

Molecular identification of *Chironomus* spp. (Diptera) for biomonitoring of aquatic ecosystems

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Abstract Chironomidae larvae often represent a major component of the benthic fauna in inland water bodies and are used frequently as bioindicators of ecosystem health. The genus *Chironomus* is a recognised indicator of organic enrichment and has been used extensively in the northern hemisphere and New Zealand in ecotoxicological studies. However, similar use of *Chironomus* in Australia is limited due to the presence of cryptic species that restrict the collection of information on species-specific responses to environmental stress. To address the problems associated with species identification, we have used PCR-RFLP of the mitochondrial COI gene to develop DNA profiles for nine common Australian *Chironomus* species. Species-specific haplotypes were identified using reference taxa previously identified by cytological analysis, and verified with field specimens collected from seven wetlands around Melbourne. This research provides an effective tool for species identification of this ubiquitous and often abundant genus that will provide the basis of obtaining species-specific information to inform on the health of aquatic ecosystems.

Key words biological monitoring, invertebrates, water pollution.

INTRODUCTION

Chironomidae (Diptera) are the most widely distributed and frequently the most abundant group of invertebrates in freshwaters (Cranston 1995). They are a major component of biomass in lentic systems and the dominant food resource for many fish (Armitage 1995). There are few bodies of freshwater anywhere in the world where chironomids do not occur, and their ubiquitous presence has led to their inclusion in nearly all aquatic monitoring programs and assessment schemes (Warwick 1985; Johnson *et al.* 1993; Pettigrove *et al.* 1994; Hardwick *et al.* 1994; Lindegaard 1995). Together with the Oligochaeta, chironomids represent the greater part of the sediment-dwelling fauna, which makes them especially useful for sediment quality assessment (Hynes 1960), and they have been used as freshwater bioindicators since the early 1900s (reviewed in Lindegaard 1995). Some species of chironomids are tolerant to heavy metals, whereas others are sensitive (Clements *et al.* 1988), highlighting the importance of identifying chironomids beyond the family level (Clements 1991). A positive correlation between deformities of chironomid larvae and sediment contamination has been proposed (Warwick 1985), and it has been suggested that the frequency of structural abnormalities could be an effective indicator of stress from pesticides in rice agriculture (Pettigrove *et al.*

1994). Chironomids have also been used extensively in ecotoxicological studies that involve exposing an organism to a toxic material, such as heavy metals or pesticides, and determining the response (e.g., Chapman 1995; De Bisthoven *et al.* 1998).

Within the Chironomidae, the genus *Chironomus* is recognised as a taxon characteristic of organic enrichment and has been widely used as an indicator of aquatic ecosystem health (Johnson *et al.* 1993; Lindegaard 1995). The widespread use of *Chironomus* in biomonitoring studies has probably arisen because of its known tolerance to many stressors and toxins (Lindegaard 1995). The variation in sensitivity to pollution that exists between species of *Chironomus* also makes them ideal indicators of water body degradation (Johnson *et al.* 1993; Lindegaard 1995). Studies using *Chironomus* in bioassessment of freshwater aquatic ecosystems have been carried out predominantly in Europe and North America (Johnson *et al.* 1993), attributable to the extensive taxonomic research of this genus undertaken in these regions (e.g., Sublette & Sublette 1974; Wülker & Butler 1983; Sublette & Martin 1994). In contrast, no formal taxonomic keys for Australian *Chironomus* species are available (Cranston 1994) as many species are morphologically similar, particularly in the larval stage that is routinely used in biomonitoring studies.

In his comprehensive revision of the Australian Chironomidae, Freeman (1961) identified six *Chironomus* species based on adult morphology: *C. nepeanensis* Skuse, *C. vitellinus* Freeman, *C. magnivalva* Kieffer, *C. tepperi* Skuse, *C. australis* Macquart, and *C. alterans* Walker. More recent cytological studies have revealed that a number of these

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form species complexes (e.g., Martin *et al.* 1978, 1996; Martin 1979; Martin & Cranston 1994). Additional species now recognised on the basis of cytological variation include *C. duplex* Walker, *C. cloacalis* Atchley & Martin, *C. 'februarius'* Martin, *C. 'jacksoni'* Martin, *C. maddenii* Martin & Cranston, *C. 'pseudoppositus'* Martin and *C. oppositus* Walker (Bugledich *et al.* 1999). Cytological characterisation, however, requires considerable expertise and is also time consuming. Together with the inadequacy of morphological characters for identifying species of these taxa, these shortcomings have prohibited species-level identification of *Chironomus* in rapid biomonitoring studies in Australia, in contrast to their wider utilisation elsewhere (Lenat & Barbour 1994). Biomonitoring studies in Australia have, at best, identified *Chironomus* only to genus, and there is no information on the sensitivity of species' environmental stressors. This has precluded the development of species-specific bioindicators within Australian *Chironomus*, despite their widespread occurrence and the fact that many species can be successfully cultured. *Chironomus* are used widely in many parts of the globe in ecotoxicological and genetic studies and the capacity for laboratory culturing is a distinct advantage and endorses *Chironomus* as an ideal model system. To enable Australian *Chironomus* to be utilised fully, however, an accessible means of species identification is needed. Molecular techniques offer a rapid and cost effective alternative to cytological procedures and can provide robust species level identification.

DNA variation that distinguishes closely related individuals or species can be identified using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). PCR-RFLP analysis can be applied to both nuclear and mitochondrial DNA (Loxdale & Lushai 1998) and has been used in taxonomic studies (Cespedes *et al.* 1999). PCR-RFLP has been utilised to produce species-specific DNA profiles for identification of morphologically similar species in a variety of taxa, e.g., *Wiseana* moths (Brown *et al.* 1999), asteroid larvae (Evans *et al.* 1998), *Penaeus* shrimp (Gusmao *et al.* 2000) and screwworm flies (Litjens *et al.* 2001). In this study, we have applied the PCR-RFLP technique to develop species-specific profiles for nine common Australian *Chironomus* species. Given the potential for *Chironomus* species to provide valuable ecological information, this paper tests the utility of PCR-RFLP to identify *Chironomus* larvae collected from south-eastern Australia to species. The south-eastern region of Australia was selected to field trial this method as 10 of the 15 Australian species occur in the region (Cranston & Martin 1989; Martin & Cranston 1994) and many of these have cryptic larval morphologies.

MATERIALS AND METHODS

Biological samples

Nine species of *Chironomus* (including four subspecies of *Chironomus oppositus*), all laboratory reared and originally sourced from Victoria, Queensland, South Australia, New

Table 1 PCR-RFLP composite haplotypes derived for reference data

Taxon	COI haplotype	Source location
<i>C. oppositus</i>	A1 R1	Victoria/Tasmania
<i>C. cloacalis</i>	A3 R2	Western Australia
<i>C. 'februarius'</i> *	A3 R3	Victoria
<i>C. 'pseudoppositus'</i> *	A4 R1 T2	Queensland
<i>C. 'jacksoni'</i> *	A4 R1 T1	Victoria
<i>C. duplex</i>	A5 R4	Victoria
<i>C. tepper</i>	A6 R6	New South Wales
<i>C. australis</i>	A7 R4	Victoria
<i>C. maddenii</i>	A8 R1	South Australia
<i>K. 'cornishi'</i> *	A2 R5	Victoria

*Species names indicated are provisional names only (J Martin, pers. comm. 2003).

South Wales and Western Australia (Table 1), were generously provided by J Martin. All laboratory samples previously had been identified cytologically and constituted our reference *Chironomus* taxa. A closely related chironomid, *Kiefferulus 'cornishi'* (Martin *et al.* 1996), was included for outgroup comparison. Additional chironomid larvae were collected in August and December 2002 from 11 wetland sites in the Melbourne urban area using a hand-held dip net with a 300 mm by 300 mm opening and 250 µm mesh to sweep the substratum. The anterior and posterior region of each specimen were stored for morphological examination in 70% ethanol, and the remaining portion was stored for DNA extraction in 100% ethanol at -20°C. Collected specimens were morphologically identified to genus using the taxonomic key of Cranston (1994).

DNA preparation

Genomic DNA was extracted following a modified version of the Chelex method (Carew *et al.* 2003). Body segments of chironomid larvae were crushed to a fine powder in liquid nitrogen in a 1.5-mL Eppendorf tube. 500 µL Chelex 5% w/v was added and the mixture incubated at 90°C for 30 min. The solution was then shaken for 15 s and centrifuged for 2 min at 14000 r.p.m. and stored at -20°C. DNA preparations were thawed and centrifuged at 14000 r.p.m. for 2 min prior to use.

PCR amplification

A 710-bp fragment of the mitochondrial cytochrome oxidase subunit (COI) was amplified using primers 911 (5'-TTTCTA CAAATCATAAAGATATTGG-3'; Guryev *et al.* 2000) and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; Folmer *et al.* 1994). Reactions contained 1 × *Taq* buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 µL of each primer, 0.5 µL *Taq* DNA polymerase (Gibco), 5 µL DNA template and H₂O to 50 µL. Amplifications were carried out in a Gene Amp PCR system 2700 (Applied Biosystems) using the following profile: 3 min at 94°C, 35 cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 1 min and a final extension step of 5 min at 72°C.

Sequencing and restriction endonuclease simulation

To confirm the identity of the amplified PCR fragments, products from all *Chironomus* reference taxa were sequenced commercially (Macrogen Inc.) in both directions using an ABI3700 automatic DNA sequencer. Sequence data were assembled using Sequencher computer software and aligned using LICOR AlignIR Version 2. Simulated restriction enzyme digestion was conducted on COI sequence data for all reference taxa using the simulation program NEBcutter (available from URL <http://www.neb.com>). Simulated digests were carried out with the following restriction enzymes with four base pair recognition sites: *Bfa*I, *Apo*I, *Mse*I, *Hpa*II, *Hpy*ch4IV, *Dra*I, *Ase*I, *Hpy*ch4V, *Mbo*I, *Psi*I, *Mn*I, *Mse*I, *Taq*I, *Rsa*I, *Alu*I, *Tsp*5091 and *Hha*I, and those yielding diagnostic species-specific profiles were identified for experimental use.

Restriction endonuclease digestion

Between 10–15 µL of each PCR reaction was digested with the restriction enzymes *Alu*I and *Rsa*I (New England Biolabs) according to the manufacturer's recommendations in a total volume of 20 µL. Additional digests with *Taq*I restriction enzyme were carried out for two reference taxa (*C. oppositus* and *C. 'jacksoni'*). PCR-RFLP fragments were visualised following electrophoresis through 3% agarose gels and ethidium bromide staining.

RESULTS

Species-specific DNA identification

Six-hundred-and-thirty base pairs of reliable sequence data were obtained for all *Chironomus* reference taxa (data not

shown) and confirmed to represent the expected 5' region of the mitochondrial COI gene following alignment with previously published *Chironomus* sequence data (Guryev *et al.* 2000). Simulated digestion identified few useful restriction enzymes. Most enzyme recognition sites were either not present or invariant across all taxa. Simulated diagnostic profiles for the *Chironomus* reference taxa were identified with the restriction enzymes *Alu*I and *Rsa*I and these enzymes were used subsequently to digest the COI fragment amplified from each reference species. Unique band profiles were observed for *C. duplex*, *C. australis*, *C. maddenii*, *C. tepperi*, *C. oppositus* and *K. 'cornishi'* following *Alu*I digest (Fig. 1) while distinguishing *Rsa*I profiles were obtained for *C. 'februarius'*, *C. cloacalis*, *C. tepperi* and *K. 'cornishi'* (Fig. 2). Of the remaining reference taxa *C. 'pseudoppositus'* and *C. 'jacksoni'* shared similar *Alu*I and *Rsa*I profiles, as did the *C. oppositus* subspecies. Distinguishing DNA profiles were obtained for *C. 'pseudoppositus'* and *C. 'jacksoni'* following an additional digest with the *Taq*I restriction enzyme (data not shown). Subspecies of *C. oppositus* could not be distinguished. Species were allocated alpha-numeric haplotypes for *Alu*I (Ax), *Rsa*I (Rx) and *Taq*I (Tx), where x denotes the haplotype number. Haplotypes were based on fragments greater than 100 base pairs as smaller fragments could not be identified unambiguously. We derived 10 unique composite haplotypes corresponding to the 10 reference species (Table 1).

Validation of DNA identification

The PCR-RFLP haplotypes of 39 *Chironomus* specimens (morphologically identified to genus) collected from seven wetland sites across Melbourne were compared with the reference species haplotypes. Five *Chironomus* species were identified from the seven sites surveyed, including four species from Settlers Orchard wetland and three from Warrandyte

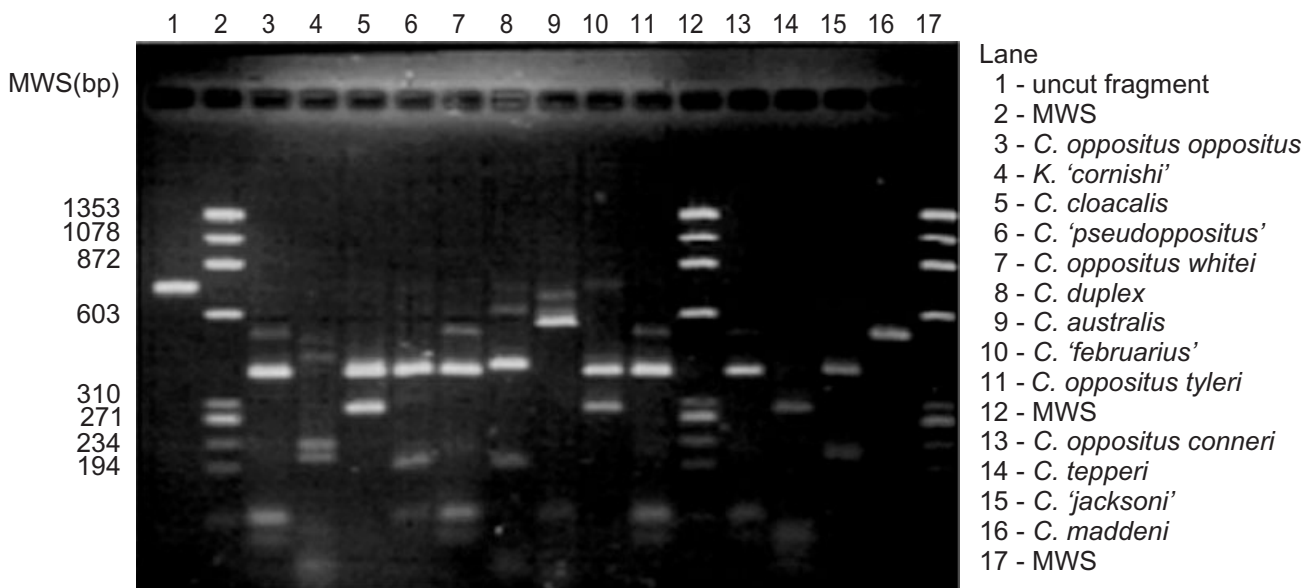


Fig. 1. *Alu*I PCR-RFLP results for reference taxa: gel electrophoresis of *Alu*I digested PCR COI amplicons, taxa listed on the right with corresponding lane number. MWS(bp): molecular weight standard, sizes in base pair.

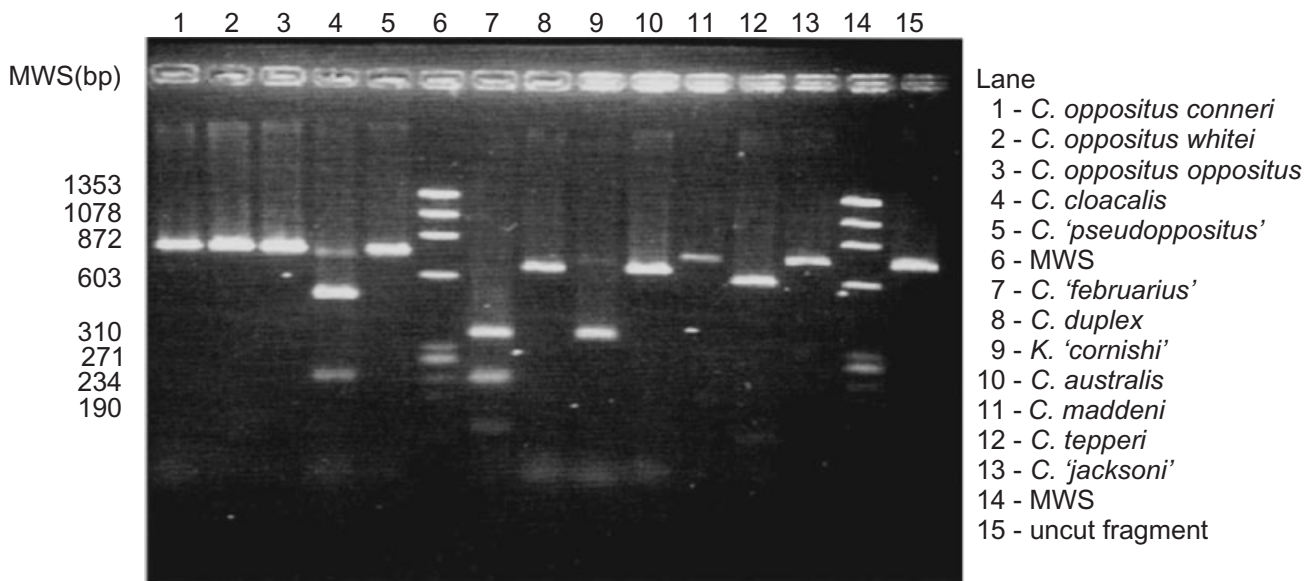


Fig. 2. *RsaI* PCR-RFLP results of reference taxa: gel electrophoresis of *RsaI* digested COI PCR amplicons, taxa listed on the right with corresponding lane number. MWS(bp): molecular weight standard, sizes in base pairs.

Table 2 Distribution of *Chironomus* sampled from seven Melbourne urban wetland sites

Taxon	La Trobe wetlands	Warrandyte State Park	La Trobe University	Settlers Orchard	Gilmore Park	Yarranga Reserve	Skeleton Creek	Total
<i>C. australis</i>	–	2	–	1	–	–	–	3
<i>C. cloacalis</i>	–	–	–	2	–	–	–	2
<i>C. duplex</i>	–	6	–	–	1	–	1	8
<i>C. 'februarius'</i>	8	–	7	3	–	–	–	18
<i>C. oppositus</i>	–	1	–	5	1	1	–	8
Total	8	9	7	11	2	1	1	39

Table 3 Results of independent PCR-RFLP and taxonomic specimen analysis for chironomid samples collected from four urban Melbourne wetland sites

Site	n	DNA profile	n	Species ID:DNA	Genus ID:taxonomic	Concordance
1	25	A1 R1	14	<i>C. oppositus</i>	<i>Chironomus</i>	100%
		A2 R2	2	<i>C. 'februarius'</i>	<i>Chironomus</i>	100%
		Unknown	9	Non- <i>Chironomus</i>	Non- <i>Chironomus</i>	100%
2	13	A1 R1	11	<i>C. oppositus</i>	<i>Chironomus</i>	100%
		A2 R2	1	<i>C. 'februarius'</i>	<i>Chironomus</i>	100%
		Unknown	1	Non- <i>Chironomus</i>	Non- <i>Chironomus</i>	100%
3	26	A2 R2	1	<i>C. 'februarius'</i>	<i>Chironomus</i>	100%
		Unknown	25	Non- <i>Chironomus</i>	Non- <i>Chironomus</i>	100%
4	15	Unknown	15	Non- <i>Chironomus</i>	Non- <i>Chironomus</i>	100%

State Park (Table 2). *C. 'februarius'* was the most abundant species present (18 specimens from three sites), followed by *C. duplex* and *C. oppositus* (8 specimens from three sites), *C. australis* (3 specimens from 2 sites) and *C. cloacalis* (2 specimens from one site). No intraspecific RFLP variation was observed from these field data.

Seventy-nine chironomid specimens from four additional sites were subjected to independent PCR-RFLP and morphological identification (Table 3). All samples with composite PCR-RFLP haplotypes that matched one of the reference spe-

cies were identified independently as belonging to the genus *Chironomus* by morphological examination, and specimens with unrecognised PCR-RFLP haplotypes were identified as belonging to chironomid genera other than *Chironomus*.

DISCUSSION

The only method available to reliably distinguish Australian *Chironomus* taxa has been cytological analysis of the large

polytene chromosomes (Martin *et al.* 1978; Martin 1979; Martin & Cranston 1994). Although definitive, cytological analysis has limitations. The banding patterns and chromosomal rearrangements of the polytene chromosomes are relatively complex and expertise in detecting and deciphering these is required. In addition, the analyses are time consuming and can be carried out only on specimens in the late developmental stage (Lentzios *et al.* 1980). In contrast, the molecular-based methods that we have developed are relatively rapid and simple, can be used at any life stage and provide the same level of *Chironomus* species characterisation. We have developed species-specific DNA profiles for common Australian *Chironomus* and demonstrated that field collected samples can be accurately identified to the species level.

Six of the 10 reference species could be characterised following digest with a single restriction enzyme, *AluI*, while an additional digest with *RsaI* distinguished two of the remaining taxa. Only two species, *C. 'pseudoppositus'* and *C. 'jacksoni'*, required additional digestion with *TaqI* to identify unique profiles. Unique DNA profiles were not obtained for the *C. oppositus* subspecies reflecting the low level of sequence variation seen among these taxa. In all, 10 composite haplotypes were produced corresponding to the nine *Chironomus* reference species investigated and the related *K. 'cornishi'*. These haplotypes were based only on fragments larger than 100 base pairs as smaller fragments could not be clearly distinguished with the electrophoresis conditions used. However, simulation analysis indicated that higher resolution conditions would allow all species to be differentiated following a single digest with the *AluI* restriction enzyme.

Field studies confirmed the robustness of the species specific DNA profiles by testing for the presence of (a) intraspecific variation and (b) shared intergenic profiles. In the first study, *Chironomus* specimens were identified from field collections to genus and subject to PCR-RFLP analysis. Five species were subsequently identified based on the resulting DNA profiles and there was no intraspecific variation found in the analysis of 39 individuals from different populations. In addition, the *C. cloacalis* DNA profiles observed for both Melbourne field samples and the Western Australian reference sample were identical. This is an important observation as it provides evidence that the DNA profiles are robust over wide geographical distances. In the second study, 79 chironomids sampled from four locations across Melbourne were subjected to independent morphological and molecular analysis. There were no shared DNA haplotypes observed between *Chironomus* and non-*Chironomus* genera with all specimens being identified clearly as *Chironomus* or not, in total accordance with the morphological analysis.

Our results indicate that PCR-RFLP offers a reliable method for species-specific identification with several advantages over traditional methodology. A major advantage is that the method can be used at any developmental stage to provide accurate identification. The relationship among different morphological life stages of the one species is not always apparent but, as an individual's DNA does not change, molecular profiling could be used to assist taxonomic studies. This has been

demonstrated in asteroid species where correspondence between larval and adult forms, previously found to be very difficult due to errors associated with morphological identification, was accomplished successfully using PCR-RFLP (Evans *et al.* 1998). Molecular-based identification offers a cost-effective alternative to taxonomic and/or cytological methods. The methods are relatively simple and expertise can be acquired readily, thus keeping training and labour costs down. In addition, molecular methods facilitate rapid assessment, from field collection to report presentation in the recommended 5 d (Lenat & Barbour 1994), while attaining species level resolution. Several authors have advocated the use of species-level data in biomonitoring programs to allow for more accurate determination of ecosystem health (Resh & Unzicker 1975; Bailey *et al.* 2001; Lenat & Resh 2001; King & Richardson 2002) and the molecular methodology presented here will permit more extensive utilisation of *Chironomus* in these programs.

In a wider context, DNA-based identification will allow for the determination of adequate baseline information required for the selection of indicator species. Baseline information includes the biology, life history, taxonomy of the organism, tolerance levels to known pollutants, and the establishment of the correlation between the taxon's response to impacts and ecosystem change (Johnson *et al.* 1993; Hilty & Merenlender 2000). For effective biomonitoring, the cause and effect of various pollutants need to be determined. At present, there are little reliable data available in Australia on the effect of various pollutants on individual *Chironomus* species. Field-based microcosm studies that have revealed valuable data on the relative effects of different pollutants and other physico-chemical variables on the abundance and development of chironomids have been limited because individual species could not readily be identified (Pettigrove, unpublished data). However, with rapid species-specific identification of *Chironomus* now possible, ecotoxicological studies can be expanded to determine the variation in sensitivities within the genus and identify those *Chironomus* that will be useful indicator species of specific pollutants. Broader application to include other chironomid genera is also being explored (Carew *et al.* 2003).

This study represents the first comparison of Australian *Chironomus* using PCR-RFLP of the cytochrome oxidase I (COI) gene. It has been suggested recently that this same fragment has the potential to distinguish all metazoan taxa, providing a DNA barcoding system for animal life (Hebert *et al.* 2003a,b). Hebert *et al.* were able to place taxa into higher taxonomic categories and distinguish closely related species across the animal kingdom based on COI sequence variation. Within the *Chironomus* genus phylogenetic relationships were investigated recently using this COI fragment and it was found that Australian *Chironomus* species represent two monophyletic clades (Guryev *et al.* 2000). We have extended their study by producing unique species profiles for additional *Chironomus* taxa and demonstrated that PCR-RFLP of COI provides an accurate method for identifying morphologically similar *Chironomus* species. Although molecular based identification methods do not have the potential to supplant traditional tax-

onomic identification, the use of this technique will complement the expansion of rapid biomonitoring programs through the facilitation of accurate species identification and allow more complete utilisation of *Chironomus* in Australian ecotoxicological studies.

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