset for the *Cx. coronator* complex range from 0.00 to 2.67%, denoting intraspecific variation. The values do not reach 1.0% for the five nominal forms that are currently recognized as species. The largest relative divergence is less than half of the sequence threshold proposed by Hebert *et al.* (2004) to separate specimens that belong to different species. Results similar to those reported here were obtained when Demari-Silva (2014) used the *COI* barcode region to distinguish Brazilian specimens morphologically identified as *Cx. coronator* and *Cx. usquatus*. The authors obtained an interspecific *COI* distance of 0.96% (0.91% was obtained in the present study). More recently, Demari-Silva *et al.* (2015) sequenced the mitochondrial genomes of males from Brazil morphologically identified as *Cx. camposi* (one from Mato Grosso do Sul State), *Cx. coronator* (one from Rio Grande do Sul State), *Cx. usquatissimus* (one from Acre State and one from Rôndonia State) and *Cx. usquatus* (one from São Paulo State). Separate Bayesian analyses of 13 protein-coding genes and the *ATP6*, *ATP8* and *NADH5* protein-coding genes yielded trees in which each of the

Figure 18. Holotype male of *Culex camposi* Dyar. A, microscope slide bearing the dissected genitalia. B, phallosome and gonocoxopodites. C, detail of the gonocoxopodite (lateral aspect). Abbreviations: ACl, apical cluster of setae; DA, dorsal arm; Gc, gonocoxopodite; Gs, gonostylus; LA, lateral arm; RSe, rod-like setae; SL, subapical lobe; sRSe, shorter rod-like setae; sSe, single separated seta; VA, ventral arm.

I: *Cx. coronator*
II: *Cx. coronator, Cx. usquatus, Cx. ousqua*
III: *Cx. coronator, Cx. usquatus, Cx. ousqua, Cx. usquatissimus*
IV: *Cx. coronator, Cx. usquatus, Cx. ousqua, Cx. usquatissimus, Cx. camposi*
V: *Cx. coronator, Cx. usquatus, Cx. usquatissimus*
VI, VII, VIII: *Cx. coronator, Cx. usquatus*
IX: *Cx. usquatus, Cx. camposi*
X: *Cx. usquatissimus, Cx. camposi*

Figure 19. Map of Bram (1967: fig. 11A, modified only by adding genus abbreviations) showing the ten areas (I–X) where the 227 specimens he studied were collected.

four forms were supported by posterior probabilities of 1. However, the authors concluded that 'the low levels of diversity shown herein, demonstrates that the utility of sequences of the mitochondrial gene [*lapsus* for genome] needs further evaluation in future studies employing a larger sample size and other species of the subgenus *Culex*'. This was done in the present study. As noted in the Results section, the unresolved relationships within the clade comprising members of the *Cx. coronator* complex recovered in the Bayesian analysis of *COI* sequences neither support nor refute the specific status of the nominal species and forms, and

the phylogenetic analysis of the mitochondrial protein-coding genes failed to provide support for recognizing the five nominal forms of the complex as separate species. In concert with these results, Demari-Silva *et al.* (2017) recently conducted a study that included a Bayesian analysis of a dataset consisting of two mitochondrial genes (*COI, NADH5*) and two nuclear genes (*CAD, hunchback*) and Bayesian inference of demographic parameters under an Isolation-Migration model to examine population evolution. The analyses produced a tree in which morphologically identified specimens of *Cx. coronator* and *Cx. usquatus* comprised four clades, three of which included specimens of both species, and evidence of a degree of gene flow, suggesting the need for additional taxon sampling.

In many species-rich groups of animals, male genitalia provide the best means for distinguishing species. Thus, evolution of genital form is thought to be involved in the origin of species by providing a morphological impediment to successful mating between individuals of
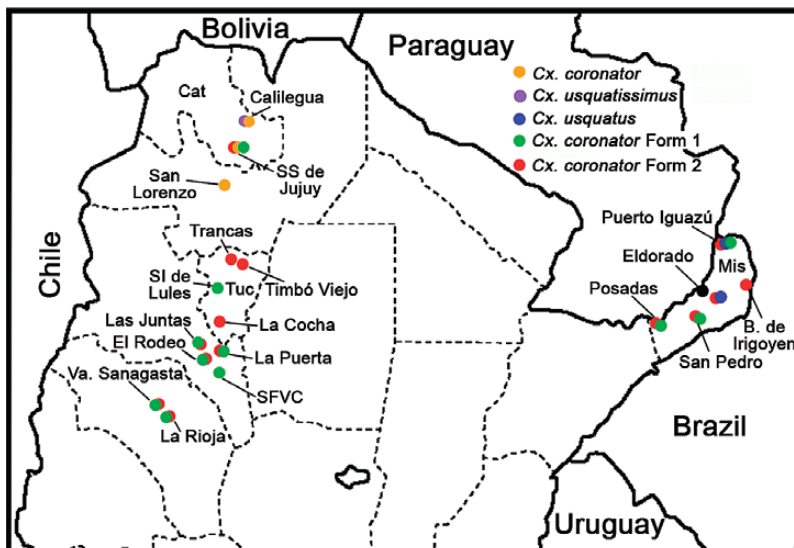
Figure 20. Localities where the three nominal species and two forms of the *Culex coronator* complex have been collected in northern Argentina since 1997.

different species. The new intermediate forms described herein provide additional evidence that various genital forms occur within and among populations and are not indicators of reproductive isolation – they are examples of intraspecific variation. In the phylogenetic study of Argentinian species of *Culex* (*Culex*) conducted by Laurito & Almirón (2013), the five nominal forms of the *Cx. coronator* complex (their "Coronator Group" excluding *Cx. covagarciai* Forattini) were recovered in an unresolved polytomy that also included *Cx. paramaxi* Duret and *Cx. brevispinosus* Bonne-Wepster & Bonne. It is likely that the inclusion of *Cx. paramaxi* and *Cx. brevispinosus* was due to the large number of morphological characters that could not be coded for these two species. Disregarding their inclusion, the *Cx. coronator* complex was recovered as monophyletic but there was no support for the specific status of the five forms.

Evidence from the morphological and molecular data obtained during the present study leads us to at least provisionally conclude that the *Cx. coronator* complex is a single polymorphic species. The morphological data appear to support the conclusion of Dyar (1925a) that 'the names in synonymy indicate various forms of the male genitalia'. Bram (1967) resurrected the nominal forms from synonymy and established the *Cx. coronator* complex based on disputable evidence, as explained above. At least two forms (*Cx. coronator* and *Cx. usquatus*) cannot be definitely distinguished due to variation in the number of setae on the subapical lobe, and they also share features, particularly the newly discovered ventrolateral seta, with two new variants that are now known to occur in certain areas (Argentina and Ecuador) within the extensive range of *Cx. coronator* (Fig. 19; also Bram, 1967: fig. 11B). Furthermore, the forms are sympatric in Colombia, Argentina (Fig. 2) and Ecuador (Linton *et al.*, 2013), and the immature stages are known to occur together in the same habitat. Although the Bayesian phylogenetic trees recovered in the mitogenomic study of Demari-Silva *et al.* (2015) indicate that specimens of the *Cx. coronator* and *Cx. pipiens* complexes comprise independent clades, the individual branches of each clade correspond to single specimens, which may or may not be representatives of different species.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of specimens used for morphological study.
Table S2. Information for specimens used for molecular analysis.
Table S3. K2P distances between 41 *COI* mtDNA sequences for specimens of the *Cx. coronator* complex from Argentina, Brazil and Ecuador.
Table S4. Mean pairwise K2P distances among four nominal species and two forms of the *Cx. coronator* complex.
Table S5. Collection areas in the Central and South Americas shared by forms of the *Cx. coronator* complex.