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A molecular phylogenetic investigation of the genera closely related to *Chironomus* Meigen (Diptera: Chironomidae)

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Abstract. The relationships among some genera previously included as subgenera of *Chironomus* Meigen have been investigated using DNA sequences from two mitochondrial genes and a nuclear gene. The different mutation rates of mitochondrial genes and the nuclear 18S rDNA gene mean that they should resolve different levels of phylogeny. The analysis in large part supports many conclusions based on morphological analyses, particularly those utilizing all life stages. *Einfeldia* Kieffer *sensu lato* is confirmed as a polyphyletic grouping of species, which seems to have evolved independently, within or close to the genus *Chironomus*. While *Einfeldia* Group C, as *Lobochironomus* Ryser, Wülker *et* Scholl, is supported as a subgenus of *Chironomus*. *Einfeldia* Group B does not belong in that subgenus but rather appears to be a separate genus, for which the name *Benthalia* Lipina should be available. *Baeotendipes* Kieffer also is closely related to *Chironomus* species near the point of origin of the subgenus *Camptochironomus* Kieffer. The inclusion of *Nilodorum* Kieffer and *Carteronica* Strand in *Kiefferulus* Goetghebuer also is supported. In addition, the phylogenies suggest the existence of two main clades among the genera studied. One comprises *Kiefferulus* in its expanded form, along with *Axarus* Roback, *Xenochironomus* Kieffer, *Goeldichironomus* Fittkau, *Lipiniella* Shilova, and the *Harnischia* complex; while the other includes *Chironomus*, with its subgenera *Camptochironomus* and *Lobochironomus*, plus *Baeotendipes,* as well as groups A and possibly B of *Einfeldia* (*s. lat.*). *Dicrotendipes* Kieffer may be a sister group to both of these clades, with *Glyptotendipes* Kieffer either in the same clade with *Dicrotendipes* or sister to all other species.

Key words: Chironomus (*s. lat.*), molecular phylogeny, *cox1*, *cob*, 18S rDNA

Introduction

The relationships and generic limits of the Chironomini genera related to *Chironomus* Meigen have long been uncertain. At one extreme, all were placed as subgenera of *Chironomus* on the basis that the females could not be differentiated (e.g., Goetghebuer 1928; Edwards 1929). On the other hand, workers studying the immature stages found characters to justify the recognition of these subgenera as separate genera. This is best exemplified in the series of studies on the Chironominae of the Holarctic region (Pinder & Riess 1983, 1986; Cranston *et al.* 1989), although problems with regard to generic limits still remained (Cranston *et al.* 1989). Sæther (1977) used this latter form of systematics in his Hennigian analysis of the phylogenetic relationships of the tribe Chironomini of the Chironomidae, which was based substantially on characters of adult females. This placed *Kiefferulus* Goetghebuer as sister to *Chironomus*, and *Einfeldia* Kieffer as sister to both these genera. Subsequently, in a study that brought together supposedly irreconcilable data from different life stages, the concept of *Kiefferulus* was broadened to include *Nilodorum* Kieffer and *Carteronica* Strand (Cranston *et al.* 1990). *Einfeldia* had long been considered a polyphyletic assemblage (Pinder & Reiss 1983, 1986) with four groupings recognized. Group A contains *E. pagana* Meigen, the probable synonym of the type species of the genus, *E. pectoralis* Kieffer (M. Spies, personal communication), and so is the true *Einfeldia*. Pinder and Reiss (1986) saw some similarities of the pupae to those

of *Kiefferulus*, but such a relationship is not supported by the larvae (Cranston *et al*. 1989). Group B was considered to be a distinct group, but subsequently Ryser *et al*. (1985) suggested that *E. dissidens* (Walker), one of the best studied species of this group, might better be placed in the subgenus *Lobochironomus* Ryser, Wülker *et* Scholl of *Chironomus* and Cranston *et al*. (1989) supported this proposal, based only on adult characters. Group C was considered to belong in *Chironomus* and Ryser *et al*. (1985) transferred the species to *C.* (*Lobochironomus*). Group D showed some affinities with *Fleuria* Kieffer. Another species that can be considered here is *C. javanus* Kieffer, in which the male terminalia resembles that of *Einfeldia*, and which is unusual in the larval stage in that the premandible has 6 teeth (Chaudhuri *et al*. 1992). A synonym, *C. vitellinus* Freeman, was placed in *Einfeldia* by Cranston and Martin (1989). However more recently, Bugledich *et al*. (1999) listed it as an unplaced *Chironomus*, while Yamamoto (2002) would place it in a separate subgenus. In addition to the uncertainty as to the correct generic placement of species placed into the broader concept of *Einfeldia*, there is also uncertainty concerning the number of species within each group and what are the valid names (Spies & Sæther 2004). In general this is beyond the scope of this study, but does impact on the Group B species used in this study. It is not certain that the Japanese specimens of *C. dissidens* available to us are the same species as the European specimens described by Walker (1856). Our material is therefore identified as *C. dissidens* (*sensu* Hashimoto 1977). There is also uncertainty as to the status of *Baeotendipes* Kieffer, since the larvae and pupae are indistinguishable from those of *Chironomus*. Consequently, Pinder and Reiss (1983, 1986) have suggested that it is no more than a subgenus of *Chironomus*.

Given this uncertainty in relationships based on morphological analyses, it is pertinent to consider whether molecular data will help to clarify the situation. Thus we have used sequences from the mitochondrial (mt) cytochrome oxidase I (*cox1*) and cytochrome b (*cob*) genes, and the nuclear 18S rDNA (18S) gene to reassess some members of these morphological groupings for their relationships to each other and to the groupings previously recognized by molecular studies in the genus *Chironomus* (Guryev *et al*. 2001). The different mutation rates between the mt genes and the nuclear gene means that they resolve different levels of the phylogenetic trees: the mt sequences resolve relationships at the tips of the branches, while 18S sequences resolve the deeper branches of the trees. By using both data sets we can obtain a better evaluation of the overall relationships within and between genera.

Material and methods

The *Chironomus* species used represent 5 of the 6 clusters recognised by Guryev *et al*. (2001), plus some other groupings (e.g., *C. maturus* and *C. whitseli* of the maturus-cytocomplex (Wülker & Martin 1974) or species from geographic regions not included in that analysis. Representatives of other genera were used as available, with *Polypedilum nubifer* included as the outgroup species. The list of the 39 species used is given in Table 1. Note that species previously in *Einfeldia, Nilodorum,* and *Carteronica* are identified as such in the table. The species of the subgenus *Lobochironomus* are identified here by the apparent generic designation "*Lo*." This is necessary to distinguish *C.* (*Lobochironomus*) *dorsalis* (Meigen), used in this analysis, from *C. dorsalis auctorum nec* Meigen, used by Guryev *et al.* (2001)

DNA sequences

Sequences from the mt and nuclear genes were obtained using the extraction techniques and PCR primers and conditions as in Guryev *et al*. (2001) and Martin *et al*. (2003). The DNA fragments were sequenced in both forward and reverse directions using the Megabase sequencer in the Physiology Department of the University of Melbourne. From these sequences, 576 base pairs (bp) of *cox1*, 675 bp of *cob* and 697 bp of 18S were used for analyses. While a longer 18S fragment would have been preferable because of the slower evolutionary rate, the primer combination 18Sai/bi could not be used because of poor results with many chironomid species (Martin *et al*. 2003). Mt sequences were aligned by eye because they contain no inserts and deletions (indels), while the 18S sequences, which include indels, were aligned using CLUSTALW as implemented on BioManager (ANGIS, Australia) and then adjusted by eye to remove a small number of obvious inconsistencies. Some *cox1* and *cob* sequences of *Chironomus* species were obtained from Guryev *et al.* (2001), while the additional sequences are in GenBank, accession numbers: *cox1* - DQ648197–230, *cob* - DQ648231–264, and 18S - DQ657907–947.

Phylogenetic trees

All analyses were performed in PAUP* v.4.10b (Swofford 2002). Maximum Parsimony (MP) analysis was performed using a heuristic search with 1000 bootstrap (bs) replicates. The Maximum

TABLE 1. List of species used in this study, with geographic source of the material, source of the DNA sequences obtained, and any relevant notes.

Species	Region	*coxI & cob*	18S	Previous genus / Notes
Tribe Chironomini				
Axarus sp. varvestris	Nearctic	This study	This study	Werle *et al.* 2004
Baeotendipes sp.	Saudi Arabia	This study	This study	
Genus *Chironomus*				
Subgenus *Chironomus*				
C. acerbiphilus Tokunaga	Japan	This study	This study	thummi cytocomplex
C. crassiforceps Kieffer	Japan	This study	This study	pseudothummi cytocomplex
C. duplex Walker	Australia	Guryev *et al.* 2001	This study	pseudothummi cytocomplex
C. entis Shobanov	Nearctic	Guryev *et al.* 2001	This study	thummi cytocomplex
C. javanus Kieffer	Japan	This study	This study	thummi cytocomplex
C. maddeni Martin *et* Cranston	Australia	Guryev *et al.* 2001	This study	pseudothummi cytocomplex
C. maturus Johannsen	Nearctic	This study	This study	maturus cytocomplex
C. riparius (= *thummi*) (Meigen)	Palearctic	Guryev *et al.* 2001	This study	thummi cytocomplex
C. transvaalensis Kieffer	Israel	This study	This study	pseudothummi cytocomplex
C. suwai Golygina *et* Martin	Japan	This study	This study	thummi cytocomplex
C. whitseli Sublette *et* Sublette	Nearctic	This study	This study	maturus cytocomplex
C. xanthus Rempel	Brasil	This study	This study	pseudothummi cytocomplex
C. inquinatus Da Silva Correia	Brasil	This study	This study	pseudothummi cytocomplex
C. sp. 2g	Nearctic	This study	This study	*C. decorus* group (Martin 2006)
Subgenus *Camptochironomus*				
C. biwaprimus Sasa *et* Kuwai	Japan	Martin *et al.* 2003	This study	
C. dilutus Shobanov	Nearctic	Martin *et al.* 2003	This study	
Subgenus *Lobochironomus*				
Lo. dorsalis (Meigen)	Nearctic	This study	This study	*Einfeldia* Gp.C. Not *C. dorsalis* (*auct.*)
Lo. dissidens (*sensu* Hashimoto)	Japan	This study	This study	*Einfeldia* Gp.B., see text
Cryptochironomus sp.	Nearctic	This study	This study	
Dicrotendipes pseudoconjunctus Epler	Australia	This study	This study	
Einfeldia sp.	Germany	This study	This study	*Einfeldia* Gp.A.
Goeldichironomus devineyae (Beck)	Nearctic	This study	This study	
Glyptotendies lobiferus (Say)	Nearctic	This study	This study	
Genus *Kiefferulus*				
K. calligaster (Kieffer)	Thailand	This study	This study	
K. "cornishi"	Australia	This study	This study	Martin *et al.* 1996
K. dux (Johannsen)	Nearctic	This study	This study	
K. intertinctus (Skuse)	Australia	This study	This study	
K. martini (Freeman)	Australia	This study	This study	
K. sp. Israel	Israel	This study	This study	
K. sp. UVI	U.S. Virgin Islands	This study	This study	
K. sp. WA	Australia	This study	This study	*K*. "tinctus" group (Cranston 2000)
K. longilobus (Kieffer)	Australia	This study	This study	*Carteronica*
K. brevibucca (Kieffer)	Israel	This study	This study	*Nilodorum*
K. tainanus (Kieffer)	Japan	This study	This study	*Nilodorum*
Lipiniella sp.	Nearctic	This study	This study	
Polypedilum nubifer (Skuse)	Australia	This study	This study	
Xenochironomus xenolabis (Kieffer)	Nearctic	This study	This study	

Likelihood (ML) analysis was performed using Quartet Puzzling with 1000 puzzling steps, using parameters estimated in MODELTEST, v.3.7 (Posada & Crandall 1998). The distance (NJ) analysis was performed by a neighbor-joining search with 1000 bs and using the ML parameters. These analyses were chosen, consistent with the previous molecular phylogenetic studies of chironomids (Guryev *et al.* 2001; Martin *et al.* 2003), because we are more concerned with determining the level of support for particular branches, than, for example, obtaining the shortest ML trees.

Results

None of the data sets were found to differ significantly in the base ratios between different samples (Homogeneity Chi-squared probability values about 0.97 or higher (data not shown)). The Adenine plus Thymine (AT) frequencies of the mt sequences (AT = 71% for both *cob* and *cox1*) are higher than in 18S (AT = 60.5%), a well-documented characteristic of insect mt genes (Simon *et al*. 1994).

Mitochondrial tree

The *cob* and *cox1* data have been concatenated as in previous studies of *Chironomus* (Guryev *et al*. 2001; Martin *et al*. 2002). Modeltest indicated that the best-fit model was GRT + I + G. These trees (Fig. 1) did not resolve the lower branches and, although the ML tree suggests a clade containing *Chironomus*, including *Lo. dissidens*, and *Einfeldia*, and another loose clade containing the other genera, these are not supported by a majority of the quartet puzzlings. The ML tree in general supported more branches than did the NJ and MP trees, although some that it uniquely supported had very weak support (Fig. 1). Eight branches were supported by all three trees: (i) *C. suwai* and *C. entis*; (ii) *C. maturus* and *C. whitseli*; (iii) *C. crassiforceps* and *C. maddeni*; (iv) *K. intertinctus* and *K.* "cornishi"; (v) *K. longilobus* and *K. martini*; (vi) *K. dux* and *K.* sp. Israel; (vii) *Glyptotendipes lobiferus* and *Goeldichironomus devineyae*; and (viii) *Lo. dorsalis* and the outgroup species *P. nubifer*. A further two branches were supported by two of the analyses: *C. biwaprimus* and *C.* sp. 2g (NJ & MP); and *C. duplex* with the *C. crassiforceps / C. maddeni* branch (ML & NJ).

Nuclear (18S) indels and tree

Phylogenetic information can be obtained from the indels of the 18S sequences. There are four indels that are parsimony informative (Fig. 2). Three of these indels, including the largest one in helix E23.13, come from the most variable region of the gene (Wuyts *et al*. 2000). The E23.13 indel points to the existence of a large grouping of *Chironomus* species, which includes all but one of the species of the pseudothummi-cytocomplex plus *C. riparius*, the maturus-cytocomplex, as well as *C.* (*Lobochironomus*) *dorsalis*, and the subgenus *Camptochironomus* Kieffer. *Baeotendipes* and *Einfeldia* Group A show some similarities with this group, but have more extensive changes which suggest they should be considered as a separate group. The remaining *Chironomus* species show many similarities, but also some differences in the middle region of the E23.23 indel. The three E23 indels all point to a grouping of those species included by Cranston *et al*. (1990) in *Kiefferulus*. However, two species, *K. martini* and *K. longilobus* are similar to each other, but differ somewhat from the rest, as seen particularly in the small E23.5 indel. *Lo. dissidens* shows some similarities with these two species at the 5' end of E23.13. E23.2 and E23.5 independently suggest a relationship of *Axarus* Roback to the main group of *Kiefferulus*, although to different species within the group. The indel of helix 29 suggests a relationship between *Xenochironomus* Kieffer and *Goeldichironomus* Fittkau as well as between *Lipiniella* Shilova and *C. entis*. However, the 3' end of E23.13 suggests *Lipiniella* has a connection more with some elements of *Kiefferulus*.

MODELTEST suggested two alternative models for analysis of the 18S sequence: Hierarchical Likelihood Ratio Tests selected HLK + I + G, while Akiake Criterion Ratio selected GRT + I + G. A Kishino-Hasegawa (KH) and a Shimodaira-Hasegawa (SH) test indicated no significant difference in the ML trees obtained using each model (Diff. -ln L = 1.29092, KH, p = 0.83, SH, p = 0.43), and the NJ trees supported the same branches with very similar bs values. Consequently, only the GRT + I + G model has been used for further analyses, as this provides consistency with the model used for the mt analyses. As with the mt trees, the lower branches are not resolved (Fig. 3), although there are supported branches for relationships between some genera: a branch with *Goeldichironomus devineyae* and *Xenochironomus xenolabis* was supported by all trees, a branch containing *Lo. dissidens* and *A.* sp. varvestris received moderate support in the ML tree, and another with *Glyptotendipes lobiferus* and *Cryptochironomus* sp. had relatively low support in the NJ tree. There is a clade related to the *Kiefferulus* species, although lacking significant support. *Kiefferulus longilobus* and *K. martini* occur on a separate branch within this group, in keeping with the evidence from the indels. This clade also contains *Axarus*, *Goeldichironomus, Xenochironomus*, and *Lo. dissidens*. *Cryptochironomus*, *Glyptotendipes* Kieffer, *Lipiniella,* and the *Einfeldia* sp. are grouped with *K.* sp. WA, attached to this clade, again lacking significant support. The species related to *Chironomus* are only loosely associated. The indel-group containing *C. suwai, C. entis, C. xanthus,* and *C. transvaalensis* appears as a separate grouping, although these branches lack significant support. This clade also includes *Dicrotendipes* Kieffer. Some branches found in the mt trees were also supported: *C. suwai* and *C. entis*; *K. intertinctus* and *K.* "cornishi"; *K. longilobus* and *K. martini*. Branches comprising *C. maturus* and *C. whitseli*, and *K. dux*

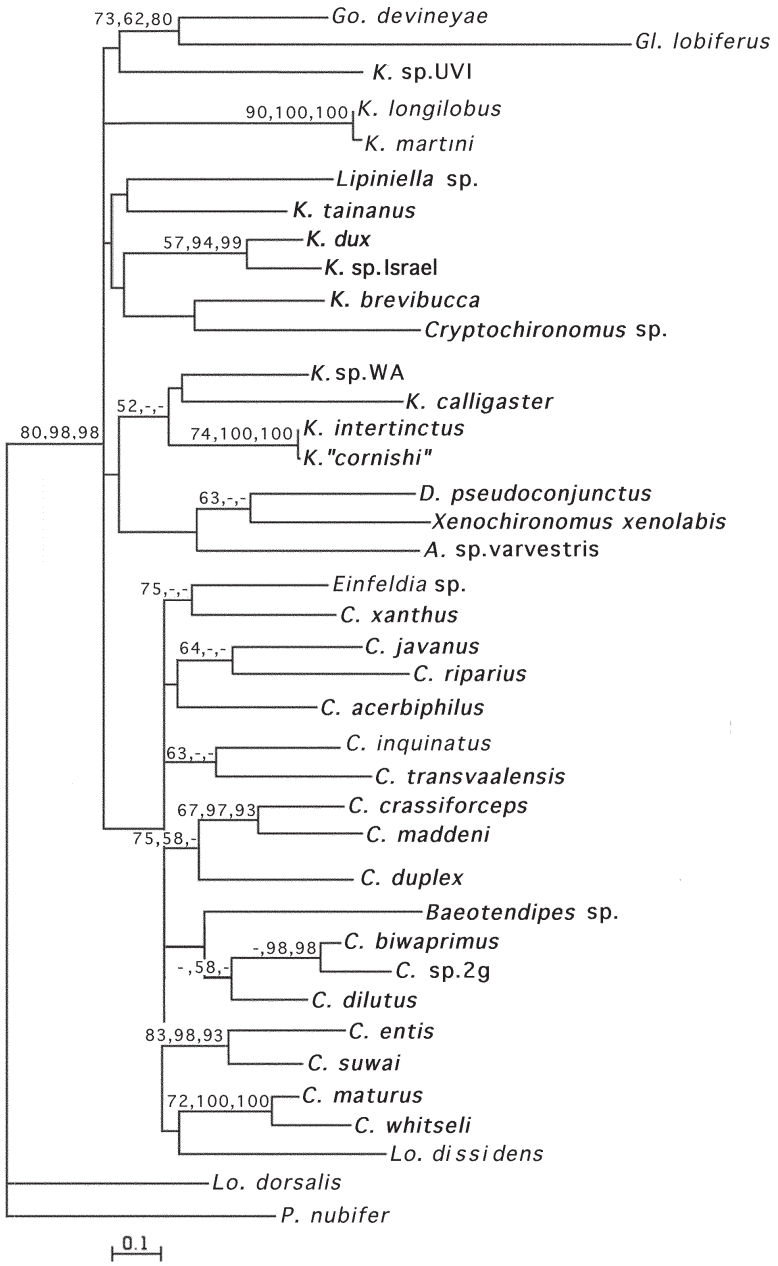

Fig. 1. ML tree (-Ln likelihood = 16431.3) inferred from the combined *cob* and *cox1* nucleotide sequence data, upon which is included the quartet puzzling score, the bootstrap score for the NJ, and for the MP tree (each for 1000 replicates). "–" indicates this branch is not supported in the particular tree. Scale represents 0.1 substitutions per site.

with *K*. sp. Israel and *K*. *calligaster* were moderately supported in the ML and MP trees.

Tests of similarity of tree topology between the mt and 18S trees were highly significant, confirming that the tree topologies are not identical. The SH and KH tests of the ML trees (Diff. -ln L = 249.01), and the KH (t = 14.790) and Templeton tests (z = -13.615) for the MP trees, all gave $p < 0.0001$.

Combined mt and 18S tree

It has been found that the combination of two data sets, that resolves different levels of the trees, gives a phylogeny that was superior to either of the genes alone (Gontcharov *et al*. 2004). Combining the mt and 18S data has also worked well in a previous chironomid study (Martin *et al*. 2003), so trees combining the data of *cox1* and 18S were constructed. Only one set of mt data was used in order

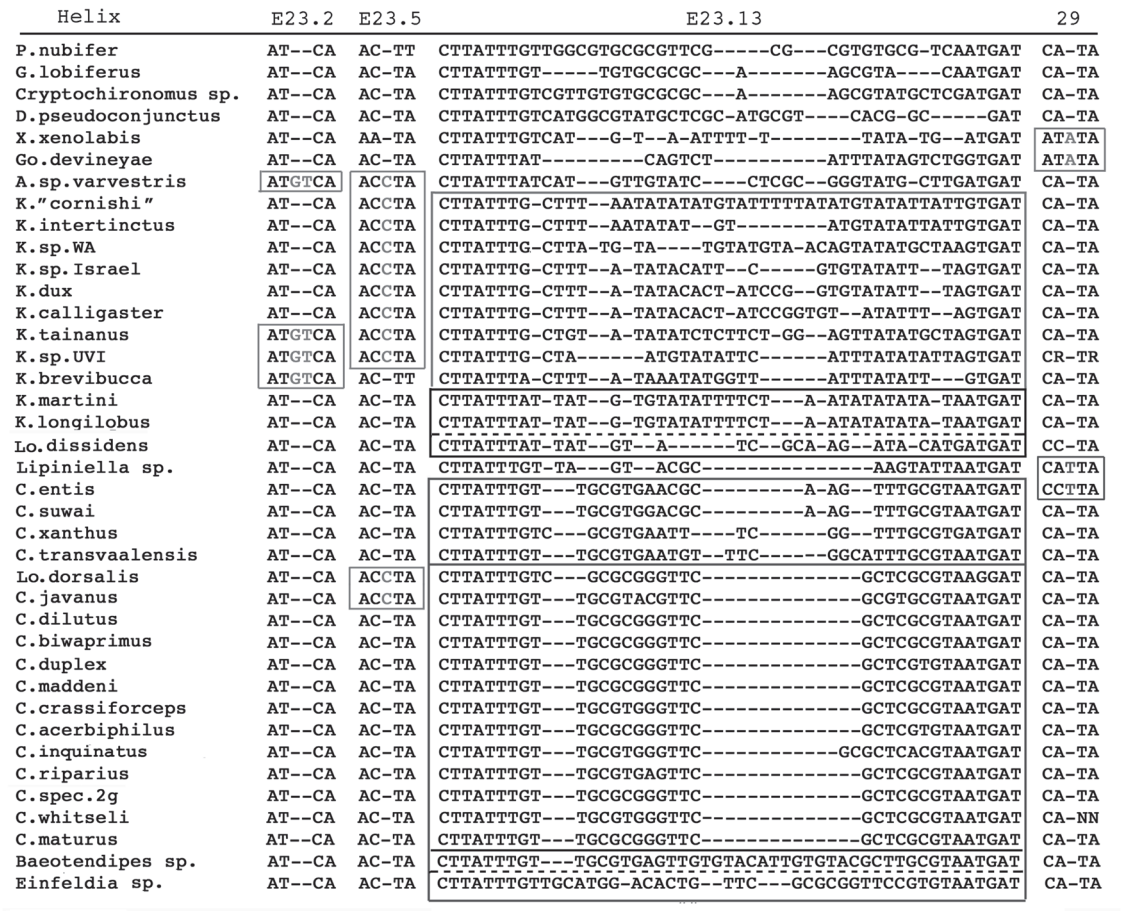

Fig. 2. The four parsimony informative indels of the 18S rDNA sequence with boxes enclosing those groups of species showing the greatest similarity in sequence. The helices in which these indels occur are numbered according to the European system (Wuyts *et al.* 2004).

to limit the dominating effect of the higher number of informative sites in the mt data (e.g., 198 vs. 55 parsimony informative characters). *Cox1* was chosen as the more commonly used mt gene in insect studies, and also as the shorter and more complete of the two mt data sets. Following Gontcharov *et al*. (2004), the averaged model (GRT + I + G) was used. The trees (Fig. 4) indicate two major groups, one of *Kiefferulus* (*sensu* Cranston *et al*. 1990), *Goeldichironomus, Cryptochironomus, Xenochironomus, Axarus,* and *Lipiniella*, as in the 18S tree; the other of *Chironomus, Baeotendipes*, and *Einfeldia* (*s. lat.*). *Dicrotendipes* and *Glyptotendipes* are sister to both these clusters. The weak support for *Glyptotendipes* as an outgroup, along with *Polypedilum* also was found in a further *cox1* / 18S tree where the Pseudochironomini taxon *Riethia stictoptera* was used as the outgroup (tree not shown). There are six supported branches, including the three common to the mt (Fig. 1) and 18S (Fig. 3) trees, and another three supported at least partially in those previous analyses. One new, highly supported, branch (Fig. 4) is that containing *K. dux* and *K. brevibucca*. There are another nine branches supported in only one of the three combined-data trees. The supported branches include the three clusters of *Chironomus* for which more than one representative was present, the two members of the maturus-cytocomplex, a branch with *C. riparius* and *C. javanus*, one with *C. xanthus* and *Einfeldia* Group A, and another with *Axarus* and *Xenochironomus*.

Discussion

Although the phylogenies are not completely resolved for the basal levels, there is sufficient support and consistency to permit some conclusions in relation to the hypothesized groupings and relationships noted in the introduction. Not

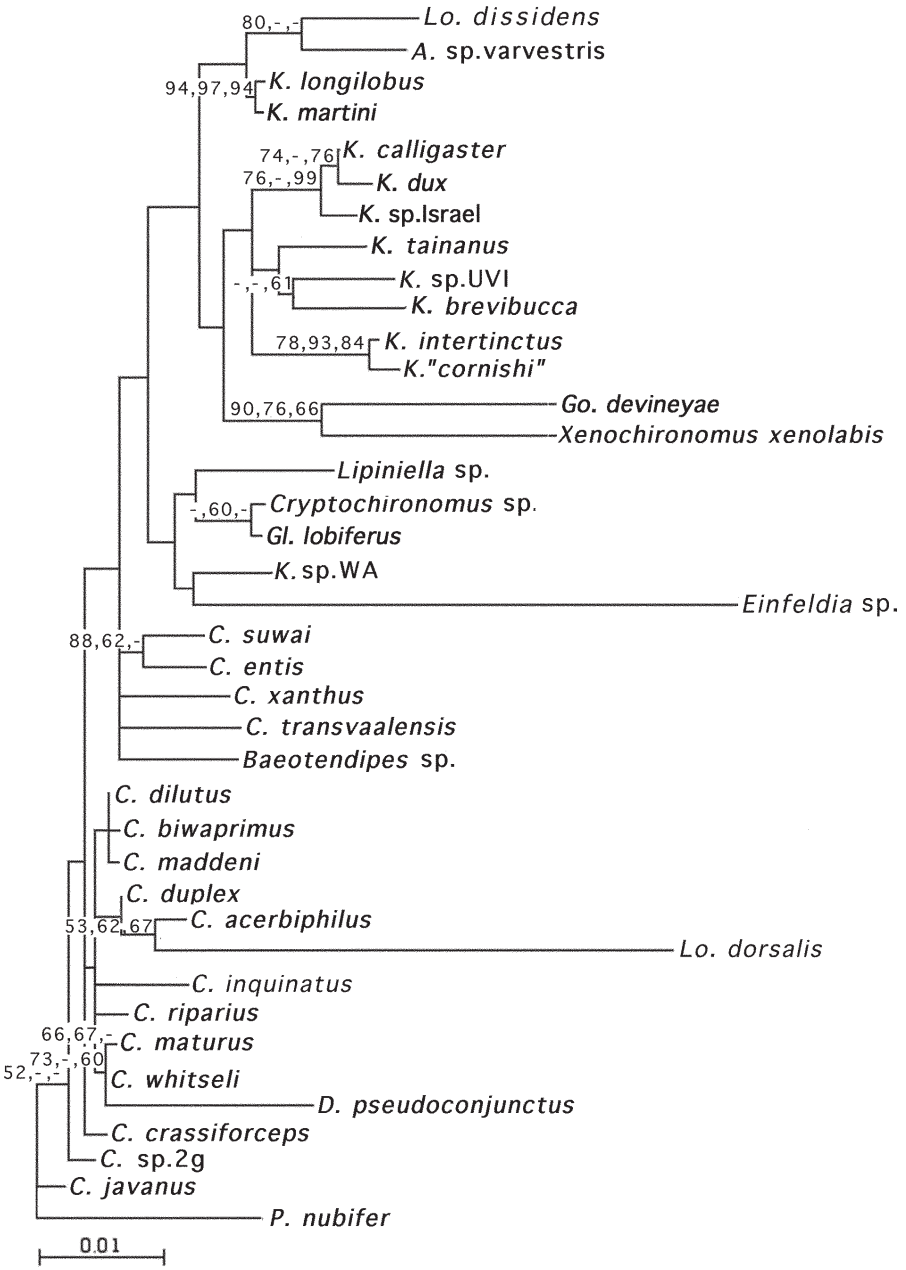

Fig. 3. ML tree (-Ln likelihood = 2582.7) inferred from 18S nucleotide sequence data, upon which is included the quartet puzzling score, the bootstrap score for the NJ, and for the MP tree (as in Fig. 1). Scale represents 0.01 substitutions per site.

surprisingly, *Einfeldia* Groups A, B, and C are shown to be paraphyletic, although all appear to be most closely related to *Chironomus*. The Group A species, i.e., *Einfeldia* (*s. str.*), clusters with the South American *C. xanthus,* largely due to mitochondrial characters (Figs. 1 & 4), but tends to be intermediate between the *Chironomus* and *Kiefferulus* clusters in the 18S data (Fig. 3). The Group C species *Lo. dorsalis* clusters with *Chironomus* species (Figs. 2–4) and the indels are typical of those of *Chironomus* species including those of the subgenus *Camptochironomus*. The Group B species *Lo. dissidens* is not consistently clustered with any species, as it is placed within the *Chironomus* group in Figures 1 and 4, but on 18S characters (Figs. 2–3) it is placed outside this cluster. *Chironomus javanus*, also at one time placed in *Einfeldia*, is clearly in *Chironomus* with very weak support for a linkage to *C. riparius* on mt sequences (Figs. 1 & 4). Therefore the true genus *Einfeldia*, as represented here by *E.* Group A species, appears more closely related to *Chironomus* than to *Kiefferulus* as was suggested by Pinder and

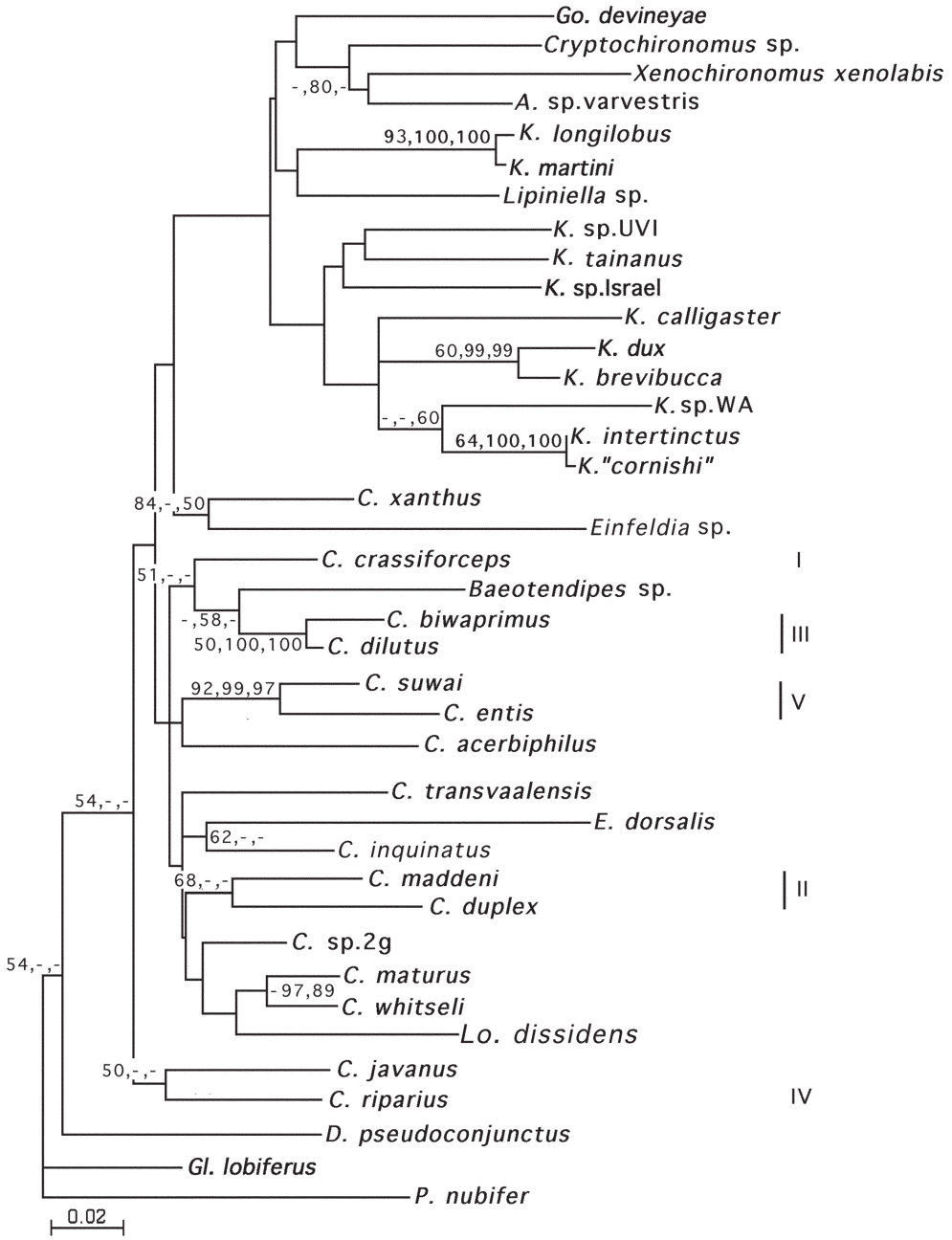

Fig. 4. ML tree (-Ln likelihood = 10080.9) inferred from combined *cox1* and 18S nucleotide sequence data. I–V, clusters recognized in analysis of Guryev *et al*. (2001). Other labelling as for Figure 1. Scale represents 0.02 substitutions per site.

Reiss (1986). The placement of Group C into *Lobochironomus* and as a subgenus of *Chironomus* by Ryser *et al*. (1985) seems justified by the observed relationships of *Lo. dorsalis*. The incorrect placement of some members of Group B, such as *Lo. dissidens,* in *Chironomus* (*Lobochironomus*) (Cranston *et al*. 1989), is shown by the molecular data. In further clarification of this point, it has become apparent that the morphological differences between the species of *Einfeldia* Group B and *E. natchitocheae* (Sublette), the only known member of Group D, are very minor and the two groups are congeneric (M. Spies, personal communi-cation). The generic name *Benthalia* Lipina, 1939 is available for the species of this combined grouping (M. Spies, personal communication). Finally, *C. javanus* clearly is a species of *Chironomus,* although the differences in the 18S indels would be compatible with its placement in a separate subgenus, as suggested by Yamamoto (2002).

One interesting point arising from the 18S indel sequences (Fig. 2) is the grouping of the species of the pseudothummi-cytocomplex, *C. riparius*, the maturus-cytocomplex and the subgenera *Camptochironomus* and *Lobochironomus*. This is consistent with cytological studies, which suggest that these cytocomplexes and subgenera all arose at about the same time from a common ancestor (Martin *et al*. 1974). *Baeotendipes* also seems to be related at about the same point, but the molecular evidence is insufficient to determine whether it should be considered as a subgenus within *Chironomus*, or as a closely related genus. The relationships of these genera and subgenera to species in the genus *Chironomus* will be investigated further in planned studies utilizing all the available *Chironomus* sequences.

The situation in the *Kiefferulus* cluster is clearer. Those genera placed into a broader concept of *Kiefferulus* by Cranston *et al*. (1990), *Kiefferulus, Nilodorum,* and *Carteronica*, are consistently grouped on neighboring branches, particularly where 18S sequences are involved (Figs. 2–4). Some branches are very well supported, including a branch with the North American *K. dux* and the African *K. brevibucca*, formerly in *Nilodorum*, which clearly supports the broader *Kiefferulus* concept. It might be noted that the three species, *K. intertinctus, K.* "cornishi" and *K.* sp. WA, which are placed in the same clade in Figure 4, are all member of what Cranston (2000) calls the "tinctus" group of Australian *Kiefferulus*, which cannot easily be recognized on the basis of morphology. However, while *K. intertinctus* and *K.* "cornishi" are sibling species that can hybridize, *K.* sp. WA is cytologically quite distinct, suggesting that it has been separated from the other two for a longer time. This is reflected in the molecular data, where the former species differ by about 0.7% in mt sequence and 0.4% in 18S sequence, while *K.* sp. WA differs from them by about 12% in mt sequence and 2.5% in 18S sequence, and is clearly a cryptic species rather than a closely related sibling species. The other noteworthy branch within the *Kiefferulus* cluster is that containing *K. martini* and *K. longilobus*, which again supports the broader *Kiefferulus* concept, since the latter species was formerly in *Carteronica*. These two species always group together but are on a separate branch from the other *Kiefferulus* (*s. lat.*) species in analyses involving 18S data (Figs. 2–4), indicating a more distant relationship and a possible linkage with the other genera in the *Kiefferulus* cluster, such as *Lipiniella.*

Overall, these molecular phylogenetic trees tend to support the morphological phylogeny of Cranston *et al*. (1990), based on characters from all life stages, rather than the earlier one of Sæther (1977), based just on characters of the female genitalia. Thus *Kiefferulus* is not the sister genus to *Chironomus*, as found by Sæther (1977), rather *Einfeldia* (*s. str.*) is supported as the sister genus, as found by Cranston *et al*. (1990), and *Dicrotendipes* and *Glyptotendipes* are sister to the other genera investigated. The molecular phylogenies also support the placement of *Lipiniella* near *Goeldichironomus*, as suggested by Sæther (1977). The resolution of the molecular trees could probably be improved by the inclusion of a more rapidly evolving nuclear gene or intron.

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