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Research Article

Phylogeny of the nominotypical subgenus of *Culex* (Diptera: Culicidae): insights from analyses of anatomical data into interspecific relationships and species groups in an unresolved tree

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The aim of this study was to produce the first objective and comprehensive phylogenetic analysis of the speciose subgenus *Culex* based on morphological data. We used implied and equally weighted parsimony methods to analyse a dataset comprised of 286 characters of the larval, pupal, and adult stages of 150 species of the subgenus and an outgroup of 17 species. We determined the optimal support by summing the GC supports for each MPC, selecting the cladograms with the highest supports to generate a strict consensus tree. We then collapsed the branches with GC support < 1 to obtain the 'best' topography of relationships. The analyses largely failed to resolve relationships among the species and the informal groups in which they are currently placed based on morphological similarities and differences. All analyses, however, support the monophyly of genus *Culex*. With the exception of the Atriceps Group, the analyses failed to find positive support for any of the informal species groups (monophyly of the Duttoni Group could not be established because only one of the two species of the group was included in the analyses). Since the analyses would seem to include sufficient data for phylogenetic reconstruction, lack of resolution appears to be the result of inadequate or conflicting character data, and perhaps incorrect homology assessments. Molecular and other biological data are needed to gain insights into the evolution of subgenus *Culex*. Nevertheless, we discuss the placement of several taxa in the current morphology-based classification of the subgenus based on insights realized during the study.

Key words: cladistics, *Culex*, mosquitoes, phylogeny, systematics, taxonomy

Introduction

The cosmopolitan genus *Culex* (Diptera: Culicidae: Culicinae: Culicini) is a large and important group of mosquitoes. It includes 770 species divided among 26 subgenera. The nominotypical subgenus includes 200 species (Harbach, 2016a) arranged in an intuitive, informal classification of species groups, subgroups, and complexes (Harbach, 2011; updated by Harbach, 2016b). A number of published and unpublished phylogenetic studies based on morphological and molecular data support the monophyly of all of the subgenera except *Culex* and *Neoculex* (Harbach & Kitching, 1998; Juthayothin, 2004; Mallampalli, 1995; Miller et al., 1996; Navarro & Liria, 2000; St John, 2007). Species of subgenera *Acallyntrum*, *Allimanta*, *Barraudius*, *Eumelanomyia*, *Kitzmilleria*, *Oculeomyia*, *Phenacomyia*, *Phytotelmatomyia*, and *Sirivanakarnius* have been placed in clades with species of subgenus *Culex* in various morphological and molecular studies (Demari-Silva et al., 2011; Deus, 2009; Harbach et al., 2012; Kitching et al., 2015; Laurito & Almirón, 2013; Rossi & Harbach, 2008; Vesgueiro et al., 2011).

Subgenus *Culex* has only been examined on a worldwide basis by Edwards (1932), who divided it into two groups: the Sitiens Group, confined to the Old World, and the Pipiens Group, represented in the Old and New Worlds. Both groups are highly complex assemblages and include species that do not readily fit into either group. Three additional groups have been recognized subsequently for species that Edwards recognized as members of the Pipiens Group: the Guiarti Group (Edwards, 1941) for several Afrotropical species, the Atriceps Group (Belkin, 1962) for three South Pacific species, and the Duttoni Group (Harbach, 1988) for the unusual Afrotropical *Culex duttoni*

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and the closely related *Cx. watti*. These groups are diagnosed principally by the presence/absence of a few salient anatomical features of the adults. The Sitiens Group (*sensu* Edwards, 1932) includes mosquitoes (50 species) that lack lower mesepimeral setae, the proboscis has a well-defined median pale band, the tarsi have narrow pale rings at the joints, and the lateral plates of the male genitalia are denticulate. Mosquitoes that have lower mesepimeral setae (usually only one) and lack the other features that characterize the Sitiens Group are classified as members of the Pipiens Group (*sensu* Edwards, 1932). The Guiarti Group includes seven African species that are distinguished from other species of subgenus *Culex* in Africa (except *Cx. macfiei*) by the more strongly verticillate antennae of females and the longer than usual first flagellomere of both sexes. Species of the Atriceps Group, which are 'isolated far out in the South Pacific' (Belkin, 1962), resemble those of the Pipiens Group but exhibit features that suggest the group 'arose through hybridization between members of the *pipiens* and *sitiens* groups' (Belkin, 1962). Species of the Duttoni Group share the presence of lower mesepimeral setae with members of the Pipiens Group and ringed proboscis and tarsi with members of the Sitiens Group, but the lateral plates of the male genitalia are uniquely developed. Heinemann and Belkin (1977, and later publications) recognized two groups in the Neotropical Region, the Declarator and Inflictus Groups, but did not diagnose them or indicate which species they include. Strickman (1990) alluded to the Declarator Group, but he also did not mention which species comprise the group.

The internal classification of the subgenus (Table S1, see online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/xx.xxxx/xxxxxxxx.xxxx.xxxxxx) is based principally on the intuitive taxonomic categorizations of Belkin (1962), Bram (1967a, 1967b), Edwards (1932, 1941), Harbach (1988), Sirivanakarn (1976) and Tanaka et al. (1979), but the inclusion of many species in groups is problematic. Most species of the Pipiens and Sitiens Groups are placed in subgroups based on overt morphological similarity and are principally polythetic assemblages diagnosed by combinations of relatively few anatomical characters. Twenty-six species of the Pipiens Group and 10 of the Sitiens Group are not placed in subgroups, and it is not known to which group, if either, nine species should be placed (Table S1, see supplemental material online). Available evidence (Demari-Silva et al., 2011; Deus, 2009; Harbach & Kitching, 1998; Harbach et al., 2012; Juthayothin, 2004; Kitching et al., 2015; Laurito & Almirón, 2013; Mallampalli, 1995; Miller et al., 1996; Navarro & Liria, 2000; Rossi & Harbach, 2008; St John, 2007; Vesgueiro et al., 2011) indicates that the subgenus is polyphyletic and has been retained solely as a 'taxon of convenience'. Hence, the aim of the present study was to investigate the relationships of the included species, their assignment to species groups and subgroups, and therefore to determine if the internal classification reflects natural relationships – in effect testing of the monophyly of the informal groups while providing a foundation for explicit hypotheses of phylogenetic relationships to be conceived and tested in future studies.

Materials and methods

Morphology

The 200 species of subgenus *Culex*, with their authorships and placement in species groups, subgroups and complexes, are listed in Table S1 (see supplemental material online). We obtained morphological data (characters listed in Appendix S1, see supplemental material online) for 196 species, including the outgroup (listed in Appendix S2, see supplemental material online). Twenty-one species of subgenus *Culex* were not available for study (denoted in Table S1, see supplemental material online) and 29 species (highlighted in Appendix S2, see supplemental material online) with insufficient data (principally unknown or unavailable life stages) were excluded from the analyses; thus, the final dataset comprised an ingroup of 150 species of subgenus *Culex* and an outgroup of 17 species. The ingroup taxa include species of all informal species groups and subgroups of subgenus *Culex* (Harbach, 2016b). The outgroup includes three species of tribes other than Culicini, i.e., *Culiseta annulata* (tribe Culisetini), *Maorigoeldia argyropus* (tribe Sabethini) and *Psorophora ciliata* (tribe Aedini), a genus of tribe Culicini other than *Culex*, i.e., *Lutzia bigoti*, and 13 species of 11 other subgenera of *Culex*, i.e., *Allimanta*, *Barraudius*, *Eumelanomyia*, *Lasiosiphon*, *Maillotia*, *Melanoconion*, *Neoculex*, *Oculeomyia*, *Phenacomyia*, *Phytotelmatomyia*, and *Sirivanakarnius*, that were recovered among species of subgenus *Culex* in our phylogenetic study of Culicini (Harbach et al., 2012).

Data for 286 characters were obtained from fourth-instar larvae (94), pupae (44), collections of water utilized by immature stages (1), adults (111), and male genitalia (36). The characters are described and explained in Appendix S1 (see supplemental material online) and the coded data for the 167 taxa included in the analyses are provided in Appendix S2 (see supplemental material online). Individually reared, pin-mounted adults with associated slide-mounted larval and pupal exuviae, as well as slide-mounted fourth-instar larvae, were studied when available. Observations of pinned adults were made under a stereomicroscope with lighting appropriately filtered to simulate natural light. Heads were removed from at least one male and one female of each species to examine structures that are not readily visible in intact specimens. Heads were cleared in 5% aqueous sodium hydroxide solution (some were then stained with acid fuchsin) and mounted frontodorsal side uppermost (ventral surface of head and proboscis lowermost) in Euparal

on microscope slides. Larval and pupal stages, and the dissected heads of adults, were studied using bright field and differential interference contrast microscopes.

In general, we examined three to six specimens (range 1−20) of each life stage of each species, and consulted literature sources for some taxa represented by few specimens or where available specimens were in poor condition or unavailable. Adult characters were derived from females unless otherwise noted. Several general methods have been proposed to construct characters from observations of features of organisms and then code these for cladistic analysis, of which the main formats are 'multistate coding', 'contingent coding' (also known as 'conventional coding') 'presence/absence coding' (also known as 'nominal variable' or 'reductive' coding) and 'Sankoff coding'. These were reviewed by Forey and Kitching (2000), who discussed the various advantages and disadvantages of each and concluded that Sankoff coding offered the most satisfactory method. However, constructing Sankoff character state transformation matrices is time consuming and their use in phylogenetic analysis significantly increases run times. As preliminary studies showed that run times for the present dataset were already going to be very long, each likely to take over 24 hours to complete, we rejected Sankoff coding as impracticable. Forey and Kitching (2000) found that the theoretical defects of presence/absence coding, in particular the redundancy that it introduced, were such that it must be rejected as a general coding method. Multistate coding can result in non-homologous states being combined into a single character if care is not taken during character construction. However, as pointed out by Sereno (2007), the logical inappropriateness of combining the absence of a feature with various states referring to transformations of that feature in a single multistate character is more problematic. He provided strong and cogent arguments for separating the neomorphic (presence/absence) and transformational elements of multistate characters, from which logically follows the need to use 'contingent coding' (Forey & Kitching, 2000). We consider the arguments of Sereno (2007) to be compelling and therefore applied this coding method to the construction of our dataset. We still found it necessary to include some multistate characters, but these consist only of sets of alternative transformational character states and so do not suffer from the logical defect highlighted by Sereno (2007).

States of continuous characters were determined by clear gaps in the observed counts or measurements (e.g., characters 93 and 103) and clear statements made explaining the disjoint nature of such data (as recommended by Simões et al., 2016). Setal characters of larval and pupal stages are coded to reflect observed intraspecific variation; hence, some such characters are coded in the form of '(0) unbranched (single); (1) branched' whereas others are coded in the form of '(0) single or double; (1) multiple branched' (or similar form) to reflect the actual development of the particular seta in question. All missing data are indicated by a question mark '?' in Appendix S2 (see supplemental material online). Characters that could not be scored due to absence of homologous structures ('dependent characters') are indicated by a dash '−', e.g., pupal seta 1-Pa (character 137) for *Maorigoeldia argyropus*, which is absent in this taxon. All multistate characters were treated as unordered. Polymorphic characters are explicitly coded as exhibiting only those states observed.

The anatomical terminology of Harbach & Knight (1980, 1982), revised and updated in the Anatomical Glossary of the Mosquito Taxonomic Inventory (http://mosquito-taxonomic-inventory.info/node/11027), is used for the character descriptions and associated information (Appendix S1, see supplemental material online). Abbreviations used for generic-level taxa follow Reinert (2009).

Specimens from collections in the following institutions were examined during the study: National Museum of Natural History, Smithsonian Institution, Washington, DC, USA; Natural History Museum, London, UK; and Laboratoire de Taxonomie des Vecteurs, Centre IRD de Montpellier, France.

Phylogenetic analyses

Parsimony analyses were implemented with TNT version 1.1 (Willi Hennig Society Edition) (Goloboff et al., 2008) using both equal weighting (EW) and implied weighting (IW) with values of the concavity constant, *K*, ranging from 1–200. The value of *K* indicates inversely the weighting 'strength' applied, with low values weighting more strongly against homoplastic characters (measured as the number of extra steps required to fit the cladogram topology in question) and higher values weighting less strongly (Goloboff, 1993). The individual character weights are summed to produce the overall 'fit' and the most parsimonious cladogram (MPC) is that with the greatest fit. Heuristic searches were conducted using the New Technology search options: sectorial searches, ratchet, tree drifting, and tree fusing. For the ratchet, the up-/down-weighting probabilities were set to 10% and the number of replicates to 100. The number of cycles of tree drifting was set to 25. All other search parameters remained at their default settings. Analyses were terminated when the MPC had been found 10 times. The maximum number of trees held was set to 10,000. Cladograms were rooted between *Cs. annulata* and the remaining taxa.

Relative support for nodes on the MPCs was assessed using symmetric resampling, as implemented in TNT, recording the frequency differences, i.e., 'Groups present/Contradicted' or GC values (Goloboff et al., 2003). This metric avoids the frequency distortions of other methods that resample to assess group support, such as the bootstrap and jackknife, and which are particularly pernicious

when applied to weighted data (Goloboff et al., 2003). For consistency, we also used this measure to assess support for the nodes on the MPCs resulting from the EW analysis. The GC values assess the difference between the absolute frequency with which a clade is found in the resampled matrices and that in the most frequent alternative topology in which the clade is not recovered. GC values range from 100, when the clade is recovered in all resampled matrices, to −100, when an alternative arrangement is found in all resampled matrices (Goloboff et al., 2003). A zero value indicates that levels of support and contradiction are equal. Due to time constraints, we calculated GC values using traditional search options, with 50,000 replicates and the default change probability, and searches were constrained to use only those groups found in the MPC in question. We then summed the GC supports across all groups on each MPC and used this as the optimality criterion to select the best topology (Goloboff et al., 2003; González-Santillán & Prendini, 2015). Cladograms were prepared and morphological character mappings investigated using WinClada ver. 1.00.08 (Nixon, 1999–2002).

An anonymous reviewer suggested that we should undertake a model-based, specifically Bayesian inference, analysis as this would allow us to present results from two different, independent types of phylogenetic analysis. Although Bayesian analysis using an Mk model (which assumes a Markov process for character change) has recently been shown to outperform parsimony (and particularly the use of implied weighting) under specific conditions and using simulated data (O'Reilly et al., 2016), the method remains undeveloped and we consider its generality undemonstrated. More importantly, we agree fully with the arguments proposed by Willi Hennig, and developed further by Farris, Nixon, Carpenter, Goloboff and others (discussed by Farris, 2008), that monophyletic groups should be based on grouping by synapomorphy and the most parsimonious cladogram is the optimal solution. From such a most parsimonious cladogram, character transformations can be explicitly studied and thus support for groups directly assessed and evaluated. Such is not the case with the results of model-based methods, where groups are recognized based on a consensus of trees and probabilities/likelihoods. Furthermore, given the nature of the data and the results obtained during the study (see below), it seemed unlikely that a Bayesian analysis would yield further insights into relationships. Consequently, we considered it was unnecessary to carry out a Bayesian analysis.

Results

First, we undertook IW analyses with values of $K = 20$–40, as previous experience had suggested that the optimal K value would probably lie within this range (Harbach & Kitching, 2016). Each IW analysis yielded a single MPC, and the EW analysis produced seven MPCs (4290 steps, CI = 0.10, RI = 0.41). In our previous study, using this approach to clade support (Harbach & Kitching, 2016), we found three peaks in summed GC values ($\sum$GC) at K values of 3, 8, and 30–33, which contrasted with the unimodal distributions, each with a clear maximum value, found in the study of González-Santillán and Prendini (2015: fig. 7). In the present study, there were two peaks within the range of $K = 20$–40, at $K = 28$ and 29 ($\sum$GC = 61 and 57), and $K = 32$–35 ($\sum$GC = 35, 25, 25 and 19) (Fig. 1), respectively. However, surprisingly, and in stark contrast to our previous study, the $\sum$GC values for the seven EW MPCs were much greater, ranging from 561 to 590. Consequently, we investigated the $\sum$GC associated with selected higher values of K. Initially, we incrementally increased the value of K in intervals of 10 from 50 to 100, which produced two further peaks at $K = 50$ ($\sum$GC = 29) and $K = 80$ ($\sum$GC = 204) (Fig. 1), followed by a decrease for $K = 90$ and $K = 100$. However, this was reversed markedly when we moved to $K = 150$ ($\sum$GC = 402), and the $\sum$GC for the final value of K investigated, $K = 200$ ($\sum$GC = 577), fell within the range of $\sum$GC for the EW MPCs ($\sum$GC = 561–590), differences that our previous analyses showed could simply be an artefact caused by the stochastic nature of the resampling procedure (Harbach & Kitching, 2016). It is, of course, expected that as the value of K increases, the results of the analysis will converge on those of an EW analysis because homoplastic features are being less strongly penalized. Thus, using $\sum$GC as the optimality criterion, we could not choose among the MPCs resulting from the $K = 200$ IW analysis and the seven derived from the EW analysis. Consequently, we consider that the strict consensus tree (SCT) of the seven EW MPCs and the $K = 200$ IW MPC represents the best summary

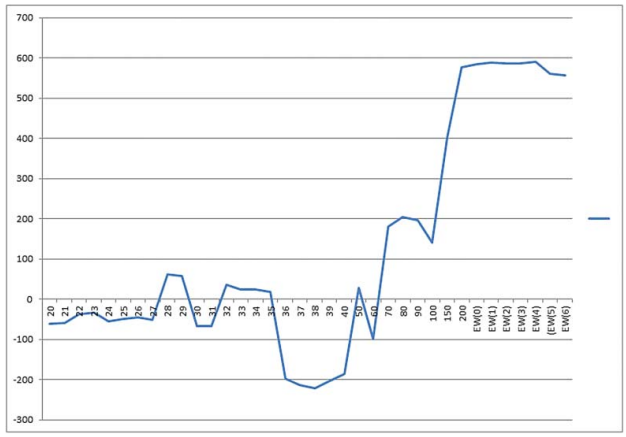

Fig. 1. Graph showing the summed GC supports for K values 20–200 of the IW analysis and, terminally, the values for each of the seven MPCs of the EW analysis (EW(0)–EW(6)). Only selected K values were investigated above $K = 40$ (see text for details).

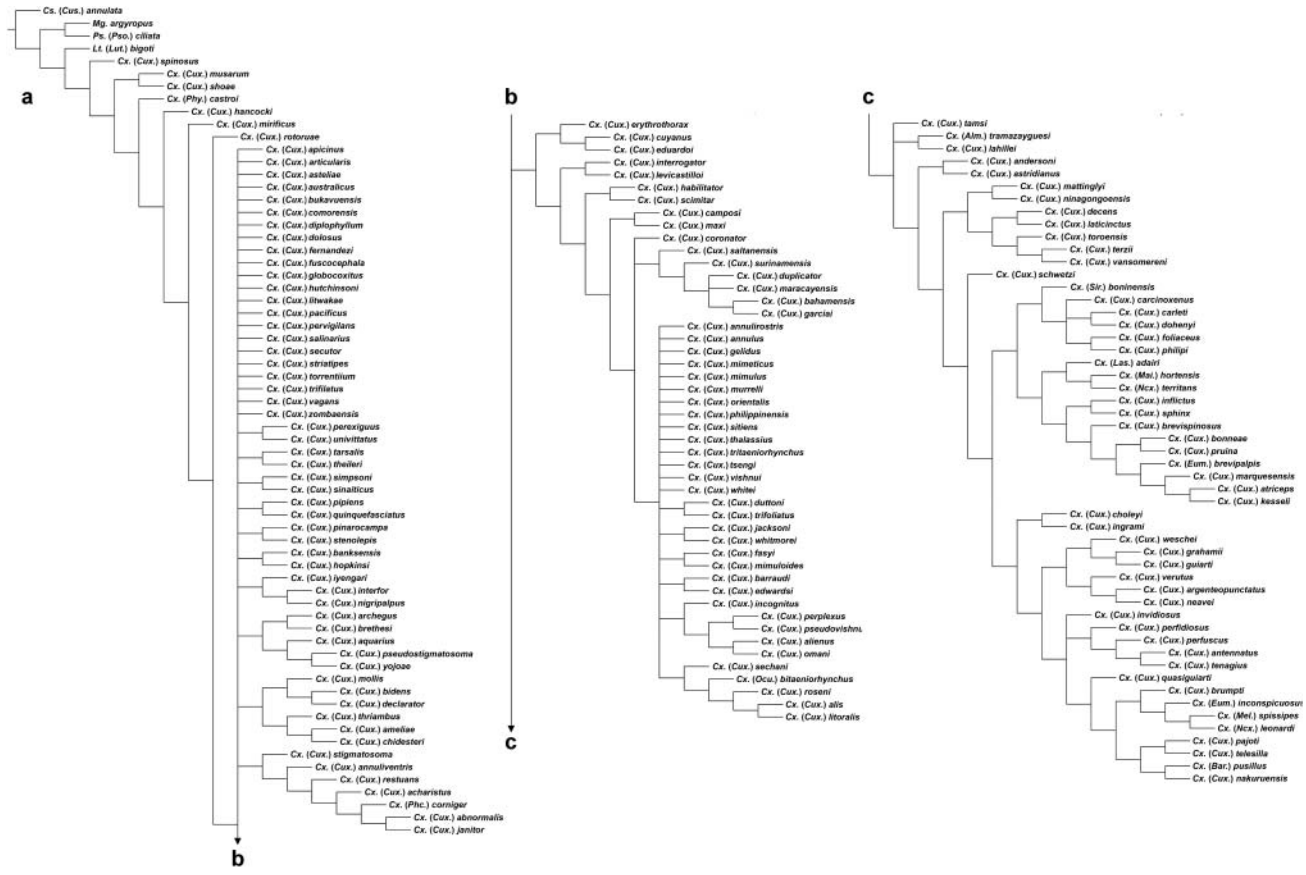

Fig. 2. Strict consensus tree of the seven EW MPCs and the $K = 200$ IW MPC. Node support values are not included as these are different for each of the eight MPCs.

and is our preferred pattern of relationships (Fig. 2). The mapping of characters onto the $K = 200$ IW MPC is shown in Fig. S1 (see supplemental material online).

The SCT of the seven EW MPCs and the $K = 200$ IW MPC (Fig. 2) collapses 55 nodes. *Lutzia bigoti* is recovered as the sister group to monophyletic genus *Culex*; however, subgenus *Culex* is polyphyletic as the members of the other 11 subgenera are scattered within it. The first species to branch off within genus *Culex* is *Cx*. (*Cux*.) *spinosus* (New World, Pipiens Group), followed serially by *Cx*. (*Cux*.) *musarum* + *Cx*. (*Cux*.) *shoae* (Afrotropical, Pipiens Group), *Cx*. (*Phy*.) *castroi* (Neotropical), *Cx*. (*Cux*.) *hancocki* (Afrotropical, Pipiens Group), *Cx*. (*Cux*.) *mirificus* (Afrotropical, Pipiens Group), and *Cx*. (*Cux*.) *rotoruae* (Australasian, Pipiens Group). The next clade contains a large polytomy comprising 34 branches. These comprise 22 individual species, six pairs, one triplet, three groups of five, six and seven species respectively, and two larger groups. The smaller of the last two clades comprises 48 species, 32 of which form a subclade that includes all species (29) of the Sitiens Group, *Cx*. (*Cux*.) *trifoliatus* (Pipiens Group), *Cx*. (*Ocu*.) *bitaeniorhynchus* (formerly a member of the Sitiens Group), and *Cx*. (*Cux*.) *duttoni* (Duttoni Group), a taxon that exhibited 'rogue' behaviour in our previous phylogenetic study of Culicini (Harbach et al., 2012). Excluding the monobasic Gelidus Subgroup, the Barraudi Subgroup (with its two known species) is the only subgroup of the Sitiens Group that remains intact. The species of the other three subgroups, the Mimeticus, Sitiens, and Vishnui Subgroups, are interspersed.

The outgroup taxon *Cx*. (*Phc*.) *corniger* is placed in the aforementioned subclade of seven taxa, but the representatives of the other eight subgenera of *Culex* included in the outgroup are all recovered within the last large subclade comprised of 53 species, with 10 outgroup species of subgenera *Allimanta*, *Barraudius*, *Eumelanomyia*, *Lasiosiphon*, *Maillotia*, *Melanoconion*, *Neoculex*, and *Sirivanakarnius* interspersed among 43 species of subgenus *Culex*.

However, the GC values show that most of these groups are effectively unsupported. Retaining only those clades with GC > 0 on any of the seven EW MPCs or the $K = 200$ IW MPC (i.e., those with positive support, however low) maximizes the number of retained groups but still leads to a considerable loss of resolution, producing the topology shown in Fig. 3, in which only 48 clades remain, some nested within others. These, together with the analyses in which they were recovered with positive GC

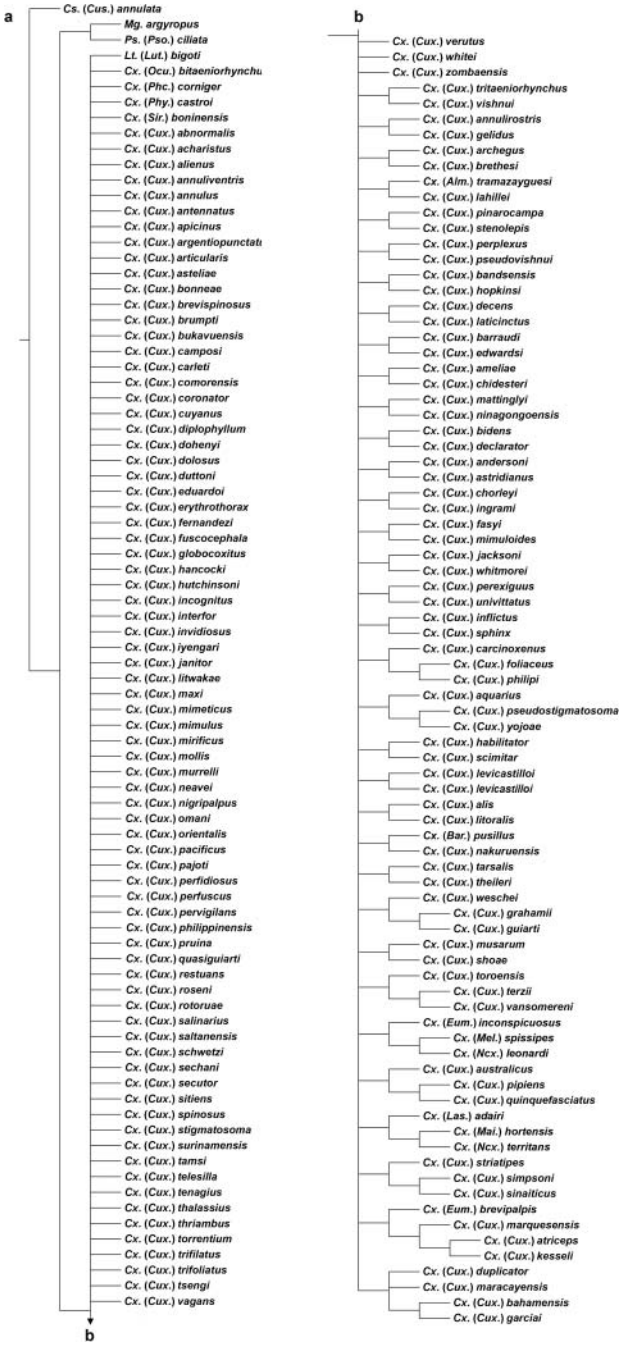

Fig. 3. Tree obtained from the strict consensus tree of the seven EW MPCs and the $K = 200$ IW MPC (Fig. 2) when branches with GC values < 1 are collapsed (only values > 0 are shown). Node support values are not included as these are different for each of the eight MPCs.

support, are listed in Table 1. Those clades that are not found in all eight of these MPCs generally have minimal GC support (GC = 1–3, occasionally 5) and stochastic effects related to the sampling employed to calculate the GC values may be partially to blame for such poorly supported groups not being consistently recovered (Harbach & Kitching, 2016).

Disregarding the basal *Cs. annulata* and the *Mg. argyropus* + *Ps. ciliata* clade, *Lutzia bigoti* and all species of genus *Culex* (outgroup and ingroup) form an enormous polytomy (GC = 6 in each of the eight MPCs) comprised of 84 individual species, 24 pairs, eight triplets, and two groups of four species. Unlike the results of previous analyses of morphological data that placed *Lutzia* outside genus *Culex* (Harbach et al., 2012; Navarro & Liria, 2000; St John, 2007), the more stringent assessment of clade support applied here again indicates that the placement of *Lutzia* relative to *Culex* based on morphology is highly equivocal, corroborating the conclusion of Kitching et al. (2015). Elsewhere in the polytomy, only three groups correspond precisely to recognized informal group taxa of subgenus *Culex*: *Cx. simpsoni* + *Cx. sinaiticus* (the only species of the Simpsoni Subgroup, Pipiens Group), *Cx. barraudi* + *Cx. edwardsi* (the only species of the Barraudi Subgroup, Sitiens Group) and *Cx. marquesensis* + (*Cx. atriceps* + *Cx. kesseli*) (the only species of the Atriceps Group). The first and last of these groups are each sister to another species. The Afrotropical Simpsoni Subgroup is the sister of *Cx. striatipes*, an Afrotropical species which is currently unplaced within the Pipiens Group, and the Australasian Atriceps Group is bizarrely paired with the principally Oriental *Cx. brevipalpis* of subgenus *Eumelanomyia*. Nine clades contain only two members, two contain three members, and one contains four members of more speciose groups, including *Cx. alis* + *Cx. litoralis* (Sitiens Subgroup, Sitiens Group), *Cx. inflictus* + *Cx. sphinx* (Apicinus Subgroup, Pipiens Group), *Cx. perexiguus* + *Cx. univittatus* (Univittatus Subgroup, Pipiens Group), *Cx. fasyi* + *Cx. mimuloides* (Mimeticus Subgroup, Sitiens Group), *Cx. australicus* + (*Cx. pipiens* + *Cx. quinquefasciatus*) (Pipiens Subgroup, Pipiens Group), *Cx. bidens* + *Cx. declarator* (Tarsalis Subgroup, Pipiens Group), *Cx. ameliae* + *Cx. chidesteri* (Apicinus Group, Pipiens Group), *Cx. perplexus* + *Cx. pseudovishnui* (Vishnui Subgroup, Sitiens Group), *Cx. pinarocampa* + *Cx. stenolepis* (Tarsalis Subgroup, Pipiens Group), *Cx. weschei* + (*Cx. grahamii* + *Cx. guiarti*) (Guiarti Group), *Cx. tritaeniorhynchus* + *Cx. vishnui* (Vishnui Subgroup, Sitiens Group), and *Cx. duplicator* and *Cx. maracayensis* in a polytomy with *Cx. bahamensis* + *Cx. garciai* (Tarsalis Subgroup, Pipiens Group).

Discussion

Subgenus *Culex* is better known than the other subgenera of *Culex* mainly because it has a cosmopolitan distribution and includes species with females that are more frequently encountered and comparatively easier to distinguish. The male genitalia and larvae generally exhibit specific differences that facilitate the identification of those species

Table 1. The 48 clades (some of which are nested within others) that remain when branches with GC values < 1 are collapsed on the strict consensus tree of the seven EW MPCs and the $K = $ IW 200 MPC (Fig. 3). The seven MPCs obtained from the EW analysis form two subsets, comprising topologies 0–4 and 5–6 respectively. The clades are listed in the order in which they appear in Fig. 3.

Clade	IW K=200	EW trees 0–4	EW trees 5, 6
All taxa except *Cs.* (*Cus.*) *annulata*	Yes	Yes	Yes
Outgroup *Mg. argyropus* + *Ps.* (*Pso.*) *ciliata*	Yes	Yes	Yes
Lutzia + *Culex*	Yes	Yes	Yes
Cx. (*Cux.*) *musarum* + *Cx.* (*Cux.*) *shoae*	Yes	Yes	Yes
Cx. (*Cux.*) *tarsalis* + *Cx.* (*Cux.*) *theileri*	Yes	Yes	Yes
Cx. (*Bar.*) *pusillus* + *Cx.* (*Cux.*) *nakuruensis*	Yes	Yes	Yes
Cx. (*Cux.*) *alis* + *Cx.* (*Cux.*) *littoralis*	Yes	Yes	Yes
Cx. (*Cux.*) *interrogator* + *Cx.* (*Cux.*) *levicastilloi*	Yes	Yes	Yes
Cx. (*Cux.*) *habilitator* + *Cx.* (*Cux.*) *scimitar*	Yes	Yes	Yes
Cx. (*Cux.*) *inflictus* + *Cx.* (*Cux.*) *sphinx*	Yes	Yes	Yes
Cx. (*Cux.*) *striatipes* + (*Cx.* (*Cux.*) *simpsoni* + *Cx.* (*Cux.*) *sinaiticus*)	Yes	No	No
Cx. (*Cux.*) *simpsoni* + *Cx.* (*Cux.*) *sinaiticus*	Yes	Yes	Yes
Cx. (*Cux.*) *perexiguus* + *Cx.* (*Cux.*) *univittatus*	Yes	Yes	Yes
Cx. (*Las.*) *adairi* + (*Cx.* (*Mai.*) *hortensis* + *Cx.* (*Ncx.*) *territans*)	Yes	Yes	Yes
Cx. (*Cux.*) *jacksoni* + *Cx.* (*Cux.*) *whitmorei*	Yes	Yes	Yes
Cx. (*Cux.*) *fasyi* + *Cx.* (*Cux.*) *mimuloides*	No	Yes	Yes
Cx. (*Cux.*) *australicus* + (*Cx.* (*Cux.*) *pipiens* + *Cx.* (*Cux.*) *quinquefasciatus*)	Yes	Yes	No
Cx. (*Cux.*) *pipiens* + *Cx.* (*Cux.*) *quinquefasciatus*	Yes	Yes	Yes
Cx. (*Cux.*) *chorleyi* + *Cx.* (*Cux.*) *ingrami*	Yes	Yes	Yes
Cx. (*Cux.*) *andersoni* + *Cx.* (*Cux.*) *bidens*	Yes	Yes	Yes
Cx. (*Cux.*) *ameliae* + *Cx.* (*Cux.*) *chidesteri*	Yes	Yes	Yes
Cx. (*Cux.*) *barraudi* + *Cx.* (*Cux.*) *edwardsi*	Yes	Yes	Yes
Cx. (*Eum.*) *inconspicuosus* + (*Cx.* (*Mel.*) *spissipes* + *Cx.* (*Ncx.*) *leonardi*)	Yes	No	No
Cx. (*Mel.*) *spissipes* + *Cx.* (*Ncx.*) *leonardi*	Yes	Yes	Yes
Cx. (*Cux.*) *decens* + *Cx.* (*Cux.*) *laticinctus*	Yes	Yes	Yes
Cx. (*Cux.*) *banksensis* + *Cx.* (*Cux.*) *hopkinsi*	Yes	Yes	Yes
Cx. (*Cux.*) *perplexus* + *Cx.* (*Cux.*) *pseudovishnui*	Yes	Yes	Yes
Cx. (*Cux.*) *pinarocampa* + *Cx.* (*Cux.*) *stenolepis*	Yes	Yes	Yes
Cx. (*Cux.*) *toroensis* + (*Cx.* (*Cux.*) *terzii* + *Cx.* (*Cux.*) *vansomereni*)	Yes	Yes	Yes
Cx. (*Alm.*) *tramazayguesi* + *Cx.* (*Cux.*) *lahillei*	Yes	No	No
Cx. (*Cux.*) *archegus* + *Cx.* (*Cux.*) *brethesi*	Yes	Yes	Yes
Cx. (*Cux.*) *weschei* + (*Cx.* (*Cux.*) *grahamii* + *Cx.* (*Cux.*) *guiarti*)	Yes	Yes	Yes
Cx. (*Cux.*) *aquarius* + (*Cx.* (*Cux.*) *pseudostigmatosoma* + *Cx.* (*Cux.*) *yojoae*)	Yes	NO	No
Cx. (*Cux.*) *pseudostigmatosoma* + *Cx.* (*Cux.*) *yojoae*	Yes	Yes	Yes
Cx. (*Cux.*) *annulirostris* + *Cx.* (*Cux.*) *gelidus*	No	Yes	No
Cx. (*Cux.*) *tritaeniorhynchus* + *Cx.* (*Cux.*) *vishnui*	No	Yes	No
Cx. (*Cux.*) *carcinoxenus* + (*Cx.* (*Cux.*) *foliaceus* + *Cx.* (*Cux.*) *philipi*)	Yes	Yes	No
Cx. (*Cux.*) *foliaceus* + *Cx.* (*Cux.*) *philipi*	Yes	Yes	Yes
Cx. (*Cux.*) *duplicator* + *Cx.* (*Cux.*) *maracayensis* + (*Cx.* (*Cux.*) *bahamensis* + *Cx.* (*Cux.*) *garciai*)	Yes	Yes	Yes
Cx. (*Eum.*) *brevipalpis* + (*Cx.* (*Cux.*) *marquesensis* + (*Cx.* (*Cux.*) *atriceps* + *Cx.* (*Cux.*) *kesseli*))	Yes	Yes	Yes

whose females cannot be distinguished. The infrasubgeneric classification of the subgenus (Table S1, see supplemental material online), however, is based principally on characters of the male genitalia and general ornamentation of the adults (Belkin, 1962; Bram, 1967a, 1967b; Edwards, 1932, 1941; Harbach, 1988).

Laurito and Almirón (2013) conducted a morphology-based phylogenetic study to determine the relationships of 53 Neotropical species of subgenus *Culex*, but no attempt has been made until now to examine the phylogenetic relationships of the species, species groups, and subgroups that comprise the subgenus as a whole. It generally has

been assumed that the infrasubgeneric groups recognized during the course of the 20th century represent natural groupings of species, but this is not supported by the results of the present study. Ironically, the inability of extensive morphological data to resolve relationships within the subgenus reflects the prediction of Harbach (2011): 'There is no doubt that the application of explicit methods of phylogenetic analysis will reveal weaknesses in the current phenetic classification of genus *Culex*'.

The results presented here strongly indicate that morphology is of little use for resolving the phylogenetic relationships of the numerous species currently classified as members of subgenus *Culex*. This may be due to a number of factors. Morphologically similar species may not actually be closely related (even though this may appear to be a reasonable *a priori* expectation), different species may possess overlapping morphology and historically distinct lineages may not exhibit morphological distinctions, or species may have arisen by hybridization (Belkin, 1962), e.g., species of genus *Orthopodomyia* often exhibit characteristics that 'can be explained only by introgressive hybridization' (Zavortink, 1968), thus leading to problems in character construction and coding. In the case of the present dataset, we agree with Scotland et al. (2003) that '… there are few characters that seem to be uncontroversial in relation to homology assessment' and 'increasing the number of characters increases the level of ambiguous or problematic characters'. We also undertook some preliminary investigations into the effects of possible 'rogue' taxa, that is, taxa of unstable position that thereby cause loss of resolution in the strict consensus tree or low support values (see Buenaventura et al., 2016 and references therein). However, although removal of a number of such taxa did yield small improvements in the resolution of the SCT obtained from an EW analysis (results not shown), the interrelationships of the remaining taxa were essentially unchanged and no improvements in agreement between the relationships in the SCT with those in the current intuitive classification were obtained. Consequently, we conclude that the lack of such correspondence is due to properties of the characters rather than of the taxa.

As noted above, the currently recognized groups and less inclusive subgroups and complexes that comprise the classification of subgenus *Culex* are based principally on few characters of the adult habitus and male genitalia, e.g., the Sitiens Group comprises species in which the proboscis has a well-defined median pale band, the tarsi have narrow pale rings, lower mesepimeral setae are absent, and the lateral plates of the male phallosome are denticulate (Edwards, 1932; Sirivanakarn, 1976). Despite the presence of lower mesepimeral setae and remarkably different development of the lateral plates of the phallosome, *Cx. duttoni* (Duttoni Group) and *Cx. bitaeniorhynchus* (subgenus *Oculeomyia*) are recovered among the species of the Sitiens Group in the strict consensus tree (Fig. 2), due, most probably, to the shared possession of a banded proboscis and ringed tarsi. For this reason it is not surprising that the latter species was classified as a member of the Sitiens Group (Edwards, 1932, 1941; Harbach, 1988; Sirivanakarn, 1976) until Tanaka (2004) resurrected *Oculeomyia* from synonymy with *Culex* for species of the Bitaeniorhynchus Subgroup of Sirivanakarn (1976), viz. the *bitaeniorhynchus* series and *bitaeniorhynchus* group of Edwards (1932, 1941, respectively). In this context, it is important to note that in the study of Laurito and Almirón (2013) *Cx. bitaeniorhynchus* and four species of the Sitiens Group formed a terminal clade within a larger clade comprised of 53 Neotropical species of the Pipiens Group. Why *Cx. trifoliatus* (Pipiens Group) is also placed among species of the Sitiens Group is more difficult to assess. These three species remain in the Group when rogue taxa are removed, but are not associated with members of the Group when the branches with GC support < 1 are collapsed to obtain the 'best' topography of relationships (Fig. 3).

Edwards (1932) and Sirivanakarn (1976) suggested that the Pipiens Group (cosmopolitan) and Sitiens Group (Old World, principally Oriental) possibly deserve to be ranked as separate subgenera. Disregarding the seemingly unsupportable inclusion of *Cx. duttoni* and *Cx. trifoliatus*, and the questionable inclusion of *Cx. bitaeniorhynchus*, the clade otherwise comprising the Sitiens Group in Fig. 2 would seem to support subgeneric status for this group but not for the Pipiens Group, which was also recovered as a polyphyletic group in the study of Laurito and Almirón (2013). If the Sitiens Group were to be afforded subgeneric rank, it is possible that it could include the former Bitaeniorhynchus Subgroup as a species group. It is less likely that the Pipiens Group is a monophyletic assemblage given the diversity of morphological forms and its worldwide distribution. Lane (1953) divided the Neotropical species of subgenus *Culex* into two groups based on the presence or absence of seta *g* on the subapical lobe of the male genitalia. The species of those two groups are interspersed among Old World species in the consensus tree (Fig. 2), an example of just one (character 266, Appendix S1, see supplemental material online) of many homoplastic characters that illustrate the morphological complexity of the Pipiens Group.

In view of the lack of support for most clades in the strict consensus tree (Fig. 2), there is little to be gained by discussing relationships among species of the Pipiens Group beyond what was mentioned in the Results. It is only necessary to point out unexpected omissions from two clades that survive in the collapsed tree (Fig. 3). These include the exclusion of *Cx. neavei*, a member of the Univittatus Subgroup (Univittatus Complex of Jupp & Harbach, 1990) from the *Cx. perexiguus* + *Cx. univittatus* clade and *Cx. globocoxitus* of the Pipiens Subgroup (Sirivanakarn, 1976) from the clade comprising *Cx.*

australicus + (*Cx. pipiens* + *Cx. quinquefasciatus*). The omission of *Cx. neavei* from the former group is unexplainable as morphological coding for this species differs little from the coding of characters for the other two members of the group. The only constant feature that distinguishes *Cx. neavei* from the other two species is the greater length of the ventral arm of the male genitalia (Jupp & Harbach, 1990), a character that is not included in the dataset. On the other hand, it is less surprising that *Cx. globocoxitus* does not appear to be closely related to the other members of the Pipiens Subgroup. Adults of this species exhibit different feeding and mating behaviours and distinctive morphological features, including short maxillary palpi and very swollen gonocoxites in males, and vestigial fourth palpomeres and different patterns of pale scaling on the proboscis and abdominal terga in females (Dobrotworsky, 1953, 1965). As far as other clades that survive in the collapsed tree are concerned, it must be assumed that they consist of species that are more closely related to one another than are other species of the groups to which they are assigned.

Based on observations made during the present study and the findings of Laurito and Almirón (2013), the composition of the Neotropical Apicinus Subgroup (Table S1, see supplemental material online) is problematic and the uncertain placement of *Cx. fernandezi* within the subgenus (Table S1, see supplemental material online) is surprising. *Culex apicinus* differs markedly (especially in features of the male genitalia) from the other species placed in the Apicinus Subgroup (see Rossi et al., 2008), which is obviously a heterogeneous assemblage of species. This in fact was noted by Edwards (1932) when he established the group (as the *salinarius-apicinus* series): 'This is hardly a natural group, but is perhaps convenient'. The morphological distinctions suggest that *Cx. apicinus* is a unique lineage that may require subgeneric rank (Laurito & Almirón, 2013). *Phalangomyia debilis* Dyar & Knab, an established junior synonym of *Cx. apicinus* (see Knight & Stone, 1977), provides an available generic-level name.

Although the results of the analyses do not indicate a relationship between *Cx. fernandezi* and *Cx.* (*Phy.*) *castroi*, the adults and larvae of these two species are very similar. In fact, *Cx. fernandezi* exhibits many of the morphological traits that diagnose subgenus *Phytotelmatomyia* (listed in Rossi & Harbach, 2008). In particular, the lateral plates of the male phallosome are remarkably similar, the development and positional relationships of the prominent larval and pupal setae are similar, and the immature stages are confined to phytotelm habitats. Further study may reveal that *Cx. fernandezi* should be transferred to subgenus *Phytotelmatomyia*.

Finally, the pairing of *Cx. nakuruensis*, unplaced in the Pipiens Group (Table S1, see supplemental material online), with *Cx.* (*Bar.*) *pusillus* (Fig. 2), and the survival of this clade in the collapsed tree (Fig. 3), indicates that it should not be classified as a member of subgenus *Culex*. When Mattingly (1951) named and described *Cx. nakuruensis*, based on three males, he noted morphological similarities with species of subgenera *Eumelanomyia* (as *Mochthogenes*) and *Neoculex*. The larva, pupa, and female of *Cx. nakuruensis* were described by van Someren (1967), who noted that the larva and female so 'closely resemble those of *Cx. antennatus*' that it was 'extremely difficult to decide on the identity of some specimens'. Based on the morphological data summarized in Appendix S2, *Cx. nakuruensis* is so distinct from species of subgenera *Culex* and *Barraudius* that it should eventually be placed in a separate subgenus. Mattingly (1951) suggested that *Cx. nakuruensis* was closely related to *Cx. mirificus* based merely on superficial similarity of the otherwise 'remarkable' male genitalia of the two species. In addition to specific differences in the male genitalia, e.g., the absence of seta *g* on the subapical lobe in males of *Cx. nakuruensis*, the two species exhibit significant differences in the adult and larval stages, indicating that they are unlikely to belong to the same lineage.

Conclusion

This study is the first attempt to reconstruct evolutionary relationships within subgenus *Culex* as a whole based on morphological data, and thus try to ascertain whether the current subgeneric classification reflects natural (genealogical) groupings of species. The results reveal two important realizations: (1) specific and supraspecific relationships within the subgenus, as currently defined, cannot be resolved unambiguously based on cladistic analysis of the morphological data collected and coded during the present study, and even differential character weighting does not seem to help; (2) the internal classification of the subgenus based on intuitive assessment of morphological similarities and differences does not seem, in large part, to reflect natural (genealogical) relationships. Although the traditional morphology-based classification of the subgenus may not correctly reflect evolutionary history, it is the only available hypothesis of specific and supraspecific relationships, based on both probable and insufficient evidence, to be further tested and modified as more information becomes available. It must be borne in mind that the present dataset may contain errors due to questionable homology assessments (e.g., patterns of pale scaling in adults and specialized setae of the male genitalia) and problematic character construction and coding (e.g., quantitative characters such as setal branching and measurements). The inclusion of new data and reinterpretation of character coding could markedly alter the topology of the most parsimonious cladogram(s), which may then more

accurately reflect the current morphology-based classification. Mitochondrial genomes and multigene approaches are becoming widely used for phylogenetic studies. In view of the results of the present study, it would seem prudent to explore the use of data from mitogenomes, nuclear DNA sequences, or transcriptomics for resolving evolutionary relationships within subgenus *Culex*.

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Supplemental data

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