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## From bands to base pairs: Problems in the identification of species using the example of *Chironomus oppositus* Walker

JON MARTIN

Department of Genetics, The University of Melbourne, VIC 3010, Australia

E-mail: j.martin@unimelb.edu.au

**Abstract** The difficulty of identification of most species of *Chironomus* at any life stage is such that they are largely ignored as biological indicators in field studies. The problem largely arises because many of the species originally identified on the basis of morphology have turned out to be complexes, sometimes of many species. Each continent has its species complex, and in this work the Australian *Chironomus oppositus* species group is used to illustrate some of the problems involved in working with such species. The group comprises four recognized species: *C. oppositus* Walker, 1856, *C. maddenii* Martin & Cranston 1995, *C. "pseudoppositus"* and *C. "jacksoni"*. Within *C. oppositus*, five forms have been recognized on the basis of the complement of inversion sequences and differences in ecological preferences. Within one of the forms, form whitei, there are populations in which the chromosomal location of the dominant male determiner (MD) differs. These populations could indicate that MD location is an intraspecific polymorphism, or it could indicate the existence of additional cryptic forms. These data are considered in relation to the specific status of the forms, and the application of the species concept in *Chironomus*, when low levels of hybridization occur between these forms, and have also been reported between some recognized species. It is shown that, while DNA barcoding might be useful for identifying most *Chironomus* species, introgression can be a problem with mitochondrial sequences, so that they may not provide resolution where species complexes are an important component of the fauna of a study area.

**Key words:** Chironomidae, *Chironomus*, speciation, DNA barcoding

### Introduction

Most *Chironomus* species are notoriously difficult to identify morphologically, in any life stage. Not only are useful morphological characters often difficult to find, but many of the species originally identified on the basis of morphology have turned out to be complexes, sometimes of many species. Members of such complexes are best identified by the banding patterns of the polytene chromosomes, a specialised technique with which few field biologists have the time to become proficient. Consequently *Chironomus* species have been largely ignored as biological indicators in field studies.

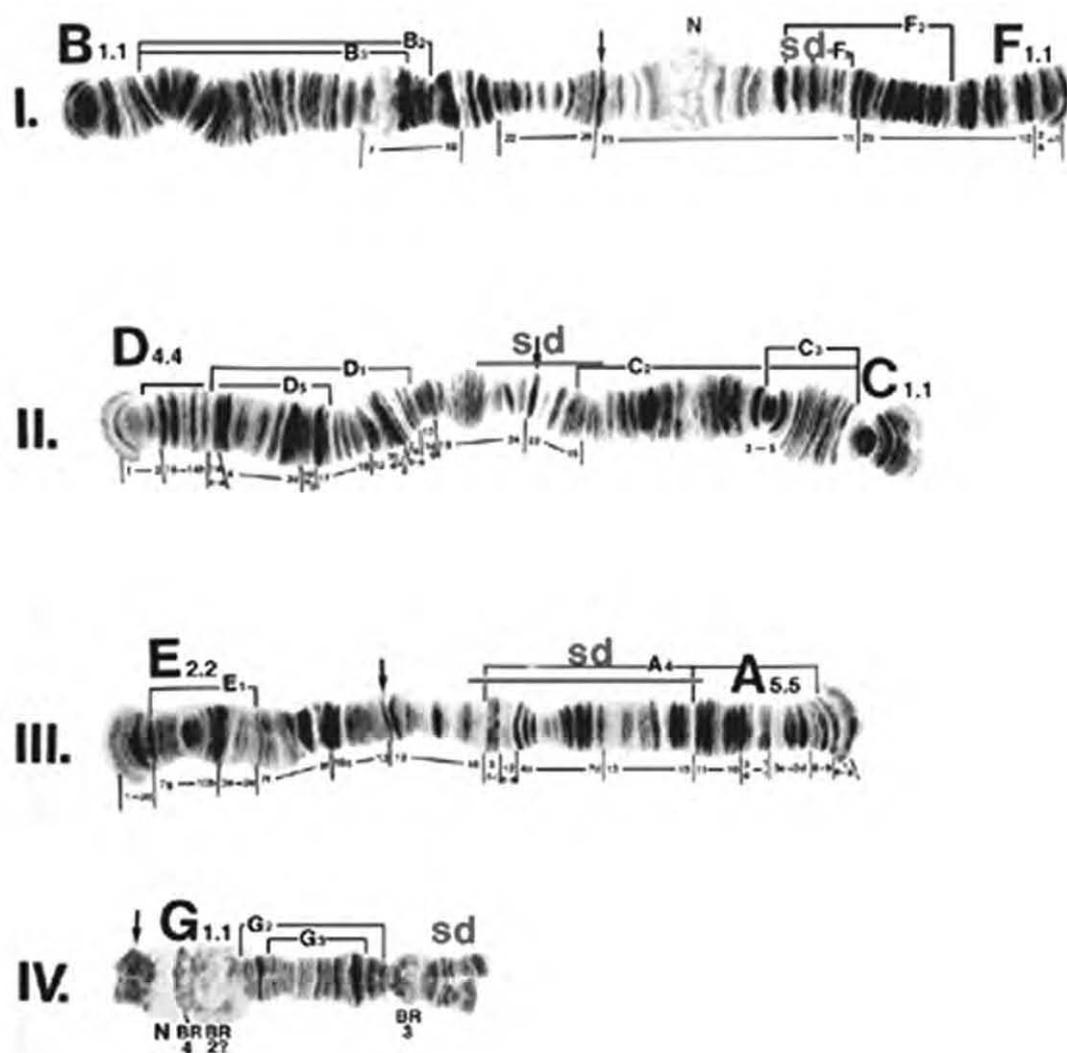


FIGURE 1. Representative photomap of the polytene chromosomes of *C. oppositus*, labeled where possible according to the Keyl/Devai system, and showing the limits of some inversions. SD – approximate sites of sex determining genes; N – nucleolus; BR – location of genes associated with specific Balbiani rings, which may or may not be expressed.

When I began my studies of Australian species in 1959, Paul Freeman was working on his revision of the Australian Chironomid fauna. I therefore sent him adults from the various larval types that I was collecting. Amongst these were those reared from medium size *Chironomus* larvae, some of which were plumosus-type, some bathophilus-type, some had darkened head capsules, others had pale head capsules. To my surprise Freeman (1961) identified all these specimens as *Chironomus alternans* Walker, 1856, although noting that I had obtained specimens from different larval types.

This led to the erection of the *C. alternans*-group, the Australian equivalent of the *C. decorus*-group in North America, the *C. thummi*- and *C. plumosus*-groups in the Palearctic, and undoubtedly similar groups in Asia, Africa and South America. The morphological difference in the larvae made it simple to divide the *C. alternans*-group into at least two sub groups, with the immediate problem of which species might be *C. alternans*, and which one of the synonyms listed by Freeman. An unpublished multivariate analysis of available types and adults from known larval types (Martin, 1966) showed that the measurements used were insufficient to separate the adults. Other specimens in the list of synonyms no longer existed and ultimately *C. alternans* and all other available names, except *C. oppositus*, were classed as *nomina dubia* (Cranston and Martin, 1989). The type of *C. oppositus* Walker is an unusually large specimen collected from Tasmania, where large specimens with a bathophilus-type larva breed in the lakes of the central highlands. The two species with plumosus-type larvae therefore required new names: one has been described as *C. cloacalis* Martin, 1971, the other, still formally undescribed, is *C. "februarius"*, and they will be referred to only briefly below. Since the species of the *C. alternans*-group are the most common *Chironomus* in semipermanent water bodies in southeastern Australia, the ability to differentiate them is important.

Subsequently, material considered as *C. oppositus* proved to be comprised of several species: *C. maddeni* Martin and Cranston, 1995, *C. "pseudoppositus"* and *C. "jacksoni"* (previously *C. alternans* c, Martin and Lee, 1984) – the broader *C. oppositus*-group. All of these species were identified on the basis of the banding patterns of the four salivary gland chromosomes. Typically there are four polytene chromosomes, three of which are metacentric and a small acrocentric chromosome (Fig. 1). The arms of these chromosomes are named A to G, and can be combined in various combinations in the chromosome complement. Species differences are usually most easily seen in arm G, the small fourth chromosome (Fig. 2). *C. oppositus* larvae have a subterminal nucleolus, while the other species have one nearer the middle of the arm, or not developed at all in some specimens of *C. "jacksoni"*. The other obvious identification aid is the position or number of the large puffed regions called the Balbiani rings (BR).

A study of the inversion polymorphism in *C. oppositus* has been carried out over the years with the assistance of various students and colleagues. It quickly became apparent that we were not dealing with simple populations. The basic analytical tool for population genetics is the Hardy Weinberg (HW) equilibrium, which predicts the relationship between genotypes in a population at equilibrium. One characteristic of the HW expectation values is that the frequency of heterozygotes

is always higher than at least one of the homozygous genotypes. In population after population we found that we had polymorphic inversions, often in multiple arms, where there were two different homozygous genotypes, but no heterozygotes at all. The simplest explanation of such results, subsequently confirmed by other research, was that these populations were mixtures of multiple forms, each with the same basic chromosome patterns, but differing in the combinations of inversions that were present. Four forms of *C. oppositus* have previously been recognized (Martin and Lee, 1984) and a fifth, form *edwardi*, is recognized here. There are also ecological differences, such as seasonal abundance, preferred depth, and overall size (when obtained from the same location at the same time) (Kuvangkadilok 1982, 1984), so that there are advantages to identifying the form present in a study area.

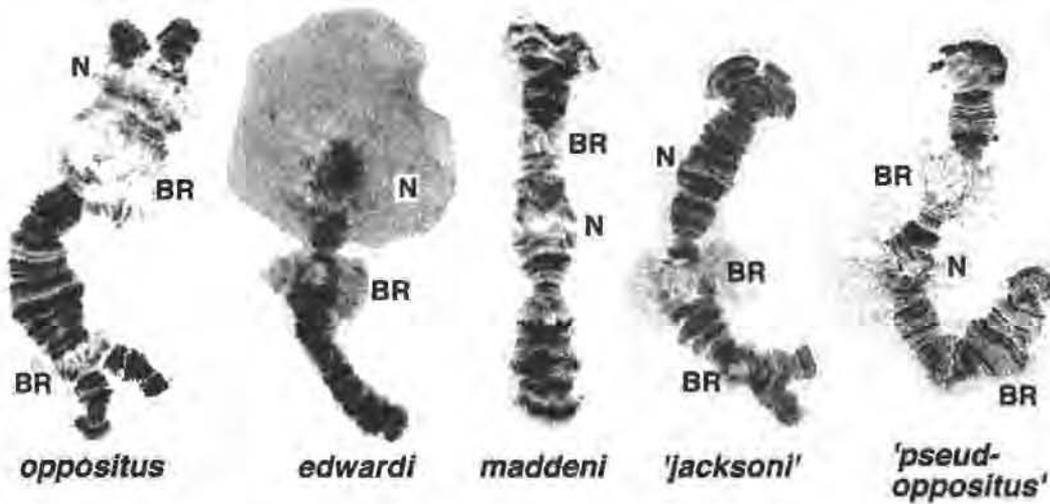


FIGURE 2. Arm G of species of the *Chironomus oppositus*-group. Symbols as in Fig. 1.

The most common sequence combinations are summarized in Table 1. Other sequences are found in low frequency, some of which such as E1 in forms *connori*, *oppositus* and *whitei*, E2 in *f. tyleri*, A1 and A2 in *f. whitei*, are most likely due to introgression following hybridization. There are also differences in the location of the dominant male determining (MD) gene mentioned in the table and shown in Fig. 1. With the exception of *f. whitei*, only one of these sites can be demonstrated in each form. Martin and Lee (1988b) suggested that populations of *f. whitei* with different MD locations may represent still further cryptic forms. There is other evidence for subgroups of *f. whitei* in a number of populations, where the same deficiency of heterozygotes as mentioned previously, is observed. Table 2 shows data from a population at Eildon, Victoria, where both arms B and D show polymorphism. There is a marked deficiency of some heterozygotes, and certain combinations of sequences in arm B and arm D occur together more frequently than expected by chance. Thus, there seems to be one group where B1 occurs more commonly with D1 and D3, and another group where

B2 occurs more commonly with D2. Note that D2.3 heterozygotes did not occur, although over 30 would have been expected. This is strong evidence that non-random mating is occurring between these two cytological groupings.

**TABLE 1.** Most common banding patterns found in each chromosome arm of the forms of *Chironomus oppositus*. Others may occur in low frequency due to introgression following hybridization between forms

Form/arm	A	B	C	D	E	F	G
<i>connori</i>	2, 3 & 51	2	2	1 & 2	2	1	1
<i>oppositus</i>	1 & 2	1	1 & 22	1 & 22; 3(SA)	2	1	1
<i>tyleri</i>	1 & 2	1	3 & 1	4, 1 & 5	1	1 & 2	1 & 2
<i>whitei</i>	5 & 44	2 & 1	1 & 24	1, 2 & 34	2	1 & 34	1 & 34
<i>edwardi</i>	4	edw5	1	ausD1*	1	1	3?

<sup>1</sup> f. *connori*: A2 (& A3)(males only), A5 (both sexes); <sup>2</sup> f. *oppositus* sequences in arms C and D may appear sex linked; <sup>3</sup> f. *tyleri*: G2 often sex-linked; <sup>4</sup> f. *whitei*: sequences in arms A (A4), C, D, F (F3) and G may appear sex linked in different populations; <sup>5</sup> f. *edwardi*: Be (inv of B2); \*ausD1 - as D1 of *C. australis*.

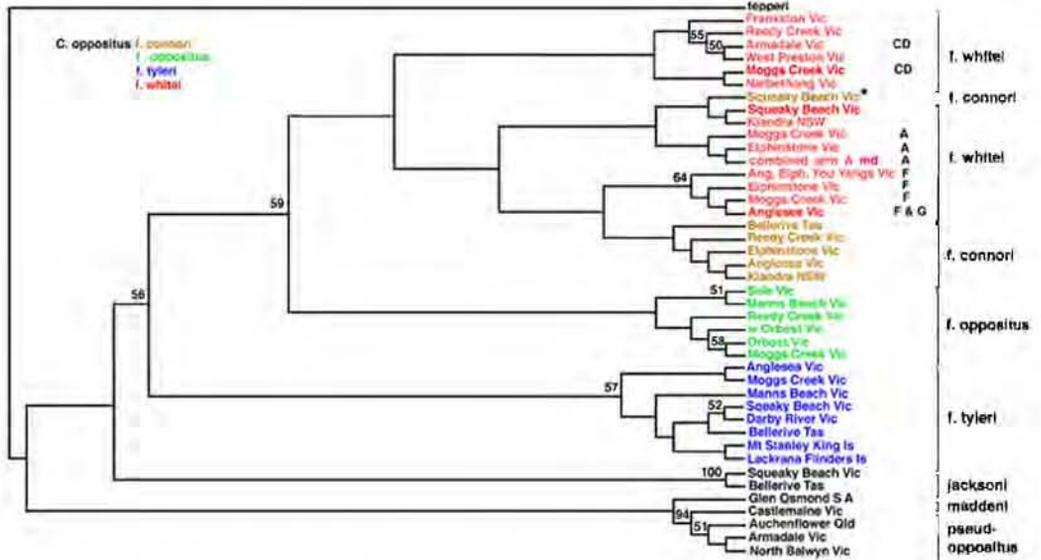
**TABLE 2.** Association of sequences of arms B and D in the Eildon, Victoria population of *C. oppositus* f. *whitei*. Expected values for random association are calculated from the marginal totals, while Hardy Weinberg (HW) expected values for each arm are shown in the Totals row (for arm B) and column (for arm D)

Sequence	B1.1	B1.2	B2.2	Total
D1.1 o.	115	53	16	184
e.	82.55	45.19	56.26	138.89
D1.3 o.	55	31	13	99
e.	44.41	24.32	30.27	73.92
D3.3 o.	7	5	1	13
e.	5.83	3.19	3.97	9.83
D1.2 o.	0	4	0	4
e.	1.79	0.98	1.22	119.11
D2:2 o.	2	5	92	99
e.	44.41	24.32	30.27	25.54
Total	179	98	122	399
HW e.	130.09	195.48	73.43	

Note: The heterozygote D2.3 is not included in the table because none were found and therefore none would be expected from the marginal totals for random assortment. However, 31.71 were expected under HW, so HW expected values shown for arm D do not sum to 399.

Although defined by cytological criteria, a UPGMA tree (as employed by Christidis and Schodde, 1997) of the relationships of the forms of *C. oppositus* based on inversion frequencies, does not separate populations accurately into the various forms, with only f. *tyleri* and most of f. *oppositus* forming discrete clusters (Fig. 3). Consequently allozyme polymorphisms were investigated, for a smaller data set, and it was found that only f. *tyleri* produced a discrete cluster (data not shown), but when the two data sets are combined (Fig. 4), there is only one sample, f. *oppositus* from Squeaky Beach, Victoria, that is not with the other members of its form. It should be noted that while consistent clustering of populations has been found in a number of analyses of





**FIGURE 4.** UPGMA tree of the relationships between populations of members of the *Chironomus oppositus*-group based on the combined frequencies of inversion sequences and allozyme alleles. Labelling as for Fig. 3.

All members of the *C. oppositus*-group, with the exception of the males of *C.* "pseudoppositus", will breed in the laboratory to variable extents (Martin *et al.* 1980; Martin and Lee, 1981, 1984, 1988a) under the conditions specified in Martin and Lee (1981). This permitted hybridization to be investigated when adults of different members are available at the same time. Martin and Lee (1981) reported some preliminary data, and more extensive data are shown in Table 3. The absence of data for some crosses generally reflects either the low frequency of successful laboratory mating in these forms, or a paucity of contemporaneous adults. Where successful crosses were obtained, the level of fertility was high, and backcrosses could be performed in many cases. Fertility of hybrids was high, with even crosses between the recognized species (using only females in the case of *C.* "pseudoppositus"), producing some egg masses with fertility above 80%. In *C. maddeni* X *C. oppositus* crosses, one egg mass showed quite low fertility, while some embryos from the other egg masses showed abnormal development and the rate of hatching was reduced compared to that of other hybrid crosses.

Martin *et al.* (1978) showed the existence of natural hybrids between the forms of *C. oppositus*, while noting the difficulty of detecting individual hybrids in a sample from a natural population. Much of this difficulty can be overcome by studying larvae from unhatched egg masses collected in the wild, or laid by gravid females collected at light. The presence of F1 hybrids can be readily detected, as all larvae will be heterozygous for inversion sequences that would not usually occur in any form. It is also possible to pick up many instances that are most easily explained as resulting

from the backcross of a hybrid to one of the parental forms. Form *edwardi* has not been included in this analysis as only one egg mass has been seen, and that from Western Australia where the other forms are not known to occur. About 520 egg masses have been analysed, of which 150 were f. *whitei*, 147 f. *connori*, 99 f. *tyleri*, 96 f. *oppositus* and the other 15 egg masses were either F1 hybrids (6) or backcrosses (9). This is an overall hybridization frequency of 2.9%. All forms have been found to be involved. The frequency of hybridization between particular forms varies from about 0.3% between f. *whitei* and f. *connori* (although these egg masses did not include any from Queenstown, Tasmania, where Martin and Lee (1981) found evidence of such hybridization), to about 1.6% between f. *tyleri* and f. *connori*.

TABLE 3. Laboratory hybridization within the *C. oppositus*-group

Female Male	f. <i>oppositus</i>	f. <i>tyleri</i>	f. <i>connori</i>	f. <i>whitei</i>	<i>maddeni</i>	<i>jacksoni</i>	<i>pseudoppositus</i>
f. <i>oppositus</i>	+	n.t.	+	+	n.t.	+	n.t.
f. <i>tyleri</i>	n.t.	+	+	+	n.t.	+	n.t.
f. <i>connori</i>	n.t.	+	+	+	n.t.	+	n.t.
f. <i>whitei</i>	n.t.	+	+	+	+	+	n.t.
<i>maddeni</i>	n.t.	n.t.	n.t.	+	+	+	+
<i>jacksoni</i>	n.t.	+	+	+	+	+	+
" <i>pseudoppositus</i> "	—	—	—	—	—	—	—

+ - fertile hybrid egg masses, obtained; — -no laboratory matings; n.t. - not tested.

The egg masses of the other species in the *C. oppositus*-group were also investigated, although these were relatively low in number, and two potential hybrids were noted among 13 egg masses of *C. "jacksoni"*. As well, a hybrid between *C. "jacksoni"* and f. *oppositus* has been reported from Bellerive, Tasmania (Martin and Lee, 1981), and a small sample from Whitemark, Flinders Island appeared to be hybrids of f. *whitei* and *C. "jacksoni"*. This apparently high frequency of hybridization may be one of the reasons that *C. "jacksoni"* is so rare and is found in water subjected to estuarine tidal flows that the *C. oppositus* forms do not generally use.

Given these data, we can ask whether any of the forms of *C. oppositus* should be given specific status. The answer to this question is not of major importance here, rather it provides an opportunity to investigate what definition of a species is usually used in studies of chironomids in general and *Chironomus* in particular, where many species are recognised on the basis of the banding patterns of the polytene chromosomes. If the biological species concept is used, *C. oppositus* would be just a single species, with the exception of f. *edwardi*, where there is not enough evidence to decide one way or the other. However, when the literature on other *Chironomus* species is examined it can be seen that hybridization occurs at low frequency in nature between a number of closely related species. For example, *C. riparius* and *C. piger* can be readily crossed in the laboratory (the reason

that they were initially considered to be only subspecies (Keyl and Strenzke, 1951)), and natural hybrids have also been reported (Goldschmidt, 1942). *C. tentans* and *C. pallidivittatus* also breed readily in the laboratory (Kiknadze *et al.*, 1998), with considerable evidence of past hybridization found in the mitochondrial (mt) DNA sequences, although only a single natural hybrid has been reported (Martin *et al.*, 2002). Hybridization although very rare, has been recorded for single instances between four members of the *C. plumosus*-group in the Palaearctic (Butler *et al.*, 1999) and inferred from the mt DNA sequence of a fifth species (Polukonova *et al.*, 2009). From such examples it can be inferred that the biological species concept is not the main basis for species determination. This also applies to situations where species are allopatrically distributed so that natural hybridization cannot occur, but instances of laboratory hybridization show that it is still possible (Martin *et al.*, 1980) even though these allopatric species are cytologically distinct. In general, specific status is determined by cytological differentiation that is maintained even when two taxa occur together in the same habitat. This raises the question of how much differentiation is required, particularly if the taxa do not ever occur in the same locality due to allopatric distribution. Some European workers (Gunderina *et al.*, 1996; Kiknadze *et al.*, 1998, 2000; Butler *et al.*, 1999; Michailova *et al.*, 2005) have attempted to resolve this question by using a measure, based on Nei distance, that they call Cytogenetic Distance ( $D_N$ ). They obtained a  $D_N$  value of 1.65-2.50 between sibling species, but less than 0.80 between populations within species. However, such a measure can only be used between relatively closely related species since it will go to infinity if there are no sequences in common between the species being compared. The value is also very dependent upon the amount of polymorphism, increasing when polymorphism is more frequent. Hence there is no  $D_N$  value that can be used as a standard criterion of whether the taxa being compared are sufficiently different to be considered different species. This is amply illustrated when such a criterion is applied to the data for the *C. oppositus*-group (Table 4). Here it is readily seen that the  $D_N$  values between the recognised species, including the outgroup *C. tepperi* Skuse, 1889, are all less than one, values that would put them as only differentiated populations in the cases studied by Kiknadze *et al.* (2000). This difference is readily explained by the low level of polymorphism within and between these Australian species, compared to that in the studied Holarctic species. When it comes to comparing the  $D_N$  values between the relatively monomorphic species in this study and the more polymorphic forms of *C. oppositus*, it is seen that the values increase considerably, and all forms of *C. oppositus* would appear to be distinct from the other species of the *C. oppositus*-group. However, looking at the differences between the forms of *C. oppositus* reveals a much more complex picture (Table 4). For a start, there is much variability within forms, but surprisingly in view of the previous suggestion of the possibility of additional subgroups of *f. whitei*, it is *f. tyleri* that shows the highest values. This is due to marked geographic variation in this form, particularly in western Tasmania. The Western Australian *f. edwardi* has relatively high values despite the lack of known polymorphism, suggesting that this is a distinct species. For the other forms of *C. oppositus*, the almost continuous divergence and degree of reproductive isolation makes it very likely that we are looking at relatively recently diverged taxa that are undergoing ecological

speciation (Rundle and Nosil, 2005). Consequently it is very difficult to determine exactly where they are along this continuum (Nosil *et al.*, 2009), and at what point they can be considered as separate species.

TABLE 4. Cytological Nei distances ( $D_N$ ) within and between forms and species of the *C. oppositus*-group, on and below the diagonal, and uncorrected pairwise distance for *Cox 1* sequences above the diagonal. Values for *C. oppositus f. edwardi* (in brackets) are for only 318 bp.

	tepperi	maddeni	jacksoni	pseud-oppositus	f.connori	f.oppositus	f.tyleri	f.whitei*	f.edwardi
tepperi	0.00	0.11	0.10-0.11	0.12	0.10-0.11	0.10-0.11	0.10	0.10-0.11	(0.10)
maddeni	0.85	0.00	0.02-0.03	0.06	0.02-0.03	0.02-0.03	0.02-0.03	0.02-0.03	(0.04)
jacksoni	0.53-0.58	0.80-0.82	0.00-0.01	0.05-0.06	0.00-0.01	0.01-0.02	0.00-0.01	0.00-0.02	(0.03-0.04)
pseud-oppositus	0.56-0.57	0.79-0.85	0.75-0.82	0.00-0.02	0.05	0.05-0.06	0.05	0.05	(0.05)
f. connori	1.17-1.92	1.23-1.92	1.06-1.89	1.15-1.92	0.00-0.37	0.00-0.02	0.00-0.01	0.00-0.01	(0.03)
f. oppositus	1.78-1.95	1.12-1.95	1.05-1.92	1.14-1.95	0.17-0.73	0.00-0.50	0.01-0.02	0.00-0.02	(0.02-0.03)
f. tyleri	0.80-1.25	1.16-2.00	0.74-1.19	0.74-1.29	0.59-1.91	0.16-1.93	0.00-1.11	0.00-0.01	(0.03)
f. whitei	0.86-2.00	1.16-2.00	0.82-1.97	1.10-2.00	0.01-0.54	0.13-1.00	0.33-1.91	0.00-0.79	(0.03)
f. edwardi	0.82	1.21	0.71-0.81	1.16-1.21	1.42-1.91	1.15-1.88	0.80-1.78	1.01-1.99	0.00

Of more importance is whether it is possible to unequivocally identify the various members of the *C. oppositus*-group without becoming an expert on karyosystematics. The obvious candidate is DNA barcoding (Hebert *et al.*, 2003), which is being increasingly used in various groups of the Chironomidae (e.g. Newburn and Krane, 2002; Carew *et al.*, 2003, 2007; Sharley *et al.*, 2004; Ekrem *et al.*, 2007; Pfenninger *et al.*, 2007; Sinclair and Gresens, 2008). To use it effectively there must be strong taxonomic support, i.e. the DNA sequence, usually mt *cox 1*, must match to sequence from taxonomically recognised specimens (Ekrem *et al.*, 2007). From cases where such taxonomic support exists, we know that introgression can present problems. Table 5 is based on the data for *Camptochironomus* species from Martin *et al.* (2002), and shows the corrected pairwise distance values for *cox 1* sequences. Note that the values for Nearctic *C. pallidivittatus* are larger for the comparison to Palearctic *C. pallidivittatus* than for the comparison to Nearctic *C. dilutus*. In fact, given that the values for the interspecific comparison to *C. dilutus* are within the range of intraspecific variation in that species and outside the range of intraspecific variation of Palearctic *C. pallidivittatus*, these Nearctic specimens would be identified as *C. dilutus* without cytological confirmation that the samples are *C. pallidivittatus*. This, then, is a possibility that must be borne in mind in comparisons of closely related species.



I am not a great advocate of the use of distance measures for barcoding purposes, particularly for a genus like *Chironomus* that is very old and so has a very great variation in separation times between species, from over 60 MY to less than 5 MY (Martin *et al.*, 2002). DNA distances between species can therefore be expected to be quite variable. A table of pairwise distances for the *C. oppositus*-group (Table 6) provides little help for distinguishing most forms and species. *C. tepperi*, the outgroup, is obviously well separated, and the values for *C.* "pseudoppositus" are consistently about 0.05-0.06, suggesting a separation time of about 7-8 MY, based on the method used by Martin *et al.* (2002). *C. maddeni* is consistently about 0.02-0.03, suggesting a separation time of about 3-4 MY, while *C.* "jacksoni" has low and variable values, presumably due to introgression since the highest values are for *C. maddeni* and *f. edwardi* where introgression is unlikely. Form *edwardi*, based on a shorter length of *cox 1*, shows values of 0.02-0.03, and higher for *C.* "pseudoppositus" and *C. maddeni*, suggesting that it has been isolated since before the development of the present desert barrier between the east and west of Australia, starting about 2.5 MYA.

**TABLE 6.** Uncorrected pairwise distances, based on the *cox 1* sequences, of the members of the *C. oppositus*-group. The outgroup is *C. tepperi*. The values for *f.* "edwardi" (in brackets) are based on just the 5' end of the *cox 1* fragment.

	<i>tepperi</i>	<i>maddeni</i>	"jacksoni"	'pseudopp'	<i>f.connori</i>	<i>f.oppositus</i>	<i>f.tyleri</i>	<i>f.whitei</i>	<i>f.edwardi</i>
<i>tepperi</i>	0.00								
<i>maddeni</i>	0.11	0.00							
"jacksoni"	0.10-0.11	0.02-0.03	0.01						
"pseudopp"	0.12	0.06	0.05-0.06	0.01					
<i>f. connori</i>	0.10-0.11	0.02-0.03	0.00-0.01	0.05	0.00-0.01				
<i>f. oppositus</i>	0.10-0.11	0.02-0.03	0.01-0.02	0.05-0.06	0.00-0.02	0.00-0.02			
<i>f. tyleri</i>	0.10	0.02-0.03	0.00-0.01	0.05	0.00-0.01	0.01-0.02	0.00-0.01		
<i>f. whitei</i>	0.10-0.11	0.02-0.03	0.00-0.02	0.05	0.00-0.01	0.01-0.02	0.00-0.01	0.00-0.01	
<i>f. edwardi</i>	(0.10)	(0.04)	(0.03-0.04)	(0.05)	(0.03)	(0.02-0.03)	(0.03)	(0.03)	(0.00)

DeSalle *et al.* (2005) have suggested that, rather than looking at distances, specific base differences that characterize related species should be sought. For the *C. oppositus*-group, the 59 variant bases in the *cox 1* sequences (Table 7) were compared. As would be expected from the distance values, there are several bases that are unique to *C.* "pseudoppositus"; for *C. maddeni* there are four in our data, but based on specimens from only one locality; and for *C.* "jacksoni" there is just one in the current three samples – shared with *C. pseudoppositus* and *f. edwardi*, with which it is not likely to be confused because of other sequence differences. It should be noted that the *f. edwardi* sequence comes from two 45-year-old dehydrated specimens, so some of the observed differences may be artefacts due to DNA degradation. For the other *C. oppositus* forms there are really no consistent differences, just some indicators of introgression, such as the A at base 28, that occurs in three forms in Tasmania, but only in one population of *f. tyleri* on the mainland.



of closely related species complexes, the ribosomal intergenic region, particularly ITS-2 (Asari *et al.*, 2004; Kani *et al.*, 2006; Kaga *et al.*, this volume). With data for only three members of the group so far (Table 8), compared here to *C. duplex* sequence from GenBank (AJ296776), there are differences between *f. tyleri*, *f. edwardi* and *C. pseudoppositus*.

**TABLE 8.** Aligned nucleotide sequence for the ITS-2 region of four Australian *Chironomus* species. Identical bases are indicated by dots. Gaps required to best align the sequences are indicated by dashes.

<i>C. duplex</i>	gattacaattataagtagtgaGATCTCTCTCTGTAGTATATCCCATTACACAATAGAGTGTTCATCAAAGCCGCCGTCGGC
<i>C. opp. f. tyleri</i>	..... T.-----,--- TA.----- TG.... C.... C... TTG.... T. A.... A
<i>C. opp. f. edwardi</i>	..... T.--- A. A. ,-----,----- TT... T.. A
<i>C. pseudopp.</i>	gattacaactataagtaatgaTATTT---A. A. ,-----,----- TT... T.. A
	end of 5.8S>
<i>C. duplex</i>	---TA-----TGTGGATGGGGATTATGACTAAAATGCTAATC-TAAA--TGTCAGTTTACGC-CTATTTTAAAT
<i>C. opp. f. tyleri</i>	CCTC. A-----,--- G... G.... T. T. T. . T. A... G... C. C. TGG....--- C. C. T. . T.... T.. A
<i>C. opp. f. edwardi</i>	---. -TCACACATA.....
<i>C. pseudopp.</i>	---. -TCACACATA..... A.....
<i>C. duplex</i>	AAATGGGGAGA-AAGAGTGAA-AACTTCAAAATTCGC-----TCGC--ATGTACTATAT-GT-ATG-----TGTGA-TA
<i>C. opp. f. tyleri</i>	. GG..... RG.... --- R.... T. C.... C. CCCCACCC.... GC.... GAG. G. GA. -C.----- A. ---
<i>C. opp. f. edwardi</i>	..... A. A. -- CC. TGGG.... ,--- G... AGTCT.... G..
<i>C. pseudopp.</i>	..... A. A. -- CC. TGGG.... ,--- G... AGTCT.... G..
<i>C. duplex</i>	TGA -----G-----TCT---CTCTGGCGCTAACTTT--ACACGT---TGTGTC-T---CGTTAGTTGCTCCGTGACTC
<i>C. opp. f. tyleri</i>	. A.-----A----- T. TTA... TG..... AC. MC... TG---... TG. --- T.... A.... TC....
<i>C. opp. f. edwardi</i>	. A. ATTTTCATT. AAAATTT... ---..... A. CAT.... G- TGT.....
<i>C. pseudopp.</i>	. A. ATTTTCATT. AAAATTT... ---..... A. CAT.... G- TGT.....
<i>C. duplex</i>	GTTGACGTTGATTTTGGAGATAGTAAAAAGTAGTCTTCTCTAATGTTTATAAATACTGAAGTAATTT-T-TG-ATATACATA
<i>C. opp. f. tyleri</i>	... T. ,-----,---..... AA.T... Y... TA. A. . CT. TA..... A. A. A. T.... -..
<i>C. opp. f. edwardi</i>	... T. A. ,-----,---..... G... C..... R... A. A. A. --..... G
<i>C. pseudopp.</i>	... T. A. ,-----,---..... A..... A... C.....
<i>C. duplex</i>	T-TAA--AGACAAAttcgggacctcaactcatgttgactacccctgaatttaagcatattaatt
<i>C. opp. f. tyleri</i>	-A.....
<i>C. opp. f. edwardi</i>	. A... TA. T. A. . C..... a.
<i>C. pseudopp.</i>	.....
	<start of 28S

To conclude, the use of DNA barcoding can make it much easier to identify the majority of *Chironomus* species, often without the necessity for detailed sequencing, as for example in the techniques of Carew *et al.* (2003) and Sharley *et al.* (2004) using combinations of restriction

enzymes to provide unique patterns of bands from *cox -I*. This method enables the differentiation of a number of Australian *Chironomus* species, including *C. cloacalis* from *C. "februarius"* (Sharley *et al.*, 2004). They could not, as would be expected from the results presented above, separate the members of the *C. oppositus*-group. The improved ability to differentiate species is producing some very interesting results. One example is that of Hare's group in Canada, where the ability to separate the morphologically similar *C. staegeri* Lundbeck and *C. "tigris"* (previously *C. sp. r*, as in Butler *et al.*, 1995) has permitted them to demonstrate that, in the same polluted lake, these two species show different levels of some heavy metals, apparently as a result of the larvae feeding at different levels in the substrate (Martin *et al.*, 2008).

In Australia, with the ability to differentiate the members of the *C. alternans*-group, *C. "februarius"* has been shown to be common in habitats around Melbourne, Victoria (Sharley *et al.*, 2004), appearing tolerant to zinc in microcosm studies but with field studies suggesting that other environmental factors can affect the level of tolerance (Pettigrove and Hoffmann, 2005). This apparent difference in response was suggested to be due to factors such as differential competition for suitable habitat arising from the different taxa occurring in the microcosms and in the field sites sampled.

Problems can still be expected with closely related species, due to the possibility of hybridisation and introgression. As with the *C. oppositus*-group, such closely related species complexes may not be resolved by just a mitochondrial barcode, but nuclear genes such as those mentioned above, may provide a solution.

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**This version of the paper has been modified to improve the clarity of the figures (using the color versions used in the original presentation) and, in particular to correct the errors introduced into Table 8 during typesetting.**